

Targeting cytochrome bc_1 of *Plasmodium falciparum*

Priyanka Agarwal



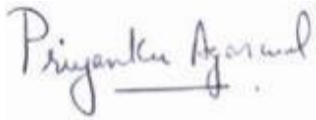
WITS
UNIVERSITY

A thesis submitted to the Faculty of Health Sciences,
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in fulfilment of the requirements for the degree
of
Doctor of Philosophy

Johannesburg, 2025

DECLARATION

I Priyanka Agarwal declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

31st day of March 2025 at Parktown

DEDICATION

To my parents, who remain in my heart, and to my supportive family.

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Published articles

1. Kavita Pal, Sahil Lala, Priyanka Agarwal, Tarosh S. Patel, Jenny Legac, Md. Ataur Rahman, Saiema Ahmedi, Nida Shahid, Sneha Singh, Kajal Kumari, Hari Madhav, Abhik Sen, Nikhat Manzoor, Bharat C. Dixit, Robyn van Zyl, Philip J. Rosenthale and Nasimul Hoda. Naphthyl bearing 1,3,4-thiadiazoleacetamides targeting the parasitic folate pathway as anti-infectious agents: *in silico*, synthesis, and biological approach. RSC Medicinal Chemistry 2023; 14(12): 2768-2781. doi: 10.1039/d3md00423f.

Student's contribution to the paper

To determine cytotoxicity of test compounds against HEK293 cell line (**Appendix F**).

2. Sabahat Samreen, Jyoti Gupta, Puleng A. Thaban, Priyanka Agarwal, Robyn L. van Zyl, Afreen Inama. Synthesis, antiprotozoal activity, molecular docking and ADME study of 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives. African Journal of Biological Sciences 2024; 6(15):10517-10541.

Student's contribution to the paper

Determination of physicochemical and toxicological properties of test compounds using *in silico* methods (**Appendix G**).

3. Priyanka Agarwal, David D. N'Da3, C. Johan van der Westhuizen, Robyn L. van Zyl A Dual *In vitro* and *In silico* Approach to Evaluate 1,4-naphthoquinone-1,2,3-triazole Hybrids Against Atovaquone-Resistant Malaria. Experimental Parasitology 2025; doi: 10.1016/j.exppara.2025.108991.

Student's contribution to the paper

Conceptualisation, methodology, data management, writing of the manuscript (**Appendix H**).

PRESENTATIONS

- Priyanka Agarwal, Robyn L. van Zyl. Design, molecular docking, drug-likeness, and ADMET prediction of chalcones as novel *Plasmodium falciparum* cytochrome *bc₁* (cyt *bc₁*) inhibitors. WRIM Postgraduate Research Day, WITS University. 25 April 2024. [Oral, **Best Presentation Award** in PhD student category]
- Priyanka Agarwal and Robyn L. van Zyl. *In silico* analysis of pyridine-based compounds targeting *Plasmodium* cytochrome *bc₁* complex. 8th Annual Southern Africa Malaria Research Conference, South Africa. 1-3 August 2023. [Poster]
- Priyanka Agarwal and Robyn L. van Zyl. Reviewing pyridine-based compounds as *Plasmodium* cytochrome *bc₁* complex inhibitors in light of SAR and molecular docking studies. School of Therapeutic Health Sciences Biennial Research Day, South Africa. 5 September 2023. [Poster]
- Priyanka Agarwal and Robyn L. van Zyl. Exploring compounds targeting *Plasmodium* cytochrome *bc₁* complex. WITS Pharmacology Research Day, South Africa. 9 November 2023. [Oral]
- Priyanka Agarwal and Robyn L. van Zyl. Molecular modelling studies and predicted pharmacokinetic properties of tetracyclic benzothiazepines as *Plasmodium* cytochrome *bc₁* complex inhibitors. WRIM Research Day, Wits University. 20th April 2022. [Oral, **2nd place prize** in PhD student presentation category]
- Priyanka Agarwal and Robyn L. van Zyl. Computational studies to identify potential lead inhibitors targeting *Plasmodium* cytochrome *bc₁* complex. 7th Southern Africa Malaria Research Conference. 2nd to 4th August 2022. [Poster]
- Priyanka Agarwal, Robyn L. van Zyl and C. Johan van der Westhuizen. A database of antimalarial compounds targeting *Plasmodium* cytochrome *bc₁*. 4th PARSA Conference on 12th September 2022. [Oral]
- Priyanka Agarwal and Robyn L. van Zyl. *In silico* studies of antimalarial compounds targeting *Plasmodium* cytochrome *bc₁*. Faculty of Health Sciences Research Day, Wits University on 15th September 2022. [Poster]

- Robyn L. van Zyl, Priyanka Agarwal, C. Johan van der Westhuizen, Jenny-Lee Panayides. *In silico* analysis of naphthoquinone compounds as potential antimalarial agents targeting the cytochrome *bc₁* complex. British Society of Parasitology, Malaria Conference. 21st -25th June 2021. [Poster]
- Priyanka Agarwal, Robyn L. van Zyl, C. Johan van der Westhuizen and Jenny-Lee Panayides. *In silico* analysis of naphthoquinone compounds as cytochrome *bc₁* complex inhibitors. 6th Southern Africa Malaria Research Conference Webinar. 3rd and 4th August 2021. [Oral]
- Priyanka Agarwal, Robyn L. van Zyl, C. Johan van der Westhuizen and Jenny-Lee Panayides. *In silico* insights into binding and pharmacokinetic properties of naphthoquinones as *Plasmodium* cytochrome *bc₁* complex inhibitors. WITS School of Therapeutic Sciences Research Day, Parktown, South Africa. September 14th, 2021. [Poster, **Best poster presentation award** in professional category].
- Priyanka Agarwal, Robyn L. van Zyl. *In silico* Insights to Optimise Naphthoquinone analogues as lead inhibitors of the *Plasmodium* Cytochrome *bc₁* Complex. Parasitological Society of Southern Africa (PARSA) Conference held on September 14th – 16th, 2021. [Oral]
- Priyanka Agarwal and Robyn L. van Zyl. A Database of Antimalarial Compounds Targeting *Plasmodium* Cytochrome *bc₁* Complex. WITS School of Therapeutic Sciences Research Day, Parktown, South Africa. October 15th, 2020. [Poster]

AWARDS

Research Grants

1. Faculty Research Committee Individual Research Grants 2021 (R 12,000).

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Award for Best Presenter

1. Priyanka Agarwal, Robyn L. van Zyl. Design, molecular docking, drug-likeness, and ADMET prediction of chalcones as novel *Plasmodium falciparum* cytochrome *bc₁* (cyt *bc₁*) inhibitors. WRIM Postgraduate Research Day, WITS University. 25 April, 2024. [**Oral, Best presentation award in PhD student category**]
2. Priyanka Agarwal and Robyn L. van Zyl. Molecular modelling studies and predicted pharmacokinetic properties of tetracyclic benzothiazepines as *Plasmodium* cytochrome *bc₁* complex inhibitors. WRIM Research Day, Wits University. 20th April, 2022. [**Oral, 2nd place prize in PhD student presentation category**]
3. Priyanka Agarwal, Robyn L. van Zyl, C. Johan van der Westhuizen and Jenny-Lee Panayides. *In silico* insights into binding and pharmacokinetic properties of naphthoquinones as *Plasmodium* cytochrome *bc₁* complex inhibitors. WITS School of Therapeutic Sciences Research Day, Parktown, South Africa. September 14th, 2021. [**Poster, Best poster presentation award in professional category**].

Training

1. Participated and completed training on “Foundations of Theoretical and Computational Science” hosted virtually in 2021 during 11th CHPC Introductory Programming School and 3rd NITheP Summer School.

ABSTRACT

Malaria is a life-threatening disease and a global health problem caused by *Plasmodium* parasite. Chemotherapy is essential to manage malarial infections, but the parasite is rapidly developing resistance against the available drugs. Thus, there is an urgent need to develop new drugs with increased potency. A promising target is that of atovaquone, inhibiting cytochrome *bc₁* (cyt *bc₁*) complex of the mitochondrial electron transport chain. The study aimed to undertake *in silico* and *in vitro* studies to identify novel *Plasmodium* cyt *bc₁* complex inhibitors with specific affinity for Plasmodia and reduced toxicity profile. To fulfil this aim, a database of small organic compounds targeting *Plasmodium* cyt *bc₁* complex was created by collating data about chemical structures and their biological activities from published research. This facilitated the distinction between active and inactive inhibitors across various chemical scaffolds, aiding in the identification of lead compounds for further optimisation. This study evaluated a series of 1,4-naphthoquinone-1,2,3-triazole hybrids for their potential as antimalarial agents targeting resistant *Plasmodium* strain through *in vitro* investigations. Additionally, *in silico* studies were performed to examine the ligand binding interactions using molecular dynamic simulations in both wild-type and mutant models, along with predicting pharmacokinetic properties and drug-likeness. Furthermore, a series of 2-(4-substituted piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl) acetamide hybrids previously reported as *P. falciparum* dihydrofolate reductase (DHFR) inhibitors were evaluated *in vitro* for their cytotoxicity against HEK293 and K562 cell lines, and *in silico* to determine their potential to target the cyt *bc₁* complex utilising molecular modelling methods involving, molecular docking, molecular dynamics simulations, and binding free energy (MM-GBSA) calculations to assess their binding affinity, stability, and key interactions. Moreover, novel chalcone compounds were designed engaging the principle of computer aided drug discovery and the ligands were virtually investigated for their antimalarial potential against atovaquone-resistant (Y279S) model using the Schrödinger software suite, followed by *in silico* evaluation of their drug-likeness and absorption, distribution, metabolism, elimination, toxicological (ADMET) properties. Overall, the thesis involved both the *in vitro* and *in silico* methods for evaluation of antimalarial potential and drug-likeness properties of compounds, highlighting the value of *in silico* approaches in early-stage drug discovery.

Keywords: Malaria, *Plasmodium falciparum*, electron transport chain, cytochrome bc_1 complex, complex III, atovaquone, 1,4-naphthoquinone, 1,4-naphthoquinone-1,2,3-triazole hybrids

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LIST OF ACRONYMS AND ABBREVIATIONS

2D:	2-dimensional
3D:	3-dimensional
$\Delta\Psi_m$:	electrochemical gradient
Å:	Angstrom
ADMET:	absorption, distribution, metabolism, excretion and toxicology
ATQ-R:	atovaquone-resistant
ATQ-S:	atovaquone-sensitive
BBB:	blood brain barrier
CADD:	computational aided drug discovery
CDC:	Centers for Disease Control and Prevention
CNS:	central Nervous System
CYP450:	cytochrome P450
cyt bc_1 :	cytochrome bc_1
DDT:	dichloro-diphenyl-trichloroethane
DEET:	N,N-diethyl-meta-toluamide
DMSO:	dimethylsulfoxide
<i>et al</i> :	et alia (and others)
ELQ:	endochin-like quinolone
ETC:	electron transport chain
FBS:	fetal bovine serum
GLUT1:	glucose transporter type 1
H-bond:	hydrogen bond
IC ₅₀ :	50% Inhibitory Concentration
IRS:	indoor Residual Spraying
ISP:	iron-sulphur protein
ITNs:	insecticide-treated mosquito nets
kDa:	kiloDalton
L:	Litre
LD ₅₀ :	50% lethal Dose

LogP:	Log partition coefficient
MD:	molecular dynamics
MM-GBSA:	molecular mechanics generalised-Born surface area
MMV:	medicine for malaria venture
µg:	microgram
µg/ml:	microgram per millilitre
mg:	milligram
mg/ml:	milligram per millilitre
µL:	microlitre
mL:	millilitre
µM:	micromolar
mM:	millimolar
ns:	nanoseconds
PBS:	phosphate buffered saline
PDB:	Protein Data Bank
<i>Pf</i> ATP4:	<i>Plasmodium falciparum</i> ATPase4
<i>Pf</i> HT:	<i>Plasmodium falciparum</i> hexose transporter
<i>Pf</i> PI3K:	<i>Plasmodium falciparum</i> phosphatidylinositol 3-kinase
<i>Pf</i> PI4K:	<i>Plasmodium falciparum</i> phosphatidylinositol 4-kinase
P-gp:	P-glycoprotein
PSAC:	plasmodial surface anion channel
RBCs:	red blood cells
RMSD:	root mean square deviation
RMSF:	root mean square fluctuation
SAR:	structure-activity relationship
SMILES:	simplified molecular input line entry system
TPSA:	topological polar surface area
v/v:	volume per volume
w/v:	weight per volume
XP:	extra precision
WHO:	World Health Organization
WT:	wild-type

CHAPTER 1

INTRODUCTION

1.1 Malaria as a global health concern

The word “malaria” has been derived from two Italian words ‘mala’ and ‘aria’, together which means bad air (Hempelmann and Krafts, 2013). It is a severe protozoal disease prevalent in 83 malaria-endemic countries resulting in 263 million malaria cases and 597,000 deaths in the year 2023, with five WHO African countries, namely Nigeria (26%), the Democratic Republic of Congo (13%), Uganda (5%), Ethiopia (4%) and Mozambique (4%), collectively contributing to more than 50% of the world malaria disease burden (**Figure 1.1**, Balmith et al., 2024; WHO, 2025). One of the major reasons for the increase in the number of malaria cases is the occurrence of resistance to the currently available chemotherapy and insecticides.

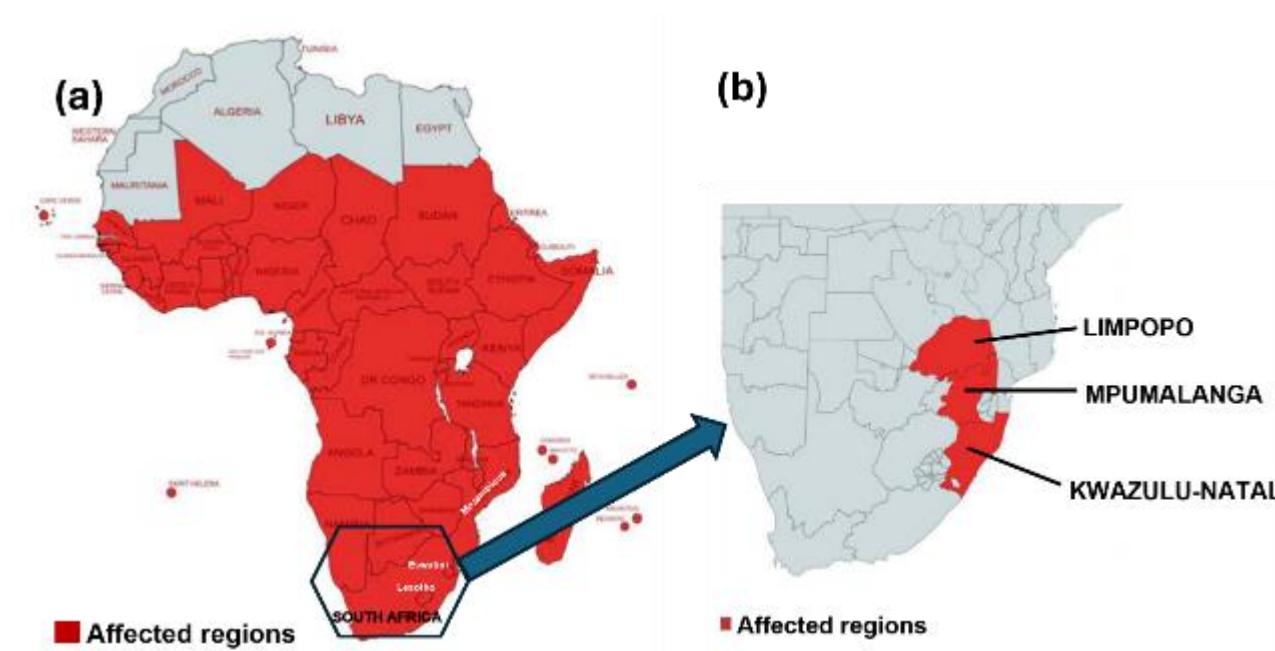


Figure 1.1:: (a) Malaria distribution in sub-Saharan Africa and (b) the malaria endemic regions in South Africa (Balmith et al., 2024).

The malaria parasite belongs to the phylum Apicomplexa and genus *Plasmodium*, with five major species infecting humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Among these, *P. falciparum* is the most virulent, responsible for severe malaria cases that can be fatal if

untreated. Areas that are mostly affected by predominantly *P. falciparum*, and to a lesser extent, *P. malariae*, *P. ovale* and *P. vivax*. in South Africa are present along the border of the country with Zimbabwe and Mozambique. More specifically, in districts of Mopani and Vhembe, districts of Capricorn and Greater Sekhukhune with few cases in Waterberg of Limpopo Province; Ehlanzeni district of Mpumalanga Province; uMknanyakude district, Zululand and districts of King Cetshwayo of Kwazulu-Natal province and Kruger National Park (**Figure 1.1**) (Balmith et al., 2024).

1.1.1 Brief History

Malaria is an ancient vector-borne disease affecting mammals, reptiles, and birds, indicating its early evolutionary origin (Carter and Mendis, 2002). References to malaria-like symptoms date back to ancient Egypt and Greece, where fever was linked to wet environments (Cunha and Cunha, 2008; Arrow et al., 2004a). The parasitic cause of malaria was discovered in 1880 by Charles Laveran, who identified pigmented granules in infected blood, earning him the 1907 Nobel Prize (Cox, 2010). Further advancements came in 1897 when Sir Ronald Ross demonstrated that *Anopheles* mosquitoes transmit malaria, leading to his Nobel Prize in 1902 (Sinden, 2007). His discovery paved the way for mosquito control strategies, including draining swamps and insecticide use, as well as the development of antimalarial drugs. Quinine, extracted from *Cinchona* bark, was historically significant, followed by synthetic drugs like chloroquine and artemisinin, which revolutionized malaria treatment (Arrow et al., 2004b; Greenwood et al., 2008; Hempelmann and Krafts, 2013; Drouin et al., 2024). Despite progress, malaria remains a major global health challenge, particularly in sub-Saharan Africa. Ongoing research focuses on novel drugs, vaccines, and innovative control strategies to combat the disease (WHO, 2025).

1.1.2 Transmission of malaria

Malaria is a vector borne disease transmitted through the bite of an infected female *Anopheles* mosquito. The mosquito acquires *Plasmodium* parasites when feeding on the blood of an infected person. After approximately a week, when the mosquito bites another person for the next meal, these parasites enter into its saliva and are injected into the bloodstream. Since *Plasmodium* parasites reside in red blood cells (RBCs), malaria can spread through blood transfusion, use of contaminated needles, organ transplant by an infected donor, and from an infected mother to her unborn child (congenital malaria) (Basu and Sahi, 2017; CDC, 2024a; Meibalan and Marti, 2017).

1.1.3 The life cycle of the malaria parasite

The *Plasmodium* parasite has a complex life cycle with two hosts: asexual phase takes place in the intermediate human hosts and sexual phase is completed in female *Anopheles* mosquitoes that act as definitive hosts (Meibalan and Marti, 2017) (**Figure 1.2**). Once sporozoites are released on the bite of infected female *Anopheles*, the parasite first invades the liver cells or the hepatocytes of the human host where it matures into merozoites. The merozoites enter the erythrocytic phase of their life cycle and infect RBCs. Here the parasite multiplies asexually by developing into ring, trophozoite and schizont stages, followed by lysis of the infected blood cells and release of merozoites that further infect other fresh RBCs. Some of the parasites enter the sexual stage and develop into male and female gametocytes, which upon ingestion during the blood meal of the female *Anopheles*, mate within the mosquito midgut. The zygotes undergo further development to produce sporozoites, a process that takes 10 to 18 days (Dixon et al., 2008). These sporozoites migrate to the salivary glands of the mosquito and as the female *Anopheles* bites a human to take the blood meal, the saliva along with sporozoites gets injected into the new host to re-infect (Cowman and Crabb, 2006). In this way, the female *Anopheles* acts as a vector and carries the disease from one human being to another, and itself remains unaffected during all this phase.

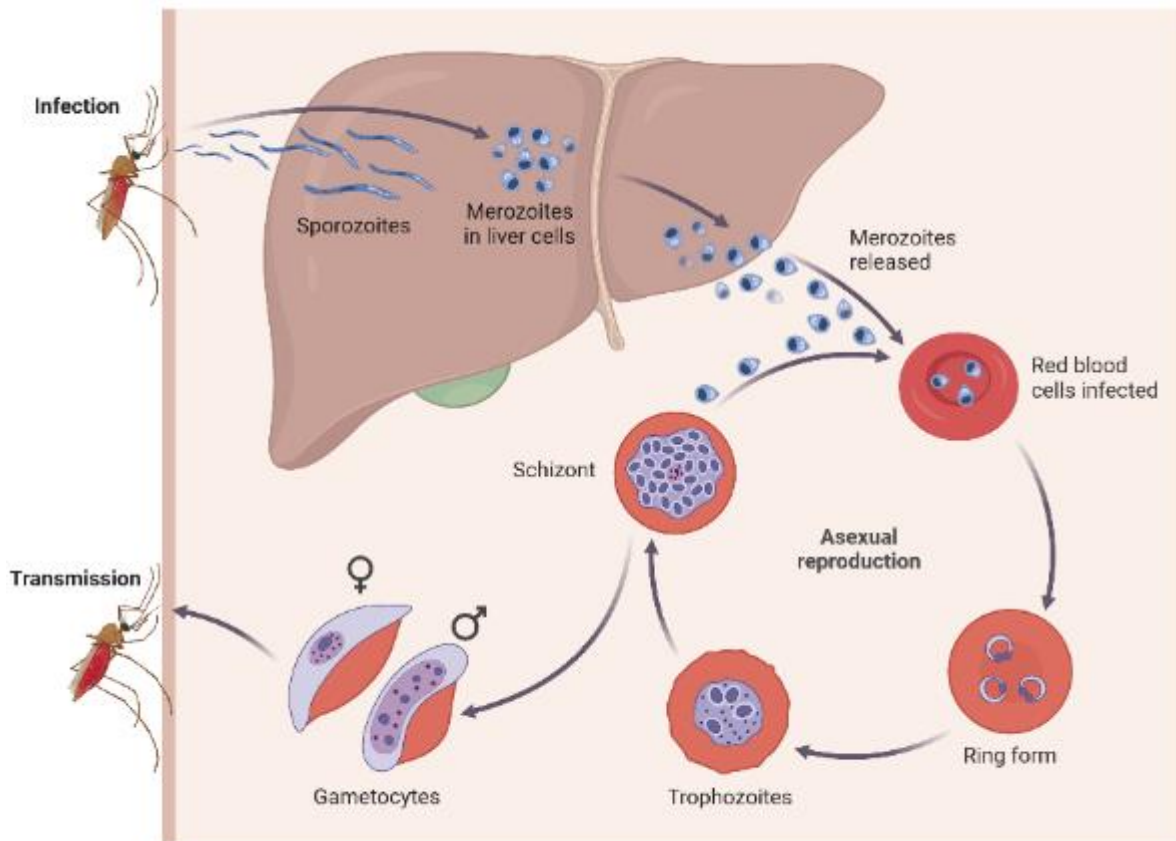


Figure 1.2: The life cycle of the malaria parasite in its human host (BioRender, 2025).

It is the blood stage or asexual erythrocytic parasites that are responsible for causing malaria symptoms. As the parasite grows in the red blood cells, many waste and toxic substances like haemozoin and glucose phosphate isomerase also accumulate in the cell. When the infected cells lyse and release merozoites in the bloodstream, these harmful and toxic substances are also released, which stimulates macrophages and other cells of the immune system to produce cytokines and other factors to produce symptoms (fever and chills) and other severe pathophysiological conditions associated with malaria (SA DOH, 2018; CDC, 2024b).

1.1.4 Symptoms of malaria

People infected with malaria parasite may display a wide variety of symptoms ranging from mild to severe, with some even asymptomatic. Malaria can be categorized into uncomplicated and complicated or severe disease. Symptoms begin to show up as early as 7 days to as late as a year. *P. vivax* and *P. ovale* can remain dormant for several years in the liver cells of the infected person and may reactivate to cause the disease months or years later. People infected with malaria develop

fever and flu-like symptoms that include chills, sweat, muscle pain, headache and tiredness. Some patients may also experience nausea, vomiting and diarrhoea (SA DOH, 2018). Since the parasite attacks red blood cells, the disease may also lead to anaemia and jaundice due to loss of blood cells. Additional clinical findings may also include thrombocytopaenia or decrease in blood platelets, elevation of bilirubin and elevation of aminotransferase (CDC, 2024b). If the condition is not treated well in time it may result in severe or complicated malaria symptoms that include seizures, mental confusion, acute kidney failures, coma, severe anaemia, haemoglobinuria or haemoglobin in the urine, acute respiratory distress syndrome due to inflammation in the lungs, low blood pressure, blood coagulation disorder, metabolic acidity or excessive acidity in the blood and tissue due to hypoglycaemia, and death. Such observations signal towards medical emergency and should be treated urgently (Mawson, 2013; Basu and Sahi, 2017; CDC, 2024b).

1.1.5 Diagnosis of malaria

The gold standard for confirming a malaria infection is the microscopic examination of blood smears to identify the presence of malaria parasites and determine the species (SA DOH, 2019). Rapid diagnostic tests (RDTs) are also commonly used in resource-limited settings to detect specific malaria antigens, providing a quick and reliable diagnosis. In addition, polymerase chain reaction (PCR)-based methods can detect and amplify the DNA of the malaria parasite, providing highly sensitive and specific results. PCR is particularly useful for detecting low-level parasitaemia or differentiating between *Plasmodium* species. Furthermore, serological assays detect specific antibodies produced by the body in response to a malaria infection, enabling the identification of past exposure to malaria and to differentiate between current and previous infections (Trampuz et al., 2003; Basu and Sahi, 2017).

1.1.6 People at higher risk to malaria

Malaria is not a contagious disease, but still anyone can be infected with malaria. People who live in or travel to malaria prone countries like the greater parts of the Africa continent, South and Southeast Asia, parts of Central and South America are at higher risk of infection. People living in malaria endemic countries develop partial immunity that reduces the risk of developing severe infection (Doolan et al., 2009). However, it does not provide complete protection. People with little or no immunity to malaria, like pregnant women, children under the age of 5 years and patients

with HIV/ AIDS are at a high risk of infection and mortality. People living in rural areas with little or no medical facility are also at a higher risk of infection (Abossie et al., 2020).

1.1.7 Reasons for increase in malaria cases

There are many factors, including human activities that pose tremendous challenges to address the issue of increased clinical cases of malaria and to successfully run malaria control programs if ultimate elimination of the disease is to be achieved. Some of these factors include:

- Population living in rural areas are economically poor and cannot afford preventive measure like indoor residual sprays, bed nets, mosquito repellents that can protect them from mosquito exposures (Gobir et al., 2014). They may also not know on how to recognize symptoms of malaria and treat it fast and correctly. Even if they recognize, they may be treating it with some traditional method which is not effective for treatment (Anyanwu et al., 2017).
- Lack of financial resources in malaria-endemic countries, lack of sufficient number of health care workers, training, supervision, lack of diagnostics equipment, medicines also add up to the increase in number of malaria cases (Macarayan et al., 2020).
- There may be people who are travelling from non-endemic countries and do not have awareness about the severity of this disease and thus may not be using any prophylactic medication or mosquito repellent (Agudelo Higueta et al., 2021; Failoc-Rojas et al., 2023).
- Developing areas for stagnant water within the house may lead to breeding grounds for mosquito larvae and thus increase in infections (Nabatanzi et al., 2022).
- Parasite is developing resistance against the currently used chemotherapy. Along with maintaining hygiene and vector control, there is a need to develop new antimalarial drugs that can overcome the drug-resistance. (Padmanaban, 2003; NICD, 2022).

1.2 Prevention and Treatment of Malaria

The risk of malaria infections can be greatly reduced by avoiding mosquito bites. Important measures include preventive (Section 1.2.1) and treatment (Section 1.2.2) methods, as both are crucial in the fight against malaria.

1.2.1 Preventive Measures

Preventive methods are essential in reducing the risk of malaria infection, especially in endemic regions. These include vector control strategies, chemoprophylaxis, environmental management or vaccination.

1.2.1.1 Vector control strategies

It aims to reduce mosquito populations and prevent mosquito bites. These include using mosquito repellents (containing N,N-diethyl-meta-toluamide (DEET), IR3535 or icaridin) in the evening, wearing long-sleeved clothes when outdoors, sleeping under insecticide-treated mosquito nets (ITNs) containing pyrethroids, using coils, vaporizers or insect sprays such as dichloro-diphenyl-trichloroethane (DDT), using window screens, and indoor residual spraying (IRS) (**Figure 1.3**) (WHO, 2019; SA DOH, 2019). Use of insects' predators like snails or larvivorous fish that can help to control mosquito larvae, use of insect traps that has got pheromones, use of genetically modifies mosquitoes that are unable to get infected with *Plasmodium* parasite.

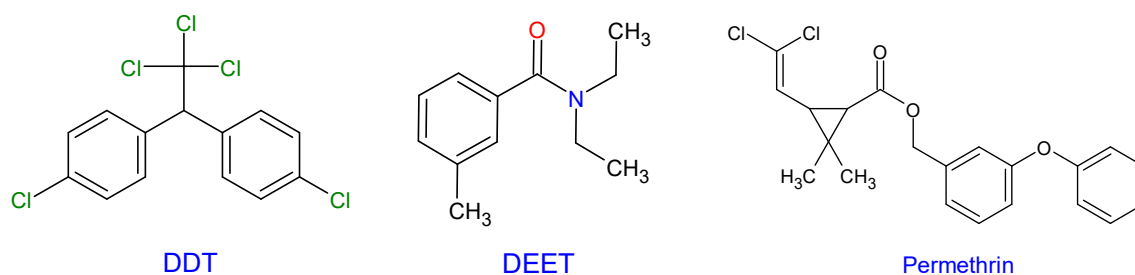
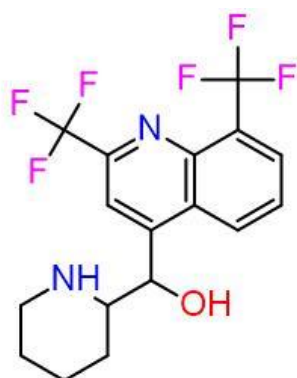


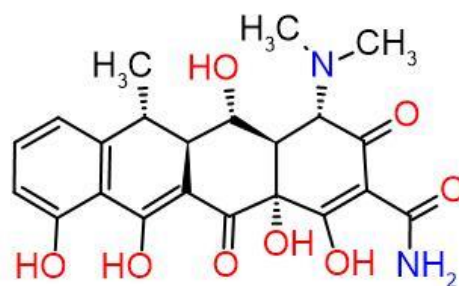
Figure 1.3: Chemical structures of the insect repellents used as preventative measures against malaria (Structure taken from NCBI PubChem 2025a, 2025b, 2025c) .

1.2.1.2 Chemoprophylaxis

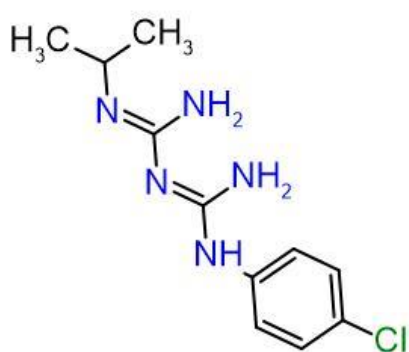
It involves taking antimalarial medications to prevent malaria infection before, during, and after travel to malaria-endemic areas. The recommended prophylactic drugs as per the South African Department of Health include atovaquone-proguanil, doxycycline and mefloquine (**Figure 1.4**) (DeVos and Dunn, 2023). The choice of chemoprophylactic drugs depends on the specific region and the drug resistance patterns of the local malaria parasites (CDC, 2024c; WHO, 2021).



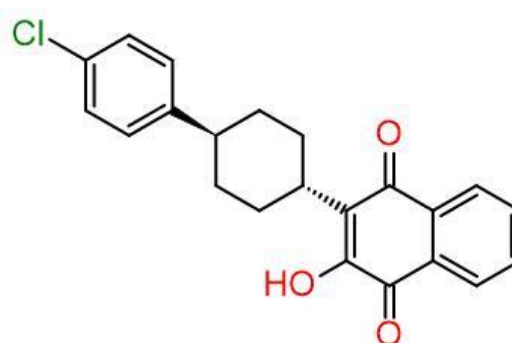
Mefloquine



Doxycycline



Proguanil



Atovaquone

Figure 1.4: Chemical structures of the antimalarial prophylactic drugs recommended for use in South Africa (SA DOH, 2019).

- **Environmental Management:** Environmental management techniques, such as larval source management, focus on eliminating or modifying mosquito breeding sites to reduce mosquito populations (Agyemang-Badu et al., 2023).
- **Vaccination:** The development of an effective malaria vaccine is an ongoing area of research. The RTS,S/AS01 (RTS,S) malaria vaccine (Mosquirix™) has been approved for use in some countries for use in children according to the WHO recommendation for its broad use among children living in regions with moderate to high *P. falciparum* malaria transmission (Laurens, 2019; Praet et al., 2022).

1.2.2 Treatment Measures

Treatment measures focus on treating individuals already infected with malaria to alleviate symptoms, prevent complications, and reduce transmission. This is achieved by killing the intra-erythrocytic parasite and inhibiting transmission by antimalarial drugs and to ensure patients receive supportive care:

- **Antimalarial Drugs:** Use of antimalarial drugs vary depending on the *Plasmodium* species and the drug resistance patterns in the specific region. In South Africa, common antimalarial drugs used to treat a malaria infection include artemisinin-based combination therapies (ACTs) combined with lumefantrine or quinine combined with clindamycin when the ACT combination is not available (SA DOH, 2019). The chemical structures of these drugs are shown in **Figure 1.5**. Chloroquine is effective in the treatment of chloroquine-sensitive non-*falciparum* species areas (SA DOH, 2019).
- **Supportive Care:** In severe cases of malaria, supportive care is essential to manage complications and provide symptomatic relief. This may include intravenous fluids to maintain hydration, treatment of anaemia, and management of complications such as respiratory distress or organ dysfunction (SA DOH, 2019).

1.2.2.1 Chemotherapeutic Management of Malaria

Three classes of drugs are considered for treatment depending on the growth stage of the parasite they target:

- **Targeting liver stage parasites** (tissue schizonticides) - these act against parasites that are dormant or developing in the liver and prevent invasion of red blood cells by the parasite. It is useful to treat or control relapse of malaria caused by *P. vivax* and *P. ovale*. Drugs used to control parasites at this stage include - primaquine, tafenoquine, pyrimethamine, cycloguanil, atovaquone (Siqueira-Neto et al., 2023).

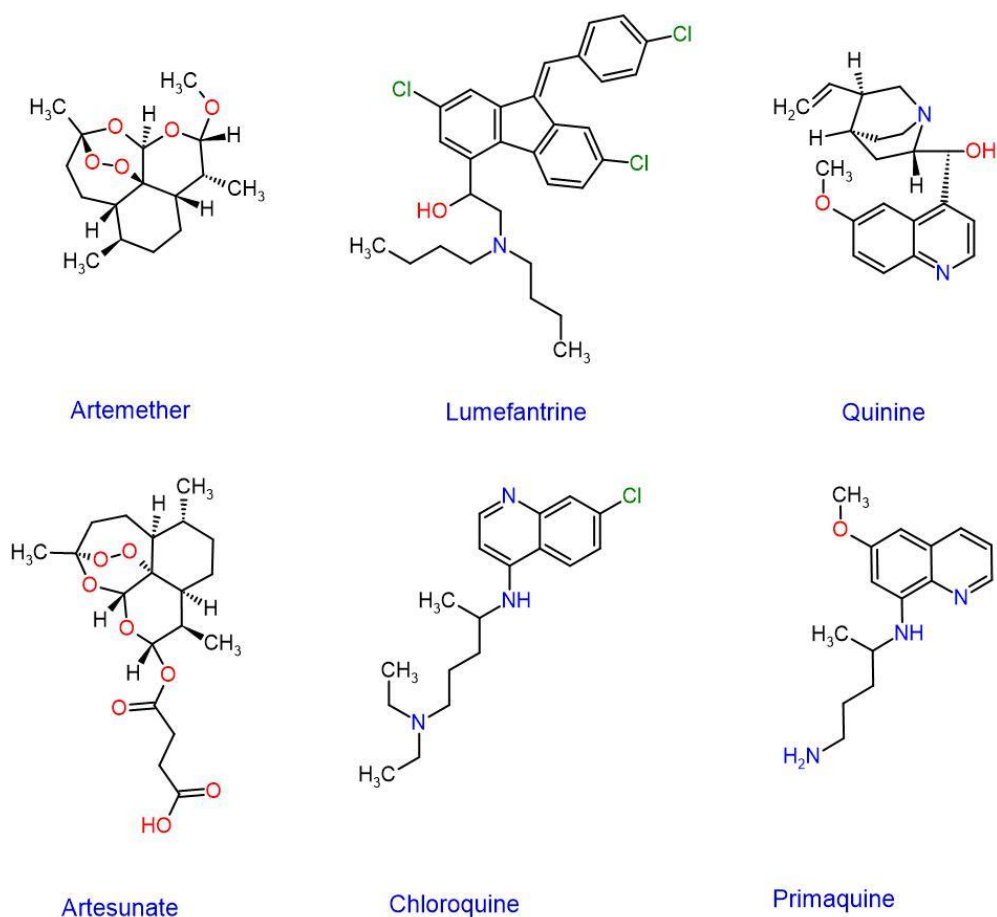


Figure 1.5: Chemical structures of the antimalarial drugs recommended for use in the treatment of a malaria infection in South Africa (SA DOH, 2019).

- **Targeting blood-stage parasites** (blood schizonticides) – these act against the asexual erythrocytic forms of the parasite and used in treating or controlling malaria symptoms. Drugs effective at this stage include - artemisinin derivatives (artesunate, artemether, dihydroartemisinin), lumefantrine, piperazine, pyronaridine, amodiaquine, mefloquine, sulfadoxine-pyrimethamine, cycloguanil, atovaquone, quinine, chloroquine (Siqueira-Neto et al., 2023)
- **Targeting sexual stages of parasites** (gametocides) - these act against the sexual stage of parasite development and prevent transmission of the gametes to the mosquito vector. Drug used at this stage is artemisinin derivatives and primaquine (SA DOH, 2019; Siqueira-Neto et al., 2023).

Malaria treatment has evolved significantly over time, particularly in the management of *P. falciparum*. Historically, quinine, derived from the *Cinchona* tree, was the primary treatment for *P.*

falciparum malaria and remained in use for centuries (Achan et al., 2011). In the mid-20th century, chloroquine became the first-line treatment due to its efficacy, affordability, and ability to clear blood-stage parasites. However, widespread chloroquine resistance, first reported in Southeast Asia and later in Africa and South America, led to its failure as an effective treatment (White, 2004). This prompted the adoption of sulfadoxine-pyrimethamine, but resistance rapidly emerged limiting its use (Bloland, 2001).

To address these challenges, newer drugs such as mefloquine, halofantrine, and amodiaquine were introduced, but resistance developed against these as well (Dondorp et al., 2009). The discovery of artemisinin, derived from *Artemisia annua*, revolutionized malaria treatment. Artemisinin and its derivatives, such as artesunate, artemether, and dihydroartemisinin, became the foundation for artemisinin-based combination therapies (ACTs), now the global standard for *P. falciparum* malaria (WHO, 2024). Common ACTs include artemether-lumefantrine, artesunate-mefloquine, artesunate-amodiaquine, and dihydroartemisinin-piperaquine (Dondorp et al., 2009). However, artemisinin resistance has emerged in Southeast Asia, characterized by delayed parasite clearance, and has now been detected in parts of Africa (Balikagala et al., 2021). Additionally, resistance to ACT partner drugs such as piperaquine and mefloquine has further complicated treatment strategies (WHO, 2024).

According to the WHO there are several medicines still available for use against malaria (WHO, 2024); and of these a limited number deemed ideal for the South African population recommended by the South Africa Department of Health (**Table 1.1**), with the others used throughout Africa and the rest of malaria endemic countries (Tse et al., 2019; WHO, 2024). Currently there have been no reports of atovaquone or ACT resistance in South Africa (Jaishree Raman, personal communication, 2023), other than the historical resistance to chloroquine, pyrimethamine and sulfadoxine against *P. falciparum*.

With the development of resistance spreading across the globe it has become imperative to better understand the malaria parasite and identify new targets or understand how the parasite has developed resistance to the drug. In doing so new targets and drugs can be designed and evaluated.

1.3 Drug targets within *Plasmodium*

Currently, most of the research done in the field of antimalarials aim at optimization of already known drugs that target the validated pathways. These antimalarial drugs can be classified based on their mechanism of action (**Table 1.1**) (Lunev et al., 2016).

Table 1.1: The antimalarial drugs recommended in South Africa - used individually or in combination (SA DOH, 2019; Tse et al., 2019; Alaithan et al., 2023; Knox et al., 2024; WHO, 2024).

Name	Mode of action	Use	References
Mefloquine	Acts primarily through disruption of haemoglobin digestion. Targets 80S ribosomes of the parasite thus inhibiting protein synthesis.	No longer widely used due to CNS toxicity. WHO recommended for use during pregnancy.	Knox et al., 2024
Atovaquone	Inhibits electron transfer in the mitochondria, thus blocking cellular respiration	To prevent malaria, atovaquone is used in combination with proguanil in a 2.5:1 ratio.	Knox et al., 2024
Proguanil	Inhibits the dihydrofolate reductase thereby blocks the biosynthesis of purines and pyrimidines essential for DNA synthesis		
Doxycycline	Inhibits protein synthesis	Used prophylactically in all but pregnant or children <8yrs. Used in combination with quinine to treat acute uncomplicated <i>P. falciparum</i> malaria	(SA DOH, 2019)
Artemether	Interacts with ferriprotoporphyrin IX or ferrous ions in food vacuole of the parasite resulting in generation of cytotoxic radical species	Artemether is used in combination with lumefantrine in a 1:6 ratio to treat acute uncomplicated <i>P. falciparum</i> malaria	Knox et al., 2024
Lumefantrine	Inhibits formation of beta-hematin, nucleic acid and protein synthesis		
Quinine	Interferes with haemoglobin digestion.	Used for treatment of uncomplicated <i>P. falciparum</i> malaria. Quinine therapy initiated with clindamycin given 2 days later.	Alaithan et al., 2023
Clindamycin	Inhibits protein synthesis		
Artesunate	Metabolised to active dihydroartemisinin that react	An artemisinin derivative used to treat severe malaria and	WHO, 2024

	with haem, generating free radicals that inhibit protein and nucleic acid synthesis	administered by IV until patient able to swallow tablets (artemether-lumefantrine)	
Chloroquine	Prevents the conversion of haem to inert haemozoin.	Recommended in areas where <i>P. falciparum</i> parasite strains are still sensitive to chloroquine. Recommended for use against <i>P. vivax</i> , <i>P. malariae</i> , and <i>P. ovale</i> .	Knox et al., 2024
Primaquine	Acts by generating reactive oxygen species. Also proposed to interfere with electron transport system of the parasite.	Following artemether-lumefantrine treatment, given to eradicate <i>P. vivax</i> and <i>P. ovale</i> . To prevent transmission of <i>P. falciparum</i> gametocytes.	Knox et al., 2024; SA DOH, 2019

1.3.1 *Plasmodium* drug targets

The continued emergence of drug-resistant malaria strains, combined with the limitations of existing treatments, highlights the urgent need for novel antimalarial drugs with new mechanisms of action, improved safety profiles, and efficacy against all malaria species, including resistant strains. The following sections explore some of the novel targets identified, highlighting their biological roles, therapeutic potential and current status in antimalarial drug discovery.

1.3.1.1 Proteases

Plasmodium protease enzymes catalyse the hydrolysis of peptide bonds that further help in cell or tissue penetration, invasion of immune system, erythrocytes, haemoglobin, breakdown of other proteins, activation of inflammation, autophagy that supports the development of the parasite. Therefore, proteases are promising targets as inhibition of these genes can inhibit haemoglobin degradation inducing parasite death (Roy, 2017; Verma et al., 2016). Some of the malarial proteases include glutamate, aspartate, cysteine, metallo, serine and threonine (Belete, 2020).

1.3.1.2 Protein kinases: *Plasmodium falciparum* Phosphatidylinositol 4-kinase (PfPI4K)

Phosphoinositide kinases or PIKs are enzymes that phosphorylate lipids and regulate proliferation, survival, trafficking and intracellular signalling. Inhibition of PfPI3K and PfPI4K have been identified for the development of antimalarial drugs that can help in the prevention, treatment and

elimination of malaria. Examples include UCT943, imidazopyrazines (KAF156) and aminopyridine (also MMV048 (Tse et al., 2019)) which target *Pf*PI4K and inhibit the development of *Plasmodium* at every stage of infection (McNamara et al., 2013; Brunschwig et al., 2018; Tse et al., 2019).

1.3.1.3 Targeting Transporters

Once the parasite has invaded the red blood cell, it requires large quantities of various substrates to obtain energy for its growth and development. Special transporters are induced by the *Plasmodium* parasite into the host RBC membranes for the uptake and removal of metabolites, making these transporters potential targets. Examples of such transporters include plasmodial surface anion channels (PSAC) that play an important role by providing nutrients (hypoxanthine, cysteine, glutamine, glutamate, isoleucine, methionine, proline, tyrosine, pantothenic acid and choline) to the intracellular parasites. Drugs like phlorizin, dantrolene, furosemide and niflumate block anion transport; while tolbutamide, meglitinide and glibenclamide specifically inhibit the choline uptake into infected RBCs (Desai, 2004; Belete, 2020).

- **Sugar transporter**

Blood stages of the parasite depend on anaerobic glycolysis for its growth and development. It converts pyruvate to lactate to produce ATP which is required for replication inside the erythrocyte. Glucose is transported inside the parasitized RBCs with the help of host cells's glucose transporter (GLUT1) and parasite-induced 'New permeability pathway'. The glucose is then transported into the parasite via *P. falciparum* hexose transporter (*Pf*HT). GLUT1 transports D-glucose, whereas *Pf*HT transports D-glucose and D-fructose. Due to differences between GLUT1 and *Pf*HT in terms of their interactions with the substrate, it has been proposed that the selective inhibition of *Pf*HT is a novel target (Slavic et al., 2011; Belete, 2020).

- **Lactate transporter**

The parasitised RBCs take up glucose 100 times faster as compared to the uninfected RBCs. Glucose is metabolized into lactic acid by the process of glycolysis in the parasite which is then exported from the parasite by the lactate:H⁺ symport mechanism into the external environment. Both glucose uptake and lactate export are essential for the energy needs, intracellular pH and osmotic stability

of the parasite. Thus, this lactate:H⁺ symport transport system is a promising target to develop novel drugs. Examples include pentafluoro-3-hydroxy-pent-2-en-1-ones, MMV007839 and MMV000972 (Belete, 2020; Nerlich et al., 2021; Davies et al., 2023).

- **Choline transport**

Phospholipids are essential for the growth and multiplication of the intra-erythrocytic parasite where they are structural components of membranes and regulate several enzymatic activities. The parasite synthesizes phosphatidylcholine by a *de novo* process using choline as a precursor. Inhibition of choline transport into the parasite, inhibits the biosynthesis of phosphatidylcholine leading to parasite death. Examples of choline transport inhibitors include albitiazolium that blocks the uptake of choline into the parasite. Similarly, the bisquaternary ammonium compound G25 and bis-cationic compound T3 interfere with phosphatidylcholine synthesis in the parasite (Peyrottes et al., 2012; Wein et al., 2012; Belete, 2020).

1.3.1.4 ATPases

- **P-type Na⁺ *Plasmodium falciparum* ATPase (PfATP4)**

In general, erythrocytes maintain a low internal Na⁺ level. The parasite increases the permeability of the erythrocyte cell membrane and facilitates the entry of Na⁺ that results in an increase in Na⁺ concentration to a level of the extracellular medium. The parasite finds itself in high Na⁺ medium and must efflux Na⁺ ions across its plasma membrane to keep low cytoplasmic Na⁺ level for survival. P-type Na⁺ *P. falciparum* ATPase4 transporter (*PfATP4*) serves as the parasite's Na⁺ efflux pump. Inhibition of this transporter increases Na⁺ levels leading to parasite death. Examples of such inhibitors include cipargamin (also known as NITD609 / KAE609) and (+)-SJ733 (Spillman and Kirk, 2015; Zagórska and Jaromin, 2023).

- **V-type H⁺-ATPase**

Similarly, the parasite also imports H⁺. To regulate the increasing concentration of these H⁺ and to stabilize the intracellular pH at 7.3, the parasite uses V-type ATPase transporter to efflux H⁺. MMV253 works by inhibiting V-type H⁺ ATPase (Beyenbach and Wieczorek, 2006; van Schalkwyk et al., 2010; Ashley and Phyo, 2018; Alaithan et al., 2023).

1.3.1.5 Aquaporin-3

Aquaporin-3 is an aquaglyceroporin that facilitates the movement of water and glycerol in host cells. Aquaporin-3 is induced in the hepatocytes in response to parasite infection and provides a pathway for the entry of glycerol into the parasite, thus playing a significant role in the replication of the parasite. Auphen reduces the intra-erythrocytic *P. falciparum* parasitaemia and *P. berghei* in the hepatic stage (Posfai et al., 2018; Alaithan et al., 2023).

1.3.1.6 Dihydroorotate dehydrogenase

Pyrimidine nucleotides are essential for the synthesis of DNA, phospholipids and glycoproteins and thus for the growth and replication of parasite. Human cells can synthesize pyrimidine either by salvage or by *de novo* process, but *Plasmodium* depends solely on the *de novo* process and inhibition of this process results in no pyrimidine for DNA replication. Dihydroorotate dehydrogenase is an important enzyme that catalyzes the oxidation of dihydroorotate to orotate, and is involved in a rate limiting step of *de novo* pyrimidine synthesis. Thus dihydroorotate dehydrogenase forms a potential target by inhibiting the synthesis of an essential nucleotide pyrimidine. Examples of dihydroorotate dehydrogenase inhibitors include DSM190 and DSM265 (Belete, 2020; Alzain et al., 2022; Gehlot and Vyas, 2023).

1.3.1.7 Isoprenoid biosynthesis

Isoprenoids are a class of hydrocarbons derived from the sequential assembly of two 5-carbon isomers – isopentenyl pyrophosphate and dimethylallyl pyrophosphate, that are used for tRNA isopentenylation and protein prenylation. It also helps in the synthesis of vitamin E, carotenoids, ubiquinone and dolichols. Isoprenoid is synthesized via methylerythritol phosphate pathway or 1-deoxy-D-xylulose 5-phosphate pathway that converts glyceraldehyde 3-phosphate and pyruvate to IPP and DMAPP and is an attractive target for novel antimalarials. Examples include phosphonic acid fosmidomycin, MMV019313 and MMV008138 inhibits deoxy-D-xylulose 5-phosphate reductoisomerase that play an important role in DOXP pathway (Guggisberg et al., 2014; Belete, 2020).

1.3.1.8 Farnesyl transferase inhibitor

Protein farnesyl transferase has emerged as a potent target for malaria treatment. It catalyzes the transfer of the farnesyl group which is a 15-carbon isoprenoid lipid unit, from farnesyl pyrophosphate to the C-terminus of proteins containing the CAAX motif. Inhibition of this enzyme kills the parasite. Protein farnesyl transferase inhibitors are well tolerated in humans, but are highly cytotoxic to the parasite. Examples: R115777, BMS-388,891 (Nallan et al., 2005; Belete, 2020).

1.3.1.9 *P. falciparum* translational elongation factor 2 inhibitor

P. falciparum elongation factor 2 (PfEF2) promotes the GTP-dependent translocation of the ribosome along messenger RNA, and is important for protein synthesis in eukaryotes. PfEF2 is isolated as a novel target for antimalarial drug development. Cabamiquine (previously known as M5717 or DDD107498) is a selective inhibitor of 80S ribosome interacting PfEF2 (Baragaña et al., 2015; McCarthy et al., 2021; MMV, 2023).

1.3.1.10 Anti-adhesive polysaccharide

The key characteristics of severe malaria include the sequestration of erythrocytes infected with parasites, inflammatory responses, and microvascular obstruction. The *P. falciparum* parasite employs heparan sulfate for adherence to endothelial cells and blood cells, leading to blockages in blood flow. The use of drugs to inhibit these abnormal cell interactions restores impaired blood flow and impedes parasite growth. Sevuparin, a polysaccharide agent derived from heparin, serves as an antiadhesive agent. It hinders merozoite invasion of erythrocytes, prevents infected erythrocyte binding to both uninfected and infected cells, and inhibits binding to vascular epithelial cells (Belete, 2020; Oleinikov, 2022; Shibeshi et al., 2020).

1.3.1.11 Lipid metabolism as novel target

with the completion of *P. falciparum* genome, it was found that the parasite contains a Fatty Acid Synthase (FAS) for the preparation of fatty acids (Gardner et al., 2002). Fatty acids serve vital functions as both building blocks for biological membranes and a significant source of metabolic energy. While humans utilize a type I fatty acid synthase (FAS-I) to synthesize fatty acids, *Plasmodium* parasites employ a distinct type II system (FAS-II) for the same purpose. The divergence

in fatty acid synthesis pathways between the human host and the parasite suggests a potential focal point for the development of novel antimalarial interventions (Ben Mamoun et al., 2010).

Of all of these targets, the electron transport chain (ETC) operating in plasmodial mitochondria has emerged as a critical target for antimalarial drug development due to its essential role in maintaining mitochondrial function and parasite survival. Among its key components, the cytochrome *bc*₁ (cyt *bc*₁) is responsible for electron transfer, sustaining ATP synthesis and pyrimidine biosynthesis, making it a vulnerable target for therapeutic intervention and focus of this study.

1.3.1.12 Mitochondrial Electron Transport Chain

The ETC operating in plasmodial mitochondria is composed of non-proton motive quinone reductases and proton motive respiratory complexes. The non-proton motive quinone reductases include dihydroorotate dehydrogenase (DHODH), malate quinone oxidoreductase (MQO), glycerol 3-phosphate dehydrogenase (G3PDH), type II NADH dehydrogenase (complex I) and succinate dehydrogenase (SDH / complex II). The proton motive respiratory complex includes cytochrome *bc*₁ complex (Complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V). Ubiquinone (coenzyme Q) and cytochrome c1 work as electron carriers between different complexes within the ETC. (Lunev et al., 2016). Drugs like atovaquone and proguanil target mitochondrial ETC of the parasite at the cytochrome *bc*₁ level, disrupting its ability to produce energy in the form of ATP and eventually leading to its death. Proguanil was first reported in 1945 and is a prodrug (Tse et al., 2019). Its active metabolite is known as cycloguanil which acts as a dihydrofolate reductase inhibitor. Atovaquone was first reported in 1991, but the parasite has developed resistance against it (Korsinczky et al., 2000). So, now a fixed-dose combination of proguanil and atovaquone (Malarone®) is used (Nixon et al., 2013a).

1.3.2 Targeting *Plasmodium* mitochondria

Mitochondria are one of the cellular organelles found in most eukaryotes, and play an important role in many biological processes like production of biosynthetic intermediates, energy transduction, and programmed cell death or apoptosis (Nunnari and Suomalainen, 2012). All these functions contribute to the maintenance of equilibrium and survival of the cell.

1.3.2.1 Structural differences between mitochondria of *Plasmodium* and vertebrates

There are differences between the mitochondria of malarial parasite *Plasmodium* and its host human that serve as an important criteria of drug target selectivity. As reported in the literature (Hikosaka et al., 2011, 2015a, 2015b), the vertebrate mitochondrion is made up of two separate membranes: an inner and an outer membrane. The inner membrane is folded into cristae and differs functionally from the outer membrane. The mitochondria also contain their own genome (mtDNA). In general, the vertebrate mtDNA is about 16 kb in size, and encodes for thirty-seven genes: two of which are for ribosomal RNAs (rRNAs), thirteen for proteins, and twenty-two for transfer RNAs (tRNAs) (Boore, 1999). Whereas, the mitochondrion in *Plasmodium* is tubular in structure possessing the smallest eukaryotic mtDNA (6-kb), it encodes for only three genes for proteins and rRNA (Das et al., 1997; Fisher et al., 2014; Hikosaka et al., 2011; Vaidya et al., 1989, 1993). Due to these significant differences between the parasite and its analogous mammalian mitochondria and its essentiality for the parasite survival, the mitochondrion has proven to be a valid and promising target to move forward on the way to develop novel antimalarial drugs.

The normal physiology of the mitochondrion includes the tricarboxylic acid (TCA) cycle, *de novo* pyrimidine synthesis and the electron transport chain (ETC). The erythrocytic stage of the parasite depends mainly on glycolysis for their energy supply, and the mitochondrial electron transport chain (mtETC) is maintained to serve for the regeneration of ubiquinone which acts as an electron acceptor for dihydroorotate dehydrogenase enzyme essential for biosynthesis of pyrimidine (Gutteridge et al., 1979; Painter et al., 2007). The electron transport chain (ETC) of *Plasmodium* contains five key enzymes, namely, NADH:ubiquinone oxidoreductase (Complex I or PfNDH2), succinate:ubiquinone oxidoreductase (Complex II or SDH) or succinate dehydrogenase, glycerol-3-phosphate dehydrogenase (G3PDH), malate:quinone oxidoreductase (MQO), and dihydroorotate dehydrogenase (DHODH) (Fisher et al., 2014). Along with these, *bc*₁ (cytochrome *bc*₁ complex), cytochrome c, aa3 cytochrome c oxidase, and F1Fo-ATPase (complex V) are also components of ETC. All these five dehydrogenase enzymes generate reduced coenzyme Q (CoQH₂) or ubiquinol, and serve partially to provide electrons to the downstream complexes of the ETC, which comprises of the following (**Figure 1.1**):

- a) Ubiquinol:cytochrome c oxidoreductase (complex III or *cytochrome bc*₁ complex)
- b) Cytochrome c oxidase (complex IV) with ubiquinone (CoQ)
- c) Cytochrome c, that acts as an electron carrier from one complex to another (complex III to IV)
- d) The ATP synthase (or complex V) (Vaidya and Mather, 2009).

1.3.2.2 Cytochrome *bc*₁ complex

The mitochondrial enzyme cytochrome *bc*₁ complex (EC 1.10.2.2), also known as ubiquinol:cytochrome 1 oxidoreductase or complex III forms an important component of the respiratory chain and is responsible for oxidation of ubiquinol to ubiquinone (Figure 1.6) (Vaidya and Mather, 2005) and catalytic transfer of electrons that maintains mitochondrial membrane potential (Kessl et al., 2003). It is a dimeric multi-subunit complex that is composed of up to 11 subunits in eukaryotes (Kessl et al., 2007). The catalytic core of cytochrome *bc*₁ complex constitutes of three subunits participating in electron transfer pathway, namely Cytochrome b (43 kDa), Cytochrome c₁ (27 kDa) and Reiske iron-sulfur protein ([2Fe2S] ISP, 21 kDa) (Nixon et al., 2013b). The *bc*₁ complex catalyses transfer of electrons from ubiquinol to cytochrome c and this transfer is coupled with vectorial translocation of protons from mitochondrial matrix to the intermembrane space. This generates transmembrane electrochemical gradient ($\Delta\Psi_m$). The series of oxidation and reduction reactions is known as Q-cycle (Mitchell, 1975).

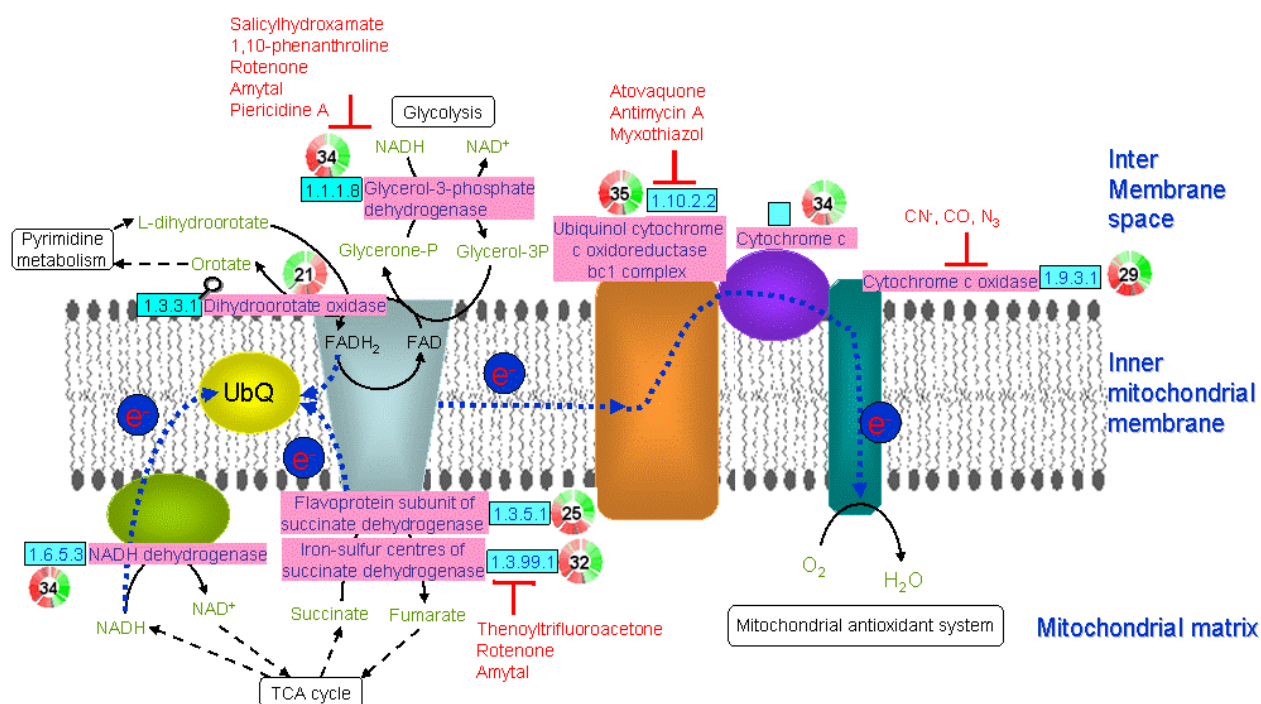


Figure 1.6: *Plasmodium* mitochondrial electron flow with targets of known inhibitors, where atovaquone inhibits ubiquinol cytochrome c oxidoreductase *bc*₁ complex (1.10.2.2) (Ginsburg, 2020).

According to the crystal structures, each monomer of this dimeric unit has two distinct sites that can bind to both quinone and quinol. The *Q*_i site is in the core of cytochrome b and topographically proximal to the matrix side of the inner mitochondrial membrane, whereas the *Q*_o site is located at

the interface of cytochrome *b* and the Rieske protein proximal to the outer surface of the inner membrane (Kessl et al., 2007). The catalytic properties and physico-chemical behaviour of these two sites have been studied extensively as they bind to different classes of compounds. Crystal structures of the cytochrome *bc*₁ complex liganded with *Q*_o-site inhibitors stigmatellin, myxothiazol, heptylhydroxydioxobenzothiazole and famoxadome are available displaying their binding modes (Esser et al., 2004; Gao et al., 2002; Hunte et al., 2000; Palsdottir et al., 2003).

1.3.3 Mitochondrial and CoQ inhibitors

Wellcome Research Laboratories investigated a hydroxynaphthoquinone compound lapinone in 1980s as an antimalarial agent (Nixon et al., 2013a). Their efforts resulted in the development of atovaquone or 2-(trans-4-(p-chlorophenyl)cyclohexyl)-3-hydroxy-1,4-naphthoquinone (**Figure 1.6**), which is a hydroxynaphthoquinone analogue of ubiquinone and acts as an inhibitor of cytochrome *bc*₁ complex (Fry and Pudney, 1992; Mather et al., 2005). It inhibits the mitochondrial electron transport chain at the cytochrome *bc*₁ complex level and also collapses the mitochondrial membrane potential across the mitochondrial inner membrane ($\Delta\Psi_m$) in the malarial parasite (Biagini et al., 2006; Srivastava et al., 1997). It was first developed in 1990s and approved by the FDA in 1995 (Kessl et al., 2005; Nixon et al., 2013a; Nixon et al., 2013b)

Atovaquone, which is a competitive inhibitor of *Q*_o binding site (**Figure 1.6**), is primarily used as a prophylactic agent against malaria (Kessl et al., 2003, 2005). It is well tolerated, metabolically stable, and a very effective broad-spectrum antiparasitic agent (Baggish and Hill, 2002). It is also used against *Pneumocystis jirovecii* (*carinii*) pneumonia (Hughes et al., 1993) and *Toxoplasma gondii* toxoplasmosis in patients with AIDS (Castro and Morrison-Bryant, 2010; Kovacs and Program, 1992; Rossiter, 2022). Structural features that contribute to the antiparasitic activity seems to be the presence of hydroxyl group at position 3 and cyclohexyl-chlorophenyl side chain at position 2 of the naphthoquinone ring (Hudson, 1993; Nixon et al., 2013a). Additionally, only the trans configuration of the cyclohexyl-chlorophenyl side chain has the potential to inhibit cyt *bc*₁ complex, whereas the cis configuration has been reported to be inactive (Fry and Pudney, 1992; Srivastava et al., 1999a). Atovaquone is recommended for paediatric use and is considered safe for treating malaria during pregnancy, as it poses no harm to the mother or fetus (Hughes et al., 2005; McGready et al., 2005; Minodier et al., 2005; Na-Bangchang et al., 2005).

Atovaquone is a lipophilic drug with low bioavailability which increases 4-fold when taken with fatty food. Its poor absorption is also a contributing factor to treatment failure in the absence of parasite

resistance (Musset et al., 2006). It is found to be highly plasma protein bound (99.9%, due to which the concentration of unbound drug is significantly low) and has a long half-life (2.2 to 3.2 days). Elimination of this drug is primarily via the liver, with over 90% excreted in the parent form in bile. It is generally well tolerated, but may cause few side effects including mild fever, vomiting, diarrhoea, abdominal pain and headache (Rossiter, 2022). The drug's lack of sensitivity to the mammalian *bc*₁ complex supports its long term low toxicity in humans (Petersen, 2003).

1.3.3.1 Atovaquone-Proguanil combination

Atovaquone is a ubiquinone analogue that inhibits electron transfer of the respiratory chain by binding to cytochrome *b* of the *Plasmodium* (Fry and Pudney, 1992). Due to development of resistance to monotherapy, it is clinically used in combination with its synergistic partner proguanil hydrochloride under the trade name Malarone® (Canfield et al., 1995) and this combination proved to be a promising antimalarial (Looareesuwan et al., 1996). Proguanil is a prodrug that is converted to cycloguanil (a dihydrofolate reductase inhibitor) by human cytochrome P450. In combination with atovaquone, proguanil acts synergistically as a biguanide rather than the dihydrofolate reductase inhibitor, to enhance the atovaquone effect. The concurrent use of proguanil with atovaquone, lowers the effective concentration of atovaquone at which it collapses mitochondrial membrane potential (Srivastava and Vaidya, 1999b). This drug combination got registered in the North America and Europe in 1996 for the prophylaxis and treatment of malaria (Musset et al., 2006). The price of this drug combination in comparison of other antimalarial drugs is very high that makes its use restrictive in the resource deficit, endemic areas (Nixon et al., 2013a).

However, resistance has been reported not only to atovaquone monotherapy, but also to the atovaquone-proguanil combination, Malarone® (Fivelman et al., 2002; Wichmann et al., 2004). Malarone® failures have been found to be associated with point mutations at amino acid 268 of cytochrome *b* (Kessl et al., 2007). According to the studies (Srivastava et al., 1999a), the presence of proguanil along with atovaquone in the drug combination did not affect the mitochondrial membrane collapse profile of atovaquone, which suggested that proguanil no longer acted as synergistic partner once the parasite developed atovaquone resistance. There could be multiple reasons for atovaquone-proguanil treatment failure that includes incorrect plasma drug dosage associated with day-of-failure parasites *in vitro* susceptible to atovaquone, failure due to resistance, an intermediate cause (Musset et al., 2006).

1.3.4 Atovaquone resistance

Atovaquone works by inhibiting the mitochondrial electron transport chain and collapsing the mitochondrial membrane potential, eventually killing the parasite by inhibiting its respiration (Fry and Pudney, 1992; Srivastava and Vaidya, 1999b). Unfortunately, the malarial parasite has developed resistance against atovaquone by mutating amino acid residues in or near the atovaquone-binding site in cytochrome *b* (Fivelman et al., 2002; Srivastava et al., 1999a).

Atovaquone resistance in *P. falciparum* has been observed to arise both *in vivo* and *in vitro* through point mutations in the mtDNA-encoded cytochrome *b* (Vaidya and Mather, 2000). Studies have shown that three point mutations at position 268 of the cytochrome *b*, namely Y268N, 268S, Y268C, are responsible for atovaquone resistance in *P. falciparum* (**Table 1.2**) (Fisher et al., 2012; Korsinczky et al., 2000; Musset et al., 2006), as these mutations alter the fitting and binding of the drug at its target site. The putative contact residues at the atovaquone-binding site in cytochrome *b* of *P. falciparum* are Ile¹¹⁹, Phe¹²³, Tyr¹²⁶, Met¹³³, Val¹⁴⁰, Ile¹⁴¹, Leu¹⁴⁴, Ile²⁵⁸, Pro²⁶⁰, Phe²⁶⁴, Phe²⁶⁷, Tyr²⁶⁸, Leu²⁷¹, Val²⁸⁴, Leu²⁸⁵, and Leu²⁸⁸ (Korsinczky et al., 2000).

Table 1.2: List of mutations identified in *P. falciparum*:

Mutation	Reference
Y268S in <i>P. falciparum</i>	(Musset et al., 2006)
Y268N in <i>P. falciparum</i>	
Y268C in <i>P. falciparum</i>	
S299N unlikely to cause atovaquone resistance as this position is in the inner mitochondrial membrane far from atovaquone binding site.	
I258M in <i>P. yoelli</i> and <i>P. falciparum</i> (I269M in yeast numbering)	(Kessl et al., 2007; Plucinski et al., 2014)
F267I in <i>P. yoelli</i> and <i>P. falciparum</i> (F278I in yeast)	(Kessl et al., 2007; Korsinczky et al., 2000)
L271V in <i>P. yoelli</i> and <i>P. falciparum</i> (L282V in yeast)	(Jacobsen et al., 2021; Korsinczky et al., 2000)
M133I (Methionine to isoleucine, in <i>P. falciparum</i>)	(Schwöbel et al., 2003)
L271F (At codon 271 in <i>P. falciparum</i> from leucine to phenylalanine)	
G280D in <i>P. falciparum</i>	(Peters et al., 2002; Schwöbel et al., 2003)

The amino acid change conferring atovaquone resistance Tyr²⁶⁸ (one of which is the main focus of our study) involved residue that is absolutely conserved in all cytochrome *b* (Srivastava et al., 1999a). Computational modelling was used to propose the mechanism of atovaquone resistance

(Akhoon et al., 2014). In the wild-type protein, Tyr²⁶⁸ is present within the atovaquone binding site. The tyrosine residue (Y) at the 268th position forms a hydrophobic contact with atovaquone at the Q_o oxidation site. However, substitution of tyrosine with the hydrophilic asparagine (N) at this position reduces the volume of the binding pocket and decreases the binding and affinity of atovaquone. Substitution of tyrosine with cysteine (C) also impairs atovaquone binding. Substitution of tyrosine with serine (S) results in a Y268S mutation, that diminishes the hydrophobic interaction between atovaquone and the binding site. Due to this mutation, an important histidine contact of atovaquone with the iron-sulfur protein (ISP) chain is lost.

Mutations at codon 268 of the *falciparum* parasite has been found to be associated with atovaquone/proguanil treatment failure *in vivo* (Schwöbel et al., 2003). Once the bulky Tyr²⁶⁸ is mutated to any of the three possible and less bulky amino acids, it alters the arrangement of the active site residues to shift far away from the drug, thus affecting the binding of the drug (Akhoon et al., 2014).

In addition to Y268S and Y268C, other mutations identified include M133I and L271F (Schwöbel et al., 2003), V284K, I258M, F267I and L271V (Kessl et al., 2005; Korsinczky et al., 2000). Currently, the South African Department of Health has not reported any resistance to atovaquone-proguanil in South Africa (SA DOH, 2018).

Since yeast cytochrome *bc*₁ complex is also inhibited by atovaquone, and has a high degree of sequence identity (about 68%) with *Plasmodium* cytochrome *b*, it is used as a model to study cytochrome *b* mutations that lead to atovaquone resistance (Kessl et al., 2003, 2005). Also, its crystal structure is available (Kessl et al., 2005; Solmaz and Hunte, 2008). Mutations I258M, F267I, Y268C, Y268S and L271V in *Plasmodium* numbering are equivalent to I269M, F278I, Y279C, Y279S and L282V in yeast numbering. I269M is located in EF loop, other mutations are in EF helix (Kessl et al., 2005). The ranking order of resistance as reported in the literature (Kessl et al., 2005) is moderate for I269M and F278I and greater resistance for Y279C and L282V. Isoleucine at 269th position is located in hydrophobic region of the binding pocket. Its mutation to 269M leads to a decrease in volume of available binding pocket which interfere with binding of the inhibitor (Kessl et al., 2005). Phe²⁷⁸ interact with the chlorophenyl ring of atovaquone. This region of the binding pocket comprises of Phe¹²¹, Phe²⁷⁸, Tyr³⁵⁹ and chlorophenyl ring of atovaquone. Its mutation F278I removes the central aromatic element and destabilizes this area of the binding pocket and interferes with the binding of the inhibitor (Kessl et al., 2005). Similarly, Tyr²⁷⁹ (yeast numbering) is also present in a stable aromatic cluster composed of His¹⁸¹ and naphthoquinone ring of the atovaquone drug.

Formation of weak hydrogen bonds between the aromatic side chain of tyrosine and the ligand is crucial. Its mutation to Y279S or Y279C (yeast numbering) replaces the aromatic group with a strong nucleophilic side chain thus decreasing the hydrophobic interactions between the drug and the binding pocket (Musset et al., 2006). Mutation S299N (serine to asparagine at position 299, *Plasmodium* numbering) is unlikely to cause high resistance levels as this position is far from the atovaquone binding site and is located in the inner mitochondrial membrane (Musset et al., 2006).

Chemotherapy is one of the most important step to control malaria, but due to the development of resistance against the available drugs, including artemisinin derivatives (Ariey et al., 2014) there is an urgent requirement of development of new drugs either with increased potency against the already approved targets or with different modes of actions. Thus, the novel inhibitors must be highly selectivity for *Plasmodium* over the equivalent mammalian target, be able to overcome atovaquone resistance mechanisms; be an ubiquinol antagonist with improved pharmaceutical and pharmacokinetic properties; are economical for use in third world countries and have an acceptable safety profile.

1.4 Problem statement, study aims and objectives

1.4.1 Problem statement

Due to emergence of resistance against atovaquone, the only drug in clinical use that kills *P. falciparum* by inhibiting mitochondrial enzyme cytochrome *bc*₁, there is a great scope and requirement to develop novel compounds that can help to resolve these issues.

1.4.2 Aims:

To undertake *in silico* and *in vitro* studies to identify novel *Plasmodium* cyt *bc*₁ complex inhibitors with potency against atovaquone-resistant strains.

1.4.3 Objectives

To achieve the aim of this project, following objectives were fulfilled.

1. To develop a comprehensive database of small organic compounds targeting the *Plasmodium* cyt *bc*₁ complex by systematically collating published data, including chemical structures and biological activities of diverse inhibitor scaffolds and analyse SAR.
2. To develop and validate *in silico* models of the WT and atovaquone-resistant (Y279S) mutant forms using crystal structure for cyt *bc*₁ from Protein Data Bank (PDB ID: 3CX5), and to investigate the binding interactions of atovaquone through molecular docking, MM-GBSA (binding free energy) calculations, and molecular dynamics (MD) simulations.
3. To use the binding profiles and interaction data of atovaquone as a reference framework to evaluate and compare the performance of test compounds, with the aim of identifying potential antimalarial agents effective against the atovaquone-resistant strain.
4. To evaluate the antimalarial activity of a series of 1,4-naphthoquinone-1,2,3-triazole hybrids against atovaquone-resistant *Plasmodium* strain through *in vitro* studies and to conduct molecular modelling analyses including MD simulation, to investigate their binding modes and predict their pharmacokinetics and drug-likeness *in silico*.
5. To assess cytotoxicity of a series of naphthyl bearing 1,3,4-thiadiazoleacetamide compounds against HEK293 and K562 cell lines. Additionally, to conduct molecular modelling studies to explore their binding modes and evaluate their potential to target the cyt *bc*₁ complex. Furthermore, to predict their pharmacokinetic and drug-likeness.
6. To design and optimise novel inhibitors of *Plasmodium* cyt *bc*₁ complex using computer-aided drug design (CADD) methodologies, with a focus on overcoming resistance associated with atovaquone.
7. To assess the pharmacokinetic properties and drug-likeness of the proposed compounds through *in silico* ADMET profiling.

1.5 Structure of the thesis

The structure of this thesis follows the format outlined in the University of the Witwatersrand, Faculty of Health Sciences guidelines. Chapters are presented in the form of manuscript submitted for publication and final manuscripts ready for submission. The general formatting and referencing styles are aligned in the chapters according.

- **Chapter 1: Introduction:** The introduction chapter discusses background information of the study, outlines its rationale and significance, and presents the research aim, objectives, and conceptual framework.
- **Chapter 2: Methods:** This chapter outlines the research design, methods, and tools employed to achieve the study's objectives. It details the procedures followed for data collection, compound selection, *in vitro* and *in silico* studies and the result analyses.
- **Chapter 3: Data collation 1:** This chapter establishes the base for this thesis and presents the systematic collation of published data related to small organic compounds targeting the *Plasmodium* cyt *bc*₁ complex. The chapter also outlines how the compiled dataset was curated and categorised based on chemical scaffolds, potency, selectivity and relevance to drug resistance. This structured compilation provides a foundation for subsequent analysis of SAR and identification of promising lead compounds. This chapter is presented in the form of written manuscript for publication and addresses the objective 1.
- **Chapter 4: Data collation 2:** This chapter analyses only those collated inhibitors from chapter 3 that displayed activity against atovaquone resistant strains.
- **Chapter 5: Published manuscript:** This chapter presents a published article titled "A Dual *In vitro* and *In silico* Approach to Evaluate 1,4-Naphthoquinone-1,2,3-triazole Hybrids Against Atovaquone-Resistant Malaria". The article has been published in Experimental Parasitology (doi: 10.1016/j.exppara.2025.108991). This chapter addresses objectives 2, 3 and 4 by integrating *in silico* and *in vitro* studies to identify most promising compounds with efficacy against atovaquone-resistant *Plasmodium* FCR3 strain (**Appendix H**).
- **Chapter 6: Manuscript draft:** This chapter is presented as a manuscript draft titled "Evaluation of naphthyl bearing 1,3,4-thiadiazoleacetamides compounds as *Plasmodium* cytochrome *bc*₁ complex inhibitors", and addresses objective 5 to determine cytotoxicity of test compounds against HEK-293 and K562 cell lines, along with *in silico* studies to explore their potential to target the cyt *bc*₁ complex, and predict their pharmacokinetic and drug-likeness (**Appendix I**).
- **Chapter 7: Manuscript draft:** This chapter presents a manuscript titled "Designing, molecular modelling, drug-likeness and ADMET prediction of chalcones as novel *Plasmodium falciparum* cytochrome *bc*₁ Inhibitors", and addresses the objectives 6 and 7 (**Appendix J**).
- **Chapter 8: Conclusion, Limitations, Recommendations:** This chapter discusses the findings, limitations and recommendations for future work.

- **Chapter 9: Appendices.** This section presents the ethical clearance certificates, supplementary information for Chapters 2 to 7, published papers, and author declarations for submitted manuscript.

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CHAPTER 2

METHODOLOGY

The methods used to achieve the aims and objectives in this PhD thesis are described below. After an intensive literature search to identify potential compounds that may target *Plasmodium* cytochrome *bc*₁ (**Section 2.1**), *in silico* molecular modelling was undertaken to verify their affinity for the selected target as described (**Section 2.2**). This was followed by evaluation of their physicochemical properties, drug-likeness, pharmacokinetic, and toxicity profiles (**Section 2.3**). Methods followed for the *in vitro* studies, including antimalarial drug sensitivity assay (**Section 2.4**) and cell viability assay (**Section 2.5**) are described thereafter.

2.1 Literature search methodology to identify potential compounds targeting *Plasmodium falciparum* cytochrome *bc*₁ complex

To advance our understanding of potential antimalarial agents, a compilation of data from previously published and tested compounds targeting *Plasmodium* cytochrome *bc*₁ (cyt *bc*₁) was done. This compilation may serve as a valuable resource for researchers and drug development initiatives seeking to identify and optimise compounds with activity against the *P. falciparum* cyt *bc*₁ complex. In this endeavour, I presented a comprehensive compilation of data, summarising research efforts by scientists who investigated various compounds for their antimalarial activities by targeting the cyt *bc*₁ complex. This compilation aimed to provide insights into the chemical diversity, structure-activity relationship (SAR), and their biological activities against atovaquone-sensitive and –resistant strains, ultimately contributing to the ongoing efforts to combat malaria and alleviate the burden it places on vulnerable populations worldwide.

2.1.1 Searching for relevant articles from academic databases: Collation of the published information into a database involved manual curation of information about chemical compounds and respective biological activities from various accredited published research literature (including Elsevier, Future Science Ltd, ACS Publications, Wiley-VCH Verlag GmbH & Co., The Royal Society of Chemistry, Springer Science) searched using academic databases PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) and Google Scholar (<https://scholar.google.com/>).

2.1.2 Keywords used to search for articles: Articles were searched using keywords like malaria, electron transport chain, cytochrome *bc*₁ complex, complex III, cytochrome *bc*₁ inhibitors, *Plasmodium* cytochrome *bc*₁ inhibitors, ELQs, endochin-like quinolones, beta-methoxyacrylates, atovaquone, naphthoquinones, quinolones, 4-pyridones, (1H-Pyridin-4-ylidene)amines, chalcones, acridones, tetracyclic benzothiazepines, and TM90-C2B strain.

2.1.3 Software used for collating data: Structures for chemical compounds were drawn and a table was compiled and organised using Instant JChem software version 20.15.0 (2020), which was later updated to version 22.19.0 (2022), from Chemaxon (<https://chemaxon.com/instant-jchem>) .

2.1.4 Organising the data: The captured data was organised in the form of a table and saved as Comma-Separated Values (CSV) files with information about chemical structure of compounds in the form of Simplified Molecular Input Line Entry System (SMILES) notations, their molecular formulas, molecular weights, various *P. falciparum* strains, biological activities (IC₅₀/EC₅₀ values or pIC₅₀/pEC₅₀), and citation of the articles that included PubMed identifiers (PMIDs), digital object identifiers (DOIs), and year of publications. Definitions used include:

- IC₅₀ is defined as the half-maximal inhibitory concentration of a compound required to inhibit a biological function by 50% in a given assay.
- EC₅₀ is the half-maximal effective concentration of a compound that produces 50% of its maximal biological effect in an assay and is used to evaluate the efficacy of agonists in stimulating a biological response.
- pIC₅₀ (or -log IC₅₀) is the negative logarithm (base 10) of the IC₅₀, and pEC₅₀ (or -log EC₅₀) is the negative logarithm (base 10) of the EC₅₀ value.

2.1.5 Analysis of data: For analysis, compounds with IC₅₀ or EC₅₀ values ≤1 μM (or pIC₅₀/ pEC₅₀ ≥6) were considered as potential candidates. Compounds (with IC₅₀ or EC₅₀ >1 μM) were considered as inactives for this study. Some of the compounds were at the borderline of actives and inactives. A SAR study was done to explain the molecular differences between active and inactive compounds.

2.2 Protocol for *in silico* molecular modelling methods

Molecular modelling studies play a pivotal role in understanding the interactions between biomolecules and ligands at an atomic level, providing insights that guide rational drug design. This

study employed a comprehensive computational approach encompassing molecular docking, molecular mechanics-generalised Born surface area (MM-GBSA) free energy calculations, and molecular dynamics (MD) simulations. These methods enabled the identification of key binding interactions and stability of protein-ligand complexes in the wild-type and mutant models.

2.2.1 Selection of PDB ID 3CX5 as the target crystal structure

No crystal structure for cyt *bc*₁ complex from *P. falciparum* is currently available. However, the cyt *bc*₁ complex of the yeast *Saccharomyces cerevisiae* has been utilised to investigate the mechanism of action of antimalarial compounds in *P. falciparum* *bc*₁ complex (Biagini et al., 2012; Carrasco et al., 2013; Cowley et al., 2012; Hill et al., 2003; Sodero et al., 2017).

- There is high sequence identity in the Q_o oxidation pocket between the two species (Jacobsen et al., 2021).
- Crystal structure of *S. cerevisiae* *bc*₁ complex with high resolutions are available in the protein data bank, whereas the same for *P. falciparum* are not available yet (Birth et al., 2014; Solmaz and Hunte, 2008).
- There is a close relationship between the inhibitory activities of the two species (Kessl et al., 2007, 2003; Vallières et al., 2013).
- In order to study the mutant model, the Y268S mutation that accounts for atovaquone resistance in *P. falciparum* is related to the Y279S mutation in *S. cerevisiae* (Jacobsen et al., 2021)

Due to the above factors, PDB ID 3CX5 was used in this work to perform molecular docking and simulation studies and analyse the binding modes of various compounds, including atovaquone.

2.2.2 Selection of software to perform molecular modelling studies

For all molecular modelling studies, the Maestro software suite by Schrödinger, Inc. was used. Studies were performed using two versions: Schrödinger Release 2021-1 (Maestro version 12.7 Schrödinger, LLC, New York, NY, 2021-1) for preliminary studies to perform Glide docking and Schrödinger Release 2023-2 (Maestro version 13.3 Schrödinger, LLC, New York, NY, 2023-2) to perform the Glide docking, Prime MM-GBSA calculations and Desmond molecular dynamic simulations for advanced studies of selective compounds (Schrödinger Release Notes - Release 2023-2). These studies were performed by submitting jobs on the Centre for High-Performance

Computing (CHPC) cluster by the National Integrated Cyberinfrastructure System (NICIS), South Africa.

2.2.3 Preparation of protein

The 3D crystal structure for *Saccharomyces cerevisiae* *bc*₁ complex PDB ID 3CX5, was retrieved from RCSB Protein Data Bank (<https://www.rcsb.org/structure/3CX5>), with co-crystallised ligand stigmatellin A and resolution 1.90 Å (Solmaz and Hunte, 2008).

2.2.3.1 Preparation of the wild-type model

The 3CX5 crystal structure was subjected to the Protein Preparation Wizard, where the protein was pre-processed and minimised. The pre-processing of the structure was done to assign the correct bond orders, add hydrogen atoms, fill in the missing side chains or loops using Prime, capping termini, and delete waters beyond 5.00 Å from the het groups. The het group referred to the heteroatom groups including ligands, cofactors and metal ions. The pre-processing step was followed by energy minimisation steps to optimise the hydrogen bonding network and minimise the system for further stability.

2.2.3.2 Preparation of the mutant model Y279S

To study the binding modes of compounds in a model that could account for one of the identified atovaquone-resistances (Y268S), a mutant model was built where tyrosine at position 279 (equivalent to position 268 in *Plasmodium* parasite) was replaced by serine to account for Y279S (equivalent to Y268S in *P. falciparum*) mutation (Jacobsen et al., 2021; Song et al., 2015). The resultant protein was again run through an energy minimisation step to stabilise the structure further.

2.2.3.3 Ramachandran Plots

To validate the cyt *bc*₁ enzyme 3D structure (post-protein preparation) and to evaluate the structural impact of mutation Y279S, the Ramachandran plots were employed for both the WT and Y279S mutant models using Maestro software (version 13.3, Schrödinger, LLC, New York, NY, 2023-2).

2.2.4 Grid generation

The binding site was determined by preparing a grid using the Glide Receptor Grid Generation program. The co-crystallized ligand molecule (stigmatellin A) was used to identify the binding site, and the grid was generated to assign the ligand binding site for the docking of compounds. The rest of the settings were kept on default. Separate grids were prepared for the wild-type and mutant models following the same method. The grid's reliability was validated by re-docking and reproducing the crystallised pose of stigmatellin in the 3CX5 binding site with the best RMSD of 0.6770 Å and docking score of -15.404 kcal/mol.

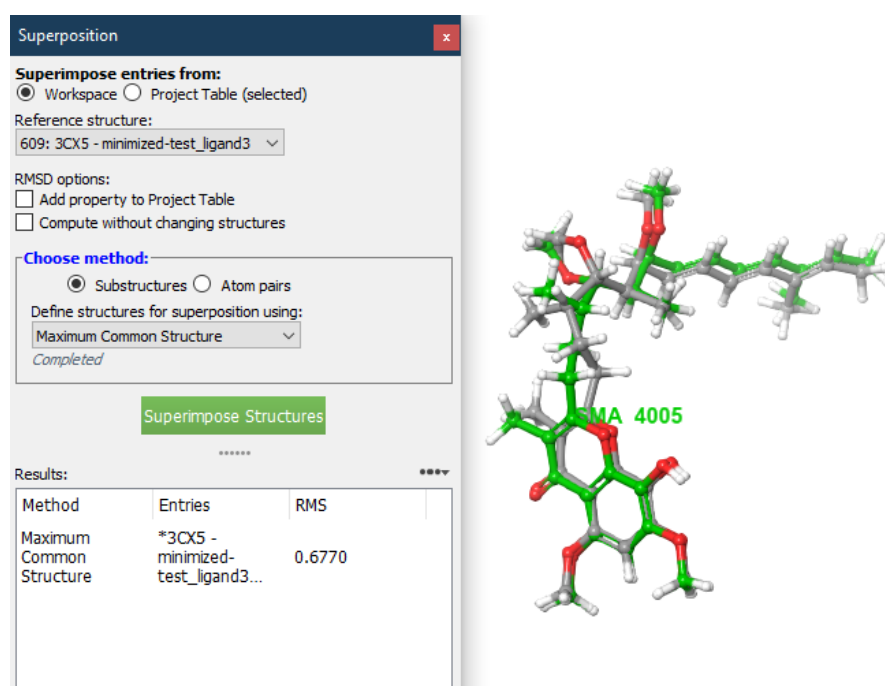


Figure 2.1: Self-docking of stigmatellin into the 3CX5 crystal structure to reproduce the crystallised pose. The RMSD = 0.6770 Å. Value less than 2.00 Å is considered good for docking (Castro-Alvarez et al., 2017; Poli et al., 2016; Ramírez and Caballero, 2018). Grey coloured ligand is the crystallised stigmatellin structure and green coloured ligand is the docked stigmatellin structure.

2.2.5 Preparation of ligands

The CSV file with SMILES notation generated in Section 2.1.4 was imported to the Maestro working directory. The ligands' 3D structures were prepared using the *LigPrep* program of Schrödinger Maestro software and the OPLS4 force field. The protonation states were predicted using Epik at a

target pH of 7.0 +/- 2.0 to generate possible ionisation and tautomeric states so that each ligand could generate a maximum of 32 stereoisomers (Shelley et al., 2007).

2.2.6 Ligand docking

Molecular docking was performed using the *Glide* extra-precision (XP) scoring function, and ligand structures were treated as flexible, which allowed for sampling of ring conformation (including input ring conformation) and nitrogen inversion to obtain five poses per ligand (Friesner et al., 2006, 2004). Strain correction terms were applied to perform post-docking minimisation. The binding modes were visually inspected based on the formation of H-bond between the ligands and His¹⁸¹ and Glu²⁷² residues and hydrophobic interactions with other binding pocket residues including Ile¹²⁵, Phe¹²⁹, Met¹³⁹, Val¹⁴⁶, Ile¹⁴⁷, Leu¹⁵⁰, Ile²⁶⁹, Pro²⁷¹, Leu²⁷⁵, Phe²⁷⁸, Tyr²⁷⁹, Met²⁹⁵, Phe²⁹⁶, Ile²⁹⁹ (Rodrigues et al., 2011).

2.2.7 Prime MM-GBSA

The Prime MM-GBSA v3.000 program available within Schrödinger Maestro version 13.3 was used to calculate the relative binding-free energy (or ΔG) for the selected ligand complexes with the VSGB or the variable-dielectric surface generalised Born solvation model and optimised potentials for liquid simulations 4 (OPLS4) force field (Genheden and Ryde, 2015; Li et al., 2011; Mulakala and Viswanadhan, 2013). It calculated the energy of optimised free receptors, free ligand and a complex of the ligand with a receptor.

$$\text{MMGBSA } (\Delta G)_{\text{Bind}} = \text{PrimeEnergy}_{(\text{Optimized Complex})} - [\text{PrimeEnergy}_{(\text{Optimized Free Receptor})} - \text{PrimeEnergy}_{(\text{Optimized Free Ligand})}]$$

Equation 2.1: MM-GBSA equation adapted from (Genheden and Ryde, 2015)

The previously docked ligand-receptor complexes were retrieved in the workspace, and the solvation model VSGB with the OPLS4 force field were used to perform the MM-GBSA job (Li et al., 2011). The distance from the ligand was changed to 5.0 Å to allow some flexibility between the binding site residues and ligands.

2.2.8 MD Simulation

Running MD simulation on the original model (developed using Schrödinger Release 2021-1, Maestro version 12.7 in Section 2.2.3) gave errors as the crystal structure was too large and the job was not reaching the completion stage. So, a new and truncated model was built with 3CX5 using Schrödinger Release 2023-2 (Maestro version 13.3, Schrödinger, LLC, New York, NY, 2023-2).

All the extra chains and ligands were deleted, keeping only chains C and P involved in the binding pocket, so that the system could become smaller and run the simulation studies smoothly. This was followed by subjecting the crystal structure 3CX5 to the Protein Preparation Wizard (Maestro version 13.3) for pre-processing that included capping termini, filling in missing side chains and loops, deleting water beyond 5 Å from the het groups, and generating het states at 7.4 pH. The protein was then optimised to assign H-bonds and sample crystal symmetry using PROPKA at pH 7.0, followed by a minimisation step to converge heavy atoms to RMSD = 0.30 Å (Rostkowski et al., 2011).

For the preparation of the Y279S resistant model, ligand preparation, receptor grid generation, ligand docking, and MM-GBSA calculations, all the steps were followed in the similar manner as done previously with version 2021-1, described in **Section 2.2.3.2** and **Sections 2.2.4 to 2.2.7**.

Once the docking poses were ranked, selected protein-ligand complexes were submitted for MD simulations using Desmond from Schrödinger Release 2023-2 suite as described in the sections below (Abdullah et al., 2023; Gohlke and Klebe, 2002; Jorgensen, 2004; Karplus and McCammon, 2002).

2.2.8.1 Preparing a membrane protein for MD simulation

For the preparation of membrane protein, the protein-ligand complex was submitted to the Desmond System builder step where DPPC membrane @325K was selected to be placed automatically. TIP4P was chosen as the solvent model with an orthorhombic boundary box whose edges were 10 Å away from the protein. The system was neutralised by adding sodium ions and sodium chloride (NaCl) salt was added at a concentration of 0.15M (similar to a biological system). The OPLS4 force field was selected to run the job.

2.2.8.2 Preparing to run MD job

Each MD simulation job was run in two batches for 50ns each for a total duration of 100 ns. The ensemble class selected was NPT, with $T = 300\text{ K}$, $P = 1.01325\text{ bars}$, and $\gamma = 2000\text{ Bar}\cdot\text{\AA}$. The rest of the settings were kept on default.

2.2.8.3 Preparing to run analysis

The resultant output files from the two batches of each run were merged and, the report was generated using the simulation interaction diagram tool within the Schrödinger suite.

2.2.8.4 Software used for this thesis

Zotero, Putty, FileZilla, MS office, ACD/ChemSketch, Instant JChem, Schrödinger Maestro.

2.2.8.5 MD Simulations of Apo-protein

The MD simulations for the apo-protein of both WT and Y279S mutant models were run for 100 ns as described in **Sections 2.2.8.1 to 2.2.8.3** using Schrödinger Release 2023-2 (Maestro version 13.3, Schrödinger, LLC, New York, NY, 2023-2). To note that for apo-protein, there was no ligand bound to the binding cavity.

2.3 Prediction of physicochemical properties, pharmacokinetic profile, drug-likeness, and toxicity

Predicting physicochemical properties, pharmacokinetic profiles, drug-likeness, and toxicity are critical steps in early-stage drug discovery to assess the potential of compounds as drug candidates. These properties provide essential insights into a molecule's solubility, permeability, stability, and bioavailability, directly influencing its efficacy and safety in biological systems. Furthermore, computational predictions help identify potential liabilities, such as toxicity or poor pharmacokinetics, early in the discovery process, thereby reducing the time and cost associated with experimental validation. This study employed advanced computational tools, including SwissADME and Chemaxon Instant JChem, to predict these properties and guide the selection and

optimisation of lead compounds for further development. The assessment methods and implications of these properties are provided in the sections below.

2.3.1 Physicochemical properties

Physicochemical properties are the fundamental physical and chemical attributes of a molecule that influence its behaviour in biological systems and its suitability as a drug candidate. The **ionisation constant (pKa)** is a crucial property that determines a drug's ionization state at different pH levels, influencing its solubility, lipophilicity, absorption, distribution, and overall pharmacokinetics (Manjooran, 2020). It plays a key role in drug absorption, as non-ionic (non-polar) forms are more readily absorbed. In contrast, ionic (polar) forms have reduced permeability and help predict whether a drug will be absorbed in the acidic stomach or the basic intestine. This makes it essential for optimising drug design and bioavailability (Manallack, 2007).

The acid dissociation constant (pKa) values of the active compounds were predicted *in silico* using Instant JChem software, version: 22.19.0 from Chemaxon (<https://chemaxon.com/instant-jchem>), to determine the degree of ionisation of the compounds at pH 7.5 to 8.0 (mitochondria), pH 7.4 (RBC cytosol) and pH 5 (parasite digestive food vacuole).

The Henderson-Hasselbasch equation (**Equation 2.2**) (Henderson, 1908; Hasselbasch, 1917) to calculate pKa is as follows

$$\text{pKa} = \text{pH} + \log \frac{\text{Acid}}{\text{Base}}$$

Equation 2.2: Henderson-Hasselbalch equation to determine the percentage ionisation for weak acids and weak bases (Henderson, 1908).

2.3.2 Measurement of pharmacokinetic properties and drug-likeness

ADMET properties, such as gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, and cytochrome P450 (CYP) enzyme interactions and molecular properties that are incorporated in Lipinski's rule of five (Ro5), along with number of rotatable bonds (RB), topological polar surface area (TPSA), and bioavailability were calculated using SwissADME program (Daina et al., 2017; SwissADME).

Lipinski's rule of five (Ro5): Lipinski first conceptualised 'drug-likeness' as the acceptable ADME/toxicity properties of the compound (Lipinski, 2002; Lipinski et al., 1997). According to

Lipinski's rule of five (Ro5), a compound is likely to be drug-like if it follows Ro5 which are: MW $\leq$ 500 Daltons (Da) or g/mol, calculated logarithm of the n-octanol/water partition coefficient (clogP) $\leq$ 5, number of hydrogen bond acceptors (#HBAs) $\leq$ 10 (these are mainly oxygen and nitrogen atoms in the molecule) and the number of hydrogen-bond donors (HBDs) $\leq$ 5 (these are the OH and NH present in the molecule).

Number of rotatable bonds (RB): An additional rule for the number of rotatable bonds (#NRB $\leq$ 10) is also often included (Veber et al., 2002). It signifies the number of single bonds that can rotate freely around their axis and determines the conformational flexibility of the molecule. For flexibility, the molecule should not have more than 9 rotatable bonds (Daina et al., 2017).

Topological Polar Surface Area (TPSA): It is a measure of the polar (hydrophilic) surface area of a molecule indicating the drug's polarity and its lipid solubility. High TPSA values indicate significant polarity, which enhances water solubility but limits membrane permeability, affecting oral absorption, bioavailability, and central nervous system (CNS) penetration. Drug development strategies often aim for an optimal TPSA range ($<140 \text{ \AA}^2$) to balance solubility and permeability for oral drugs (Lipinski et al., 1997; Veber et al., 2002).

Bioavailability score: It gives a quick estimate of the likelihood of oral bioavailability. A bioavailability score of 0.55 shows moderate bioavailability potential, whereas a score of 0.85 indicates strong potential for oral bioavailability (Martin, 2005).

BOILED Egg (or Brain Or Intestinal Estimated) Plot: The Boiled Egg Plot was generated using SwissADME, illustrated WLogP (lipophilicity) versus TPSA to assess drug-like properties such as GI absorption and BBB penetration (Daina and Zoete, 2016). Compounds in the white region were predicted to have high GI absorption, making them suitable for oral administration. In contrast, compounds in the yellow region (yolk) were expected to cross the BBB. Compounds identified as P-glycoprotein (P-gp) substrates are indicated by a blue dot, suggesting potential active efflux that may limit GI absorption or BBB entry. Compounds predicted to be non-substrates of P-gp were marked as red.

2.3.3 Toxicity

ProTox-3.0 online tool was used to predict the potential toxicity of chemical compounds. It estimated potential toxic effects, providing insights into acute toxicity (rodent) as lethal dose (LD₅₀)

in mg/kg body weight, organ toxicity, toxicological endpoints (carcinogenicity, immunotoxicity, mutagenicity and cytotoxicity), and toxicological classes, enabling the prioritisation of safe candidates for further development. (Drwal et al., 2014; ProTox-3.0).

Toxicity classes were categorised based on the Globally Harmonized System (GHS) as **Class 1**: fatal if swallowed ($LD_{50} \leq 5$ mg/kg); **Class 2**: fatal if swallowed ($5 < LD_{50} \leq 50$ mg/kg); **Class 3**: toxic if swallowed ($50 < LD_{50} \leq 300$ mg/kg); **Class 4**: harmful if swallowed ($300 < LD_{50} \leq 2000$ mg/kg); **Class 5**: may be harmful if swallowed ($2000 < LD_{50} \leq 5000$ mg/kg); **Class 6**: non-toxic ($LD_{50} > 5000$ mg/kg) (GHS, 2025).

2.3.4 Designing novel compounds

Novel compounds (**Chapter 7**) were designed applying the computer-aided drug designing method using the following two approaches.

1. **Interactive pose prediction:** Compounds were designed using the 'Interactive pose prediction' tool in the Schrödinger Maestro module. This tool helped to view the 2-D structure while designing new compounds and quickly predicted the ligand's Glide score against the selected grid.
2. **R-Group Enumeration:** The R-group Enumeration tool within Schrödinger Maestro (Release 2023-2) was used to systematically substitute the R-group (functional group or side chain) at a specific position, generating a library of analogues. For this work, 'R-groups to Create a Hydrogen Bond' was selected to develop the new compounds that could form hydrogen bonds (H-bonds) with the binding cavity residues, specifically with His¹⁸¹ and Glu²⁷².

2.4 Antimalarial drug sensitivity assay

2.4.1 *In vitro* parasite culture maintenance

2.4.1.1 Maintenance of parasite culture

The NF-54 chloroquine- and atovaquone-sensitive and FCR-3 chloroquine- and atovaquone-resistant strains of *P. falciparum* were cultured *in vitro* using the method by (Jensen and Trager, 1977) and (Freese et al., 1988) with slight modifications as per the Pharmacology Division protocol (van Zyl, 1997). A biosafety clearance certificate was obtained from the Witwatersrand Institutional Biosafety

Committee (protocol number: 20190404; **Appendix A**) for the *in vitro* laboratory studies including malaria and human cell lines.

Aseptic techniques were used in maintaining the parasites to prevent contamination, with the use of 70% ethanol (LabChem®, Johannesburg, South Africa) to clean the surfaces of the laminar flow and all reagent containers and instruments before use in the laminar flow. The parasites were maintained at approximately 5% parasitaemia and 5% haematocrit in RBCs and complete culture medium (Section 2.4.1.3). Parasite cultures were examined daily by preparing Giemsa stained slides (Section 2.4.1.5) and observed microscopically (Olympus®, CKX41 microscope) at 1000x magnification under oil immersion. Based on the microscopic observations, RBCs were added to the parasite cultures when the parasitaemia was greater than 5% trophozoites or schizonts stage and synchronised when the parasites were in the ring stage (Section 2.4.1.6). Before the parasite cultures were incubated in a fixed position at 37°C, they were supplemented with complete culture medium and gassed for 30 seconds with 2% oxygen, 5% carbon dioxide and 93% nitrogen.

2.4.1.2 Preparing incomplete culture medium

The incomplete culture medium was prepared under sterile conditions using autoclaved Milli-Q® water with 10.4 g/L Roswell Park Memorial Institution (RPMI)-1640 (Sigma-Aldrich®, Darmstadt, Germany), 5.9 g/L N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) buffer (Sigma-Aldrich®, Darmstadt, Germany), 4 g/L D-glucose monohydrate (Merck®, Darmstadt, Germany), and 44 mg/L hypoxanthine (Sigma-Aldrich®, Darmstadt, Germany) (Jensen and Trager, 1977). The solution was filter-sterilised through a 0.22 µm Durapore® membrane filter (Merck®, Darmstadt, Germany) before being stored at 4°C until required.

2.4.1.3 Preparation of complete culture medium

The complete culture medium was prepared under sterile conditions by supplementing the incomplete culture medium (Section 2.4.1.2) with 5% (w/v) sodium bicarbonate (NaHCO₃; MW 84.01 g/mol, Sigma-Aldrich®, Darmstadt, Germany) and 10% lipid rich bovine serum albumin (AlbuMAX™; Gibco®, South Africa) which was used as an alternative to human serum.

The **AlbuMAX™** (Gibco®, South Africa) was prepared in incomplete experimental medium as a 5% (w/v) solution and stirred at 168 xg for 2 hours at 35°C. The solution was filter-sterilised to remove

precipitated proteins using a 0.2 µm filter (VacuCap® 90 PF; Sigma-Aldrich®, Darmstadt, Germany), aliquoted and stored at -20°C.

2.4.1.4 Preparation of uninfected red blood cells

Human ethics was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (Protocol number: M210121; **Appendix B.1a and B.1b**) to recruit and draw human blood from healthy volunteers who were not taking antimicrobials, anticoagulants, antivirals, or antimalarial medication for less than three weeks or visited a malaria area in one month prior to donating blood. Potential volunteers were requested to complete a self-administered questionnaire and consent form to ensure they complied with the inclusion and exclusion criteria to donate blood (**Appendix B.2 and B.3**).

Human whole blood was collected in 6 ml blood tubes containing an anticoagulant, acid citrate dextrose solution-B (ACD-B; BD Vacutainer®) from healthy volunteers. The blood was washed by centrifugation at 671 *xg* for 5 minutes to remove plasma and buffy coat. The supernatant was discarded, and RBCs washed three times with phosphate-buffered saline (PBS, pH 7.4) by centrifugation. Thereafter incomplete culture media was added to maintain a 50% haematocrit and stored at 4°C for no longer than 10 days.

The PBS (pH 7.4) was prepared by dissolving one PBS tablet in 200 mL of deionised water that yielded 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride at 25 °C. The PBS solution was then autoclaved (pressure: 121 kpa, temperature: >100°C) for 30 minutes and stored at 4°C until required (van Zyl, 1997) .

2.4.1.5 Preparation of Giemsa-stained thin blood smear

Giemsa-stained thin blood smears for the parasite cultures were prepared daily on methanol cleaned slides to assess the parasite growth, morphology, and percentage parasitaemia (**Figure 2.2**). The Rapidiff staining set (Clinical Sciences Diagnostics CC) was used as directed to stain the parasites in the RBCs. The air-dried slide was dipped twice into the first solution (Rapidiff fixative, methanol) to fix the slide before being air-dried. Thereafter, the slide was dipped five times into the second solution (Rapidiff-1, eosinophil stain) to stain the RBCs and then washed with water. Lastly the air-dried slide was dipped ten times into the third solution (Rapidiff-2; Giemsa stain) to stain the parasites. The air-dried slides were viewed microscopically under oil immersion at 1000x

magnification. The percentage parasitaemia was determined by counting ten random fields over the whole slide and applying Equation 2.3.

$$\% \text{ Parasitaemia} = \frac{\text{Total number of parasitised RBCs}}{\text{Total number of uninfected RBCs} + \text{parasitised RBCs}} \times 100$$

Equation 2.3: Equation to calculate percentage parasitaemia (van Zyl, 1997).

2.4.1.6 Synchronising of the culture

The parasite culture was synchronised to a predominantly early ring stage (**Figure 2.2**) using 5% (w/v) D-sorbitol (Sigma Aldrich®, Darmstadt, Germany) (Lambros and Vanderberg, 1979). The D-sorbitol solution was prepared in autoclaved Milli-Q® water, filtered through a Sterivex™-GS 0.22 µm filter (Sigma-Aldrich®, Darmstadt, Germany) and stored at 4°C. The D-sorbitol ruptured the infected RBCs containing the more mature stages of the parasite, namely early trophozoites to mid-schizont stages (**Figure 2.1**). The parasite culture was centrifuged at 377 *xg* for 5 minutes at room temperature using ThermoScientific Heraeus™ Megafuge™ 8 centrifuge, supernatant discarded, and the parasite pellet was resuspended in 10 ml of 5% (w/v) D-sorbitol and incubated at 37°C for 10 minutes. The culture was then centrifuged at 377 *xg* for 5 minutes and the supernatant discarded. The remaining pellet was washed twice with PBS by centrifugation at 377 *xg* for 5 minutes each time, before the final pellet was re-suspended in complete culture media (Section 2.4.1.3) and gassed with 2% oxygen, 5% carbon dioxide and 93% nitrogen and re-incubated at 37°C.

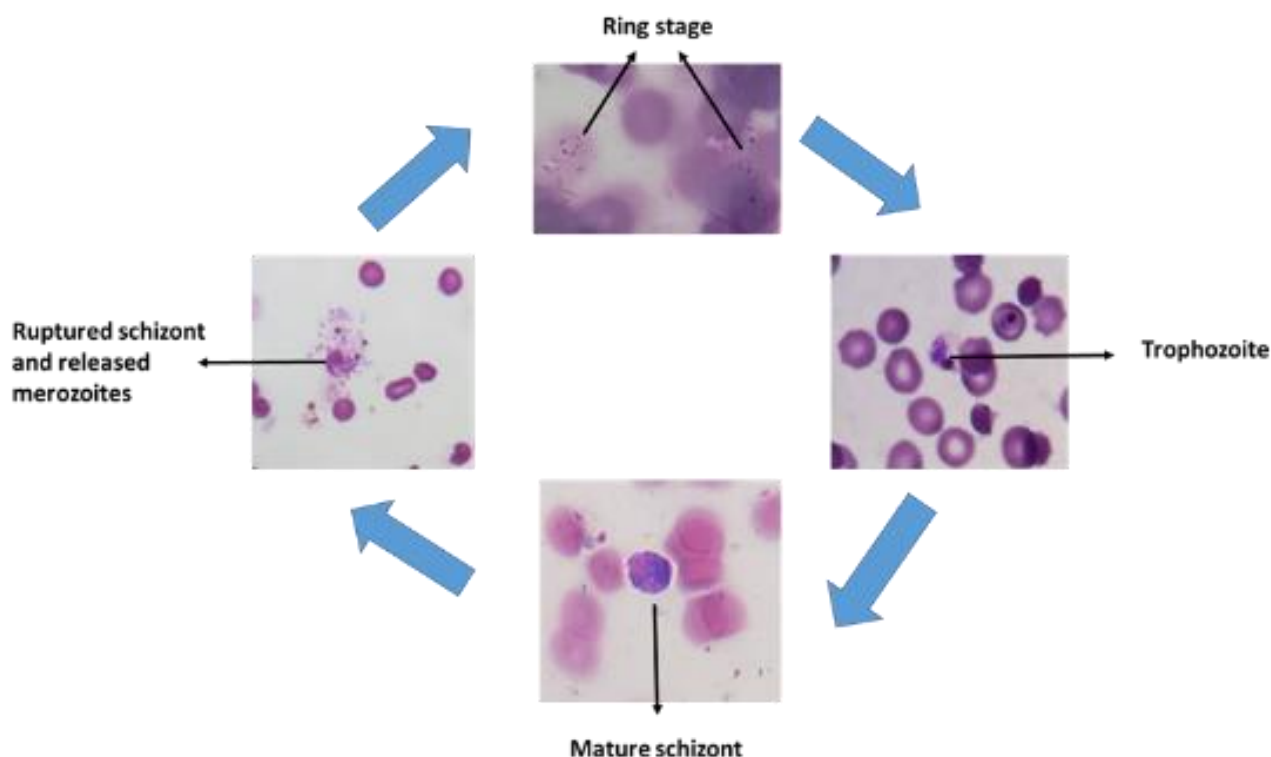


Figure 2.2: The different growth stages of *P. falciparum* parasite during the erythrocytic phase of life cycle. Pictures taken using microscope at 1000x magnification following Giemsa staining after preparing slides.

2.4.2 *In vitro* antimalarial assay

2.4.2.1 Principle of the parasite lactate dehydrogenase assay

The anti-*Plasmodium* activity of the test compounds was determined using pLDH assay as described by Makler and Hinrichs (1993) with slight modification. The underlying principle of this assay revolves around the *Plasmodium*'s capability to produce lactate dehydrogenase (LDH) as a terminal enzyme in its glycolytic pathway during the blood stage of its life cycle. The *Plasmodium* LDH (pLDH) exhibits a distinct characteristic from human LDH in its ability to utilize 3-acetylpyrimidine adenine dinucleotide (APAD) as a coenzyme for the conversion of lactate to pyruvate.

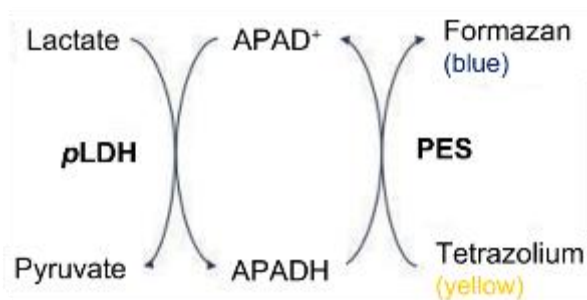


Figure 2.3: Malstat™ reaction for the detection of pLDH in viable *P. falciparum* (Markwalter, Davis & Wright, 2015). The *Plasmodium* LDH (pLDH) enzyme possesses a unique characteristic compared to human LDH in its ability to utilise 3-acetylpyrimidine adenine dinucleotide (APAD) as a coenzyme for the conversion of lactate to pyruvate. To measure pLDH activity, the Malstat™ reagent is used to permeabilize the membranes of the parasites, enabling the enzymatic reaction to take place. A substrate mixture consisting of nitroblue tetrazolium (NBT) and phenazine ethosulfate (PES) is employed in the assay. The, APAD is converted to APADH, which in turn reduces the yellow NBT to form a blue formazan salt. The formation of this blue formazan can be quantitatively measured using spectrophotometry, providing a means to assess the activity of pLDH.

2.4.2.2 Preparation of compounds

A stock solution of 10 mM of the test compounds and the reference control drug atovaquone (Sigma Aldrich®, Darmstadt, Germany; MW 366.84 g/mol) were prepared in DMSO. The compounds were first screened at four serially diluted concentrations; 0.1, 1, 10 and 100 µM for activity, with a dilution factor of 9x taken into account for this assay. Based on the screening of the compounds, the compounds were further serially diluted to concentrations varying from 100 µM to 0.0001 µM for the determination of concentration required to inhibit 50% of parasite growth (IC₅₀ value) of the test compounds.

2.4.3 Preparation of pLDH assay reagents

2.4.3.1 Phenazine ethosulfate solution

To prepare the 0.30 mM PES stock solution, 0.1 mg/mL PES (Sigma-Aldrich®, Darmstadt, Germany) was dissolved in autoclaved Milli-Q® water and sterilised through a Sterivex™-GS 0.22 µm filter

(Sigma-Aldrich®, Darmstadt, Germany) and stored for a maximum of six months at 4°C covered with foil to prevent light exposure.

2.4.3.2 Nitroblue tetrazolium solution

To prepare 2.45 mM NBT stock solution, 2 mg/mL NBT (Sigma-Aldrich®, Darmstadt, Germany) was dissolved in autoclaved Milli-Q® water and sterilised through a Sterivex™-GS 0.22 µm filter (Sigma-Aldrich®, Darmstadt, Germany) and was stored for no more than a week at 4°C covered in foil to prevent light exposure.

2.4.3.3 Malstat™ reagent

The Malstat™ reagent was prepared by dissolving 40 µL Triton X-100™ (Sigma-Aldrich®, Darmstadt, Germany), 0.18 mM calcium-L-lactate (Sigma-Aldrich®, Darmstadt, Germany) and 0.5 mM APAD (Sigma-Aldrich®, Darmstadt, Germany) in tris(hydroxymethyl)aminomethane (TRIS) buffer (27.5 mM TRIS base powder (Prolabo®, Pennsylvania, USA) dissolved in autoclaved Milli-Q® water). The pH was adjusted to 9.1 with sodium hydroxide (Sigma-Aldrich®, Darmstadt, Germany) before being sterilised through a Sterivex™-GS 0.22 µm filter (Sigma-Aldrich®, Darmstadt, Germany) and stored for no more than a week at 4°C, covered in foil to prevent light exposure (Makler & Hinrichs, 1993).

2.4.4 Drug sensitivity pLDH assay

For the pLDH assay, the synchronised ring-stage parasite culture (Section 2.4.1.6) was adjusted to a 2% parasitaemia and 2% haematocrit using washed uninfected RBCs (Section 2.4.1.4; 2.4.1.5) and complete experimental medium (Section 2.4.1.3). The parasite suspension (100 µL) was plated in a sterile 96-well microtiter plate, except for the two wells used as the uninfected RBCs control to which 100 µL of a 2% haematocrit solution of washed uninfected RBCs (Section 2.4.1.4) was plated. Thereafter, 12.5 µL diluted test compounds and atovaquone were added to duplicate wells, except to the drug-free parasitised RBC control wells, to which 12.5 µL of complete experimental media was added. The plate was then placed in a humidified sterile air-tight desiccator with an atmosphere of 2% oxygen, 5% carbon dioxide and 93% nitrogen (Jensen and Trager, 1997), before being incubated at 37°C for 72 hours. Following the incubation period, the plates were frozen at -70 °C for one hour and thereafter, thawed for 45 minutes at 37 °C. The plates were then shaken at 1,200

rpm for 2-3 minutes on an orbital plate shaker (Separations; Titramax 100). Thereafter, 25 µL of the blood lysate was transferred from each well to a new sterile 96-well microtiter plate; to which 100 µL Malstat™ reagent was added together with 20 µL of a 1:1 ratio of NBT and PES mixture (final concentrations 0.30 mM and 2.45 mM, respectively). The plate was then incubated at 37°C for 40 minutes and 50 µL of 5% (v/v) acetic acid (LabChem®, Johannesburg, South Africa) was added to each well to stop the reaction (**Figure 2.3**).

2.4.5 Data analysis

The absorbance at the test wavelength of 620 nm was determined in an ultraviolet-visible (UV-VIS) plate spectrophotometer (LabSystems iEMS Reader MF) with Ascent™ software version 2.6. Equation 2.4 was used to calculate the percentage parasite growth. Each experiment was performed at least three times for reproducibility, accuracy, and precision. Compounds that demonstrated more than 60% inhibition of parasite growth at a concentration of 10 µM were subjected to further evaluation. Dilutions of these compounds were prepared to generate a dose-response curve with a logarithmic sigmoidal shape using Graphpad Prism version 6.0 software. By analysing the curve, the IC₅₀ value was determined. The activity of the compounds was then reported as the mean ± standard deviation (s.d.).

$$\% \text{ Parasite growth} = \frac{\text{Absorbance compound} - \text{Absorbance uninfected RBCs}}{\text{Absorbance parasite control} - \text{Absorbance uninfected RBCs}} \times 100$$

Equation 2.4: Formula to calculate percentage parasite growth (Makler & Hinrichs, 1993).

The *in vitro* antimalarial activity of the compounds was classified as follows: IC₅₀ <5 µM, excellent/potent activity; IC₅₀ of 5-10 µM, good/promising activity; IC₅₀ values of 10-20 µM, moderate activity; IC₅₀ of 20-50 µM, low activity and IC₅₀ >50 µM as inactive (Batista, De Jesus Silva Júnior & De Oliveira, 2009; Phuwajaroanpong et al., 2020).

2.5 Cytotoxicity assay

2.5.1 Cell viability assay

The cytotoxicity of the test compounds was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann (1983) with modifications

according to Departmental procedures (van Zyl et al., 2002). The MTT assay is based on the conversion of the water-soluble tetrazolium salt into an insoluble purple formazan crystal by the mitochondrial enzyme succinate dehydrogenase present in the viable cells (**Figure 2.4**) (Mosmann, 1983; Bahuguna et al., 2017). The assay measures the metabolic activity of live cells, and it was used as a measure for preliminary toxicity testing of the compounds in comparison to the controls.

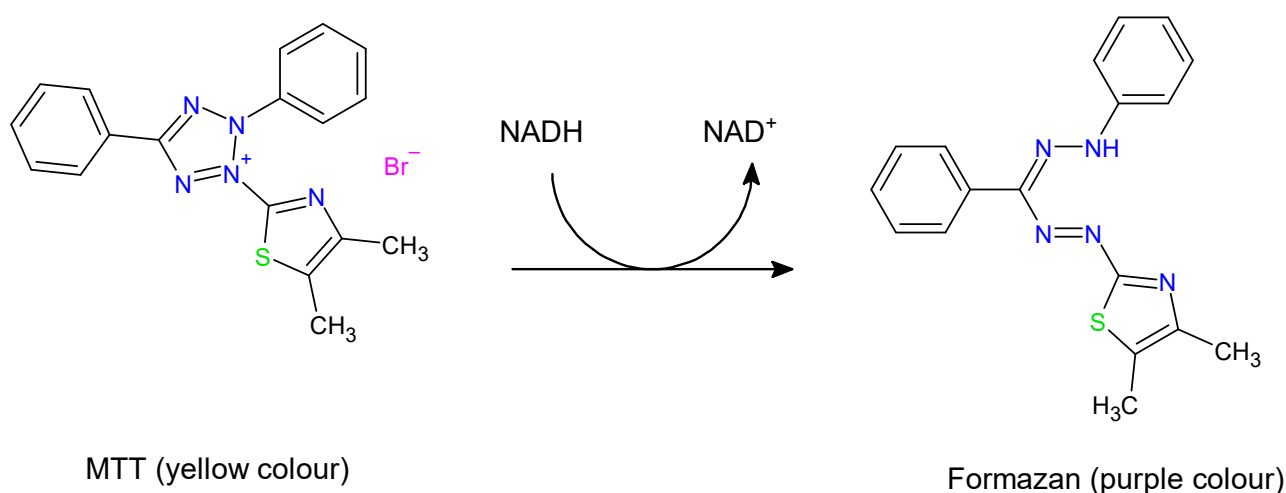


Figure 2.4: Conversion of yellow MTT to purple formazan in the MTT cytotoxicity assay (Riss et al., 2013) .

2.5.2 Cell culture maintenance

Aseptic techniques were carried out in maintaining the cell cultures to prevent contamination, with the use of 70% ethanol (LabChem®, Johannesburg, South Africa) to clean the surfaces of the laminar flow and all reagents and instruments before use in the laminar flow. Two cell lines purchased from ATCC®, were used to evaluate the cytotoxicity of the test compounds, namely the human epithelial kidney cell line (HEK-293) which are adherent cells, and the human chronic myelogenous erythroleukaemia cell line (K562) which are suspension cells. A human ethics waiver was obtained from the Human Research Ethics committee of the University of the Witwatersrand (Protocol number: W-CBP-201106-01; **Appendix C**) permitting the use of the commercial human cell lines for *in vitro* experimental purposes.

Both cell lines were continuously maintained in complete culture medium (Section 2.5.3) at 37°C in a humidified 5% carbon dioxide environment. The cells were viewed microscopically at 400x magnification (Olympus® CKX41) to determine the degree of confluency and observe cell

morphology. The cells were sub-cultured weekly and maintained in complete culture media thrice weekly when the spent medium was replaced.

When the degree of confluency of the HEK-293 cells was greater than 90%, the cells were sub-cultured by trypsinisation (Ricardo & Phelen, 2008). Briefly, the spent culture media was discarded, and the cells were washed twice with 3 mL PBS (pH 7.4), before 3 mL of 0.25% (v/v) trypsin-0.1% versene ethylenediaminetetraacetic acid (Versene-EDTA; Sigma-Aldrich®, Darmstadt, Germany) was added to the flask to detach the cells from the flask surface. The trypsin was inactivated by adding 3 mL complete culture media to the flask, thereafter the cells were sub-cultured by retaining 1 mL of the cell suspension in the flask with additional complete culture media (Section 2.5.3).

In comparison, when the density of the K562 cells was greater than 90%, the cells were gently agitated to re-suspend the cells before being sub-cultured by removing all but 1 mL of the cells in the flask and adding 14 mL complete culture media to allow for optimal cell growth.

For both cell lines, the excess cells were either discarded into containers with 5% sodium hypochlorite or transferred into a 50 mL centrifuge tube to be used for the cell viability assay (Section 2.5.5).

2.5.3 Preparation of medium and reagents for cell culturing

To prepare **complete culture medium**, Dulbecco's Modified Eagles Medium (DMEM, 1X) (gibco) and Hams F12 Nutrient mixture (1X) (gibco) were mixed together in 1:1 ratio and 10% heat-inactivated foetal bovine serum (FBS) was added. The solution stored at 4°C. The heat-inactivated FBS was prepared by heat activating the thawed FBS (at 4°C) at 56°C for 45 minutes before being aliquoted into 50 mL centrifuge tubes and stored at -20°C until required.

The 12 mM **MTT** solution was prepared in PBS (pH 7.4) and filter-sterilised through a 0.22 µm syringe filter and wrapped in tin foil and stored in the dark at 4°C.

2.5.4 Preparation of compounds

Stock solutions (10 mM) of the compounds and control atovaquone (Sigma Aldrich®, Darmstadt, Germany) were prepared in DMSO, and were further diluted to concentrations of 0.1, 1, 10, and 100 µM in complete culture medium (Section 2.5.3) with a 10-fold dilution factor taken into account for the assay.

2.5.5 Cell viability drug sensitivity assay

The cell suspension for both cell lines was prepared as in Section 2.5.2. To remove the trypsin-EDTA, the cell suspension was centrifuged at 377 $\times g$ for 5 minutes and the cell pellet resuspended in complete culture medium. To determine the cell density, 40 μL of the cell suspension was stained with 40 μL of sterile 0.2% (w/v) trypan blue solution (Sigma-Aldrich®, Darmstadt, Germany) prepared in PBS (pH 7.4), of which 40 μL was placed on the haemocytometer. The number of viable cells were counted for four fields of the haemocytometer using a light microscope at 400x magnification. The dead cells were stained blue as trypan dye penetrated through the compromised cell membranes. In contrast, the viable cells appeared clear or transparent as the cell membrane integrity was still intact and the trypan dye could not penetrate through to stain the cells (Strober, 2015). A minimum of 95% cell viability was required for the assay to be conducted and if less than 95% the cells were placed back into culture to optimise cell viability. The cell density was determined by taking into account the four counted fields from the haemocytometer and the 2x dilution factor (Equation 2.5) before being adjusted in complete culture medium such that 10,000 cells/180 μL were plated per well.

$$\text{Cell density (cells/mL)} = (\text{number of viable cells}/4) \times \text{dilution factor (2x)}$$

Equation 2.5: Determination of the cell density when counted on the haemocytometer at a 400x magnification.

Following the preparation of the cells, 20 μL of the test compounds or controls were plated out in triplicate wells, and for the untreated positive control, 20 μL of complete culture media was plated. Thereafter, the cell suspension was plated into each well, except to the background control wells (row H10 to H12) which only contained 200 μL of complete culture medium. The remainder of the wells in row H (H1 to H9) served as the untreated positive controls representing 100% cell viability. The plates were incubated for 44 hours at 37°C in a humidified environment with 5% CO_2 .

After the incubation, 40 μL of 12 mM MTT solution (Section 2.5.3) was added to all the wells and the plates were re-incubated for 4 hours at 37°C in 5% CO_2 humidified conditions.

Thereafter, the plates were centrifuged at 377 g for 5 minutes and 180 μL of the supernatant replaced with 200 μL DMSO to dissolve the purple formazan crystals (Harmse et al., 2015). The

plates were shaken in an orbital shaker (Separations; Titramax 1000) at 1,200 rpm for 5 minutes at room temperature to ensure complete dissolution of the crystals.

The absorbance of each well was determined using a UV-VIS plate spectrophotometer (Thermo Scientific; Multiskan GO). The absorbance was read at the test wavelength of 540 nm (purple formazan colour) and a reference wavelength of 690 nm (unreacted yellow MTT colour). The percentage cell viability was calculated taking the appropriate controls into account (Equation 2.6).

$$\% \text{ Cell viability} = \frac{\text{Absorbance drug (540 nm)} - \text{Absorbance drug (690 nm)}}{\text{Abs drug-free control (540 nm)} - \text{Abs drug-free control (690 nm)}} \times 100$$

Equation 2.6: Percentage cell viability taking both the test and the reference wavelengths into account (Mosmann, 1983).

2.5.6 Data analysis

For those compounds that inhibited more than 60% cell growth at 10 μ M, serial dilutions were prepared to generate a log sigmoidal dose-response curve from which the IC₅₀ values were determined using Graphpad Prism® version 5.02. The compounds which killed <60% cell growth were reported as mean of the highest concentration $\pm$ s.d. of three or more experiments. All the experiments were conducted at least in triplicate for accuracy and precision.

2.6 Statistical Analysis

All experiments were carried out in at least triplicate to produce a mean $\pm$ standard deviation (s.d.) and each experiment independently repeated three times (biological repeats). For the assays which required IC₅₀ value determination (*p*LDH and MTT), the values were calculated from log sigmoid dose response curves generated by GraphPad Prism® 5.02 (GraphPad Software, Inc., USA). The absorbances of the plates in the *p*LDH and MTT assays were read at their respective wavelengths using an UV-VIS spectrophotometer (LabSystems iEMS Reader MF) Ascent™ software version 2.6 and Thermo Scientific; Multiskan GO.

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Chapter 3

***Plasmodium* cytochrome *bc*₁ complex inhibitors database**

This chapter presents a detailed literature review that was conducted to compile a database to identify different compounds from the published literature with potential to target *Plasmodium* cytochrome *bc*₁ complex. The chapter is presented in the form of a completed review manuscript, which paved a way for the analysis of potential compounds in this study for their *Plasmodium* cyt *bc*₁ inhibitory activity and drug-likeness.

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Authors' contributions:

Contributions	Priyanka Agarwal	C. Johan van der Westhuizen	Robyn L. van Zyl
Conceptualization	✓		✓
Literature search	✓		
Database compilation and data analysis	✓		
Writing – preparation of original draft	✓		
Writing – review and editing	✓	✓	✓
Supervision		✓	✓

Compilation and Analysis of Cytochrome *bc*₁ Complex Inhibitors: A Database for Antimalarial Drug Discovery

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Abstract: The cytochrome *bc*₁ (cyt *bc*₁) complex or complex III of *Plasmodium falciparum* is a well-validated drug target for antimalarial therapy, particularly due to its role in the parasite's electron transport chain. It is a mitochondrial enzyme present in the inner mitochondrial membrane that is responsible for oxidation of ubiquinol to ubiquinone and catalytic transfer of electrons from ubiquinol to cytochrome c. This transfer of electrons is coupled with vectorial translocation of protons from mitochondrial matrix to the intermembrane space that maintains mitochondrial membrane potential. Atovaquone is a hydroxyl-naphthoquinone analogue of ubiquinone targeting *Plasmodium bc*₁ complex, where it competitively inhibits ubiquinol oxidation, thus inhibiting the mitochondrial electron transport chain at the cyt *bc*₁ complex. But the parasite has developed resistance to it and thus, there is a growing need to identify and develop novel inhibitors targeting this enzyme. As such a systematic collation and curation of reported chemical compounds that have been tested for antimalarial activity through cyt *bc*₁ inhibition was undertaken. Compounds with diverse chemical scaffolds were retrieved, categorized, and analysed based on their reported biological activities across multiple *Plasmodium* strains, including resistant isolates. Known antimalarial drugs, investigational leads, and novel small molecules were included to establish a comprehensive dataset that enables structure-activity relationship (SAR) studies. The compiled data not only highlights the structural diversity of cyt *bc*₁ inhibitors, but also provides a foundation for rational drug design and scaffold optimization toward the development of next-generation antimalarials effective against drug-resistant malaria. The compiled database contains compounds categorised according to the scaffold that includes naphthoquinones, tetracyclic benzothiazepines,

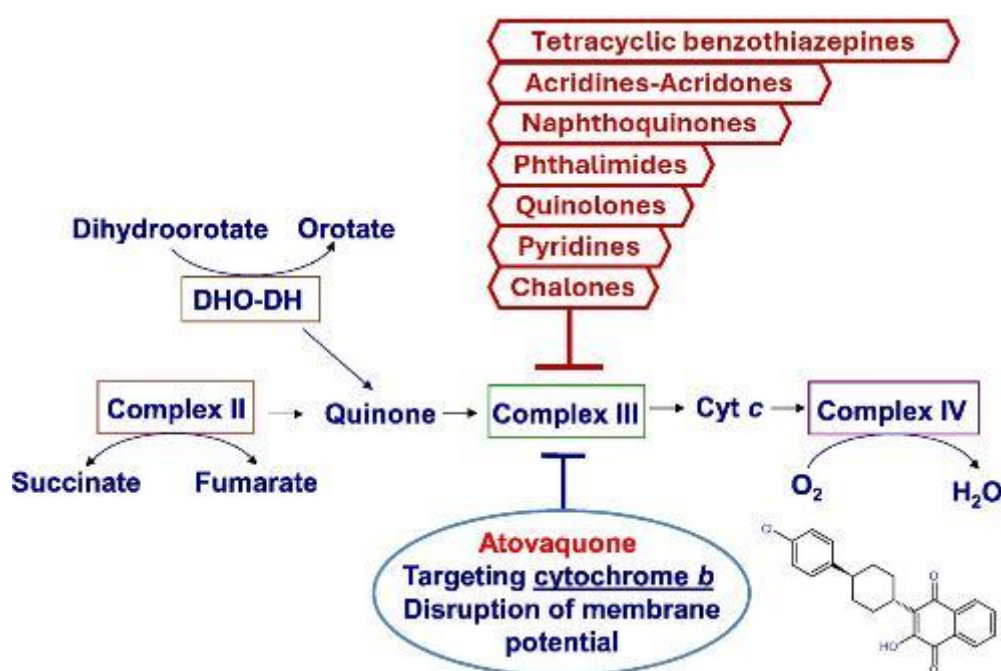
phthalimides, pyridine and pyridine-based compounds, methoxyacrylates and methoxyimino acetates, acridines and acridones, quinolones and a group of miscellaneous compounds. A total of 631 structures, including known antimalarial drugs have been reviewed.

Keywords: malaria, *Plasmodium falciparum*, mitochondria, electron transport chain, cytochrome *bc*₁ complex, cyt *bc*₁, atovaquone

Highlights:

- Collated 631 compounds published as *Plasmodium* cytochrome *bc*₁ complex inhibitors
- Quinolones emerged as the most explored, with 196 active compounds out of 257
- Phthalimides displayed no activity
- 139 compounds active against ATQ-R strains

Graphical abstract



(Adapted from Siregar, et al. 2015)

3.1 Introduction

Malaria is a severe parasitic disease prevalent in 83 countries. According to the WHO (2025), World Malaria Report 2024, there were 263 million malaria cases in the year 2023, with an estimated rise of 11 million cases from the year 2022. Most of this increased number came from the countries in the WHO African region, accounting for about 94% of all global cases, with Nigeria (26%), Democratic Republic of Congo (13%), Uganda (5%), Ethiopia (4%) and Mozambique (4%) being the top five countries (WHO, 2025). Most of the common deaths are among young children. One of the major reasons for the increase in the number of malaria cases is the occurrence of resistance to the currently available chemotherapy and insecticides.

Malaria is transmitted by female *Anopheles* mosquitoes and caused majorly by five species of *Plasmodium*, namely, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Of these *P. falciparum* is majorly responsible for severe malaria cases and deaths. The parasite has developed resistance to many of the currently used therapeutic drugs including artemisinin derivatives which are a component of the artemisinin-based combination therapies that comprise the currently used first-line therapies (Ariey et al., 2014). Thus, there is an increasing demand to develop novel anti-malarial drugs with well-defined mechanism of action. One such pathway is the *Plasmodium* mitochondrial electron transport chain (PmtETC).

Data collation is a critical step in the drug discovery process, as it provides a comprehensive foundation for understanding existing knowledge and identifying new opportunities for therapeutic development. By systematically gathering information on chemical compounds, their biological activities, mechanisms of action, and resistance profiles from the literature, researchers can build a robust database that facilitates informed decision-making. This process enables the identification of various chemical scaffolds and trends, such as structure-activity relationships (SAR), which are essential for optimizing the chemical properties of lead compounds. Additionally, data collation helps uncover gaps in research, highlighting underexplored scaffolds or novel targets that may be pivotal in addressing current challenges, such as drug resistance. It also ensures that past research efforts are not duplicated unnecessarily, saving both time and resources.

This review article examines antimalarial compounds specifically targeting the *Plasmodium* mitochondrial enzyme cyt *bc*₁ complex (EC 1.10.2.2), also known as complex III. It forms an important component of the PmtETC and is responsible for oxidation of ubiquinol to ubiquinone (**Figure 3.1 (1a, 1b)**) and catalytic transfer of electrons that maintains mitochondrial membrane potential (Vaidya and Mather, 2005, 2009). It is a dimeric multi-subunit complex that is composed

of up to 11 subunits in eukaryotes (Gardner et al., 2002; Hikosaka et al., 2015). The catalytic core of cyt bc_1 complex constitutes of three subunits participating in electron transfer pathway, namely Cytochrome b , Cytochrome c_1 and Reiske iron-sulfur protein ([2Fe2S] ISP) (Nixon et al., 2013b). The bc_1 complex catalyses transfer of electrons from ubiquinol (**Figure 3.1 (1a)**) to cytochrome c and this transfer is coupled with vectorial translocation of protons from mitochondrial matrix to the intermembrane space, which generates a transmembrane electrochemical gradient ($\Delta\Psi_m$). The series of oxidation and reduction reactions is known as Q-cycle (Mitchell, 1975) and supports maintaining a stable membrane potential for this organelle.



Figure 3.1. Structure of ubiquinol (**1a**), ubiquinone (**1b**) and atovaquone (**1c**) (Verdaguer et al., 2021).

Atovaquone (**Figure 3.1 (1c)**) is a hydroxynaphthoquinone analogue of ubiquinone (**Figure 3.1 (1b)**) that was approved for the treatment and prevention of *P. falciparum* malaria (Baggish and Hill, 2002; Nixon et al., 2013a; Osei-Akoto et al., 2005). Clinically, it is used in combination with proguanil and marketed as Malarone™ (Tse et al., 2019). It has a well-defined mechanism of action, in that it is a competitive inhibitor of ubiquinol, inhibiting the mtETC at the bc_1 complex level, resulting in loss of mitochondrial function (Biagini et al., 2006; Srivastava et al., 1997). Inhibition of the bc_1 complex also affects the electron acceptor enzyme dihydroorotate dehydrogenase (DHODH), which plays an essential role in the biosynthesis of pyrimidines. Inhibition of this enzyme complex indirectly impacts the biosynthesis of pyrimidines and the transport of electrons in the mitochondria (Rodrigues et al., 2010).

But the malaria parasite has developed resistance to this drug (Gil et al., 2003; Kessl et al., 2007; Peters et al., 2002; Schwartz et al., 2003). Genetic mutations that have caused the atovaquone resistance have been identified as point mutations where Tyr268 residue (Y268) is replaced with either serine (Y268S), asparagine (Y268N) or cysteine (Y268C) (Korsinczky et al., 2000; Musset et al.,

2006; Vaidya and Mather, 2000). These point mutations disrupt the interaction of atovaquone with the other residues at the binding site of the *bc*₁ complex. Despite the reported Malarone™ treatment failures due to resistance (Fivelman et al., 2002; Nixon et al., 2013b), this combination of atovaquone/proguanil is currently used for the treatment of multidrug-resistant malaria and also for prophylaxis in areas with chloroquine resistance (Bueno et al., 2012; Rossiter, 2022).

To advance understanding of potential antimalarial agents, the compilation of data from previously published literature was essential. In this endeavour, a comprehensive compilation of data, summarising research efforts by earlier scientists who investigated various compounds for their antimalarial activities by targeting the cyt *bc*₁ complex is presented in this publication. This compilation aimed to provide insights into the chemical diversity and anti-plasmodial activities of these compounds. It comprises of a total of **616** distinct structures and **15** known drugs that were reviewed from various research articles, and data was organised according to the chemical scaffolds. It will serve as a valuable resource to identify and optimise compounds with activity against the *P. falciparum* cyt *bc*₁ complex and ultimately contribute to the ongoing efforts to combat malaria.

3.2 Methods

The data from the published literature was collated into a database by manual curation of information about chemical compounds and respective biological activities from various accredited published research literature (including Elsevier, Future Science Ltd, ACS Publications, Wiley-VCH Verlag GmbH & Co., The Royal Society of Chemistry, Springer Science) searched using academic databases PubMed and Google Scholar. Articles were searched using different combination of keywords including malaria, electron transport chain, cyt *bc*₁ complex, complex III, cytochrome *bc*₁ inhibitors, *Plasmodium* cytochrome *bc*₁ inhibitors, endochin-like quinolones, beta-methoxyacrylates, atovaquone, naphthoquinones, quinolones, 4-pyridones, (1H-pyridin-4-ylidene)amines, chalcones, acridones, and tetracyclic benzothiazepines. Chemical structures were drawn and a table was compiled and organised using Instant JChem software, versions 20.15.0 (2020) and 22.19.0 (2022) from Chemaxon. The captured data was organised in the form of a table and saved as CSV files with information about chemical structure of compounds in the form of Simplified Molecular Input Line Entry System (SMILES) notations, their molecular formulas, molecular weights, various *P. falciparum* strains, biological 50% inhibitory activities (IC₅₀/EC₅₀), and citation of the articles that included PubMed ids (PMIDs), digital object identifiers (DOIs), and year of publications. For analysis, only compounds with IC₅₀/EC₅₀ values ≤1 μM were considered as

potential candidates. Other compounds with IC₅₀/EC₅₀ values >1 µM were considered as inactive for this study. A structure-activity relationship (SAR) study explained the molecular differences between active and inactive compounds. The general scaffolds images used to explain SAR observations were drawn using ACD/ChemSketch (Freeware) version 2019.2.1.

3.3 Results and Discussion

Several classes of compounds have been designed and evaluated against *Plasmodium* cyt *bc*₁ complex (Winter et al., 2006; Cross et al., 2010, 2011a, 2011b; Vallieres et al., 2012) including both the Qi or Qo site inhibitors, such as antimycin (Qi), myxothiazol (Qo) and stigmatellin (Qo) (Berry and Huang, 2011), acridinediones and acridones (Biagini et al., 2008; Kelly et al., 2009), quinolone (Nilsen et al., 2013; Stickles et al., 2016), and endochin-like quinolones (ELQs) (Biagini et al., 2012; Cowley et al., 2012; McConnell et al., 2018) along with other chemical scaffolds.

Each scaffold represented a unique chemical framework with distinct structural features that influenced its antimalarial activity and interaction with the *Plasmodium* cyt *bc*₁ complex. By analysing the data for each scaffold individually, the study aimed to uncover key trends and correlations that could guide future compound design and optimization. This detailed examination not only provides insights into the potency and selectivity of specific scaffolds but also highlights their potential against resistant *Plasmodium* strains, making them valuable candidates for further development.

3.3.1 Naphthoquinones

Naphthoquinones are a class of organic compounds derived from naphthalene, and exhibit a diverse range of biological activities, including antimicrobial, antiviral, and antitumor properties (Aminin and Polonik, 2020). Studies have shown that specific naphthoquinones possess potent inhibitory effects on the *Plasmodium* cyt *bc*₁ complex, making them attractive candidates for further exploration as antimalarial agents (Birth et al., 2014; Ferreira et al., 2002; Khan et al., 1998; Lanfranchi et al., 2012; Schuck et al., 2013). Furthermore, some naphthoquinones exhibit synergistic effects when combined with existing antimalarial drugs, potentially enhancing their therapeutic efficacy and overcoming drug resistance (Fivelman et al., 2002; Schuck et al., 2013; Srivastava and Vaidya, 1999). By targeting this essential mitochondrial enzyme, naphthoquinones offer a unique strategy for disrupting the parasite's energy metabolism and combating drug-resistant malaria. A

total of 71 naphthoquinones including atovaquone were collated, with anti-plasmodial activity ranging from 1.25 nM to >115 μ M.

Atovaquone displayed inhibition activities against various *P. falciparum* strains with IC₅₀ ranging from 0.1 to 8.26 nM (**Table 3.S.1**). For strains such as TM90-C2B, TM902CB, FCR3, A6, and TM93-C1088, atovaquone exhibited resistance with IC₅₀ exceeding 170 nM reaching up to 12 μ M. Results for atovaquone are compiled in **Table 3.S.1**.

Among all, naphthoquinones with esters (**Cdld A3 to A13**) or ethers (**Cdld A15 to A20**) side chains at the C2 position displayed the highest potency with the IC₅₀ values ranging from 1.25 nM to >100 nM compared to atovaquone (IC₅₀: 0.75 nM) against the ATQ-S Nigerian strain (El Hage et al., 2009). Amongst the esters and ethers, the ester-containing compounds (**Cdld A3 to A13**) were comparatively 3-fold to 10-fold more active (**Figure 3. 2**).

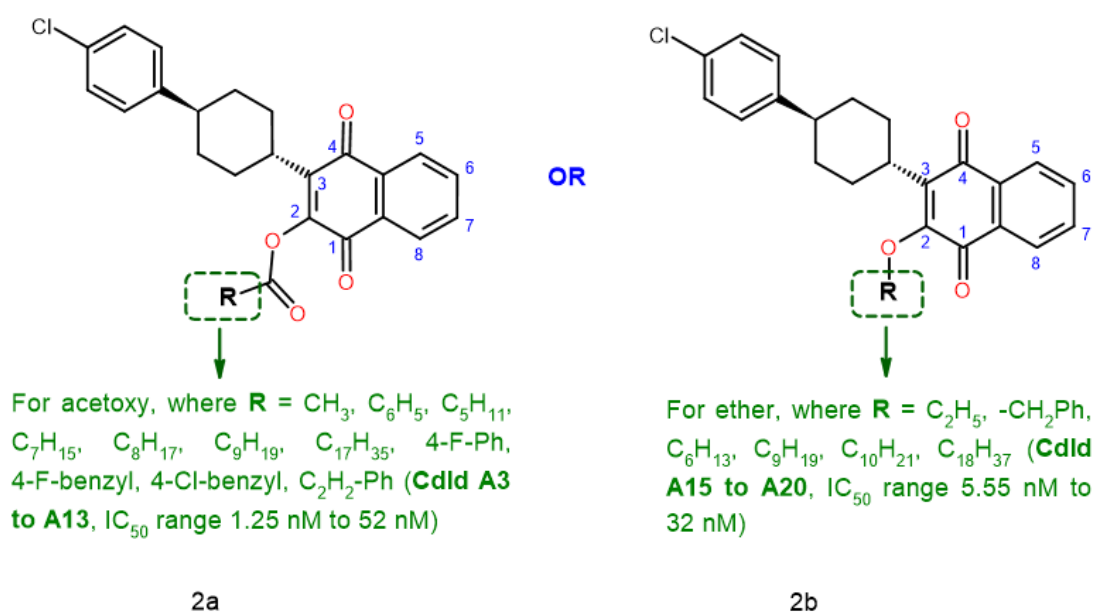


Figure 3.2: Naphthoquinones with esters (**Cdld A3 to A13; 2a**) and ethers at position 2 (**Cdld A15 to A20; 2b**) (El Hage et al., 2009).

Cdld A21 or 2-(1-hydroxyethyl)-4H,9H-naphtho[2,3-b]furan-4,9-dione (**3a**), isolated from *Tabebuia cassinoides*, displayed some activity against *P. falciparum* K1 and T9-96 strains with IC₅₀ value of 627 nM and 718 nM, respectively (**Figure 3.3**). **Cdlds A22** (Isopinnatal or (12R,13R,16S,18R)-6-hydroxy-16,18-dimethyl-2,9-dioxo-17-oxapentacyclo[11.4.1.0¹,10.0³,8.0¹²,16]octadeca-3(8),4,6,10-tetraene-18-carbaldehyde; **3b**), **A23** (Kigelinol or 4a,7-dihydroxy-1,4-dimethyl-2,3-

dihydrocyclopenta[b]anthracene-5,10-dione; **3c.1**) and **A24** (Isokigelinol or 7,10a-dihydroxy-3,11-dimethyl-1,2-dihydrocyclopenta[b]anthracene-5,10-dione; **3c.2**), isolated from *Kigelia pinnata* rootbark, exhibited diminished to no activity against the K1 and T9-96 strains (IC_{50} range from 763 nM to >16 μ M) (Moideen et al., 1999; Rodrigues et al., 2010).

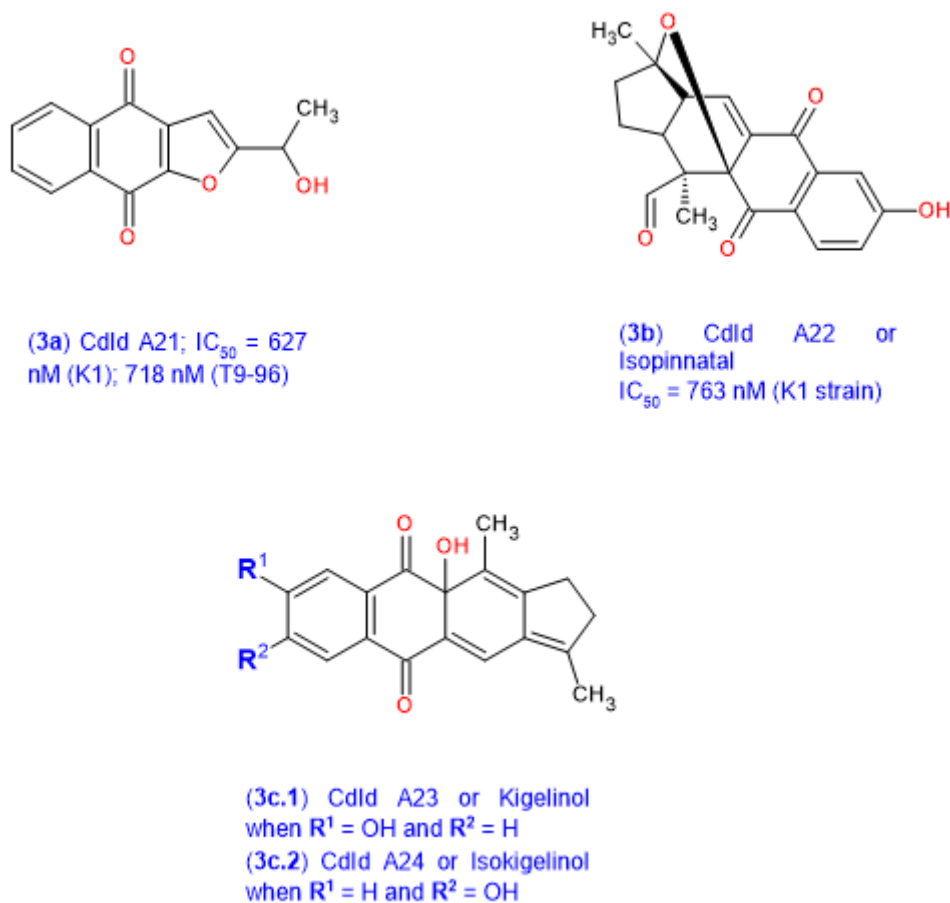


Figure 3.3: Chemical structures with antimalarial activities for compounds 2-(1-hydroxyethyl)-4H,9H-naphtho[2,3-b]furan-4,9-dione (**3a**), Isopinnatal (**3b**), Kigelinol (**3c.1**) and Isokigelinol (**3c.2**) adapted from (Moideen et al., 1999; Rodrigues et al., 2010).

Cdd A25, where $R^1 = \text{NH}_2$ and $R^2 = \text{Cl}$, exhibited $\text{IC}_{50} = 920 \text{ nM}$ (D6 strain) and 180 nM (W2 strain)

Compounds where $R^1 = \text{pyrrolidin-1-yl}$, morpholin-4-yl, phenylamino or Cl and $R^2 = \text{Cl}$ or H, exhibited diminished or no activity (**Cdd A26 to A29**, IC_{50} range $9.63 \mu\text{M}$ to $>115 \mu\text{M}$)

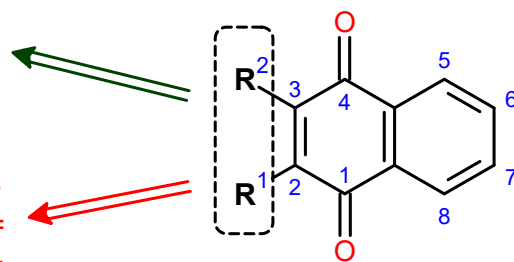


Figure 3.4: General structure for naphthoquinones with **Cdds A25 to A29** (Rodrigues et al., 2010).

Naphthoquinone **A25**, where R^1 was a primary amino group and R^2 was chlorine displayed high potency against W2 strain with an IC_{50} value of 180 nM and moderate activity against D6 strain with an IC_{50} value of 920 nM . The presence of tertiary amino groups such as pyrrolidin-1-yl or morpholin-4-yl at R^1 resulted in diminished activity against both W2 and D6 strains of *P. falciparum* compared to **A25**. Similarly the presence of a secondary amine such as phenylamino at R^1 and hydrogen at R^2 further reduced the potency of compound compared to **A25** (Rodrigues et al., 2010).

Cdd A32, where $R = \text{Ph}$, most potent with $\text{pIC}_{50} = 6.36$

Compounds where $R = \text{H}$, $4\text{-NO}_2\text{-Ph}$, 4-OMe-Ph , $\text{CH}(\text{CH}_3)_2$, or sulfanylmethyl group (**Cdd A30 to A70**) - exhibited reduced anti-plasmodial activity with pIC_{50} s from 4.05 to 6.36

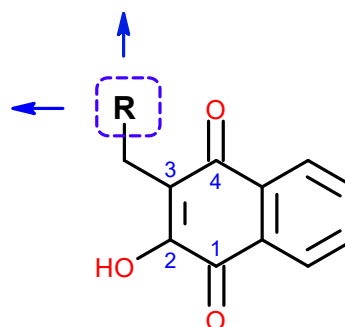


Figure 3.5: General structure for naphthoquinones with **Cdds A30 to A70** (Sodero et al., 2017).

3.3.2 Tetracyclic benzothiazepines

Tetracyclic benzothiazepines are a class of organic compounds distinguished by their unique four-ring (tetracyclic) structure, featuring a benzothiazepine core that consists of a benzene ring fused to a seven-membered azepine ring containing nitrogen and sulfur atoms. A total of 44 novel tetracyclic benzothiazepines (**Cdd B-1 to B-44**) (Dong et al., 2011; Gutteridge et al., 2023) with

structures in two tautomeric forms, keto and enol, were collated displaying antimalarial activities against *P. falciparum* (**Figure 3.6**). The enol tautomeric form tested against ATQ-S *P. falciparum* 3D7 and Dd2 strains (with IC₅₀ value ranged from 16 nM to >5000 μ M for both strains), along with cyt *bc*₁ enzyme inhibition activity against *P. falciparum* (IC₅₀ value ranged from 3 nM to >5000 M) and humans (IC₅₀ value ranged from 833 nM to >5000 nM) (Dong et al., 2011).

Of the enol derivatives, **Cdd B-3** was the most active against the *P. falciparum* 3D7 and Dd2 strains (IC₅₀ = 16 nM), whereas **Cdd B-2** displayed slightly higher potency against the Dd2 strain (IC₅₀ = 13 nM) compared to the 3D7 (IC₅₀ = 33 nM) (Dong et al., 2011).

The keto derivatives were tested against ATQ-S *P. falciparum* D6, W2 and TM91C235 strains (IC₅₀ value ranged from 12.3 nM to >29000 nM) with **Cdd B-23** displaying the best potency against all the three strains with IC₅₀ value of 12.3 nM (D6), 26.36 nM (W2) and 46.36 nM (TM91C235) (Gutteridge et al., 2023).

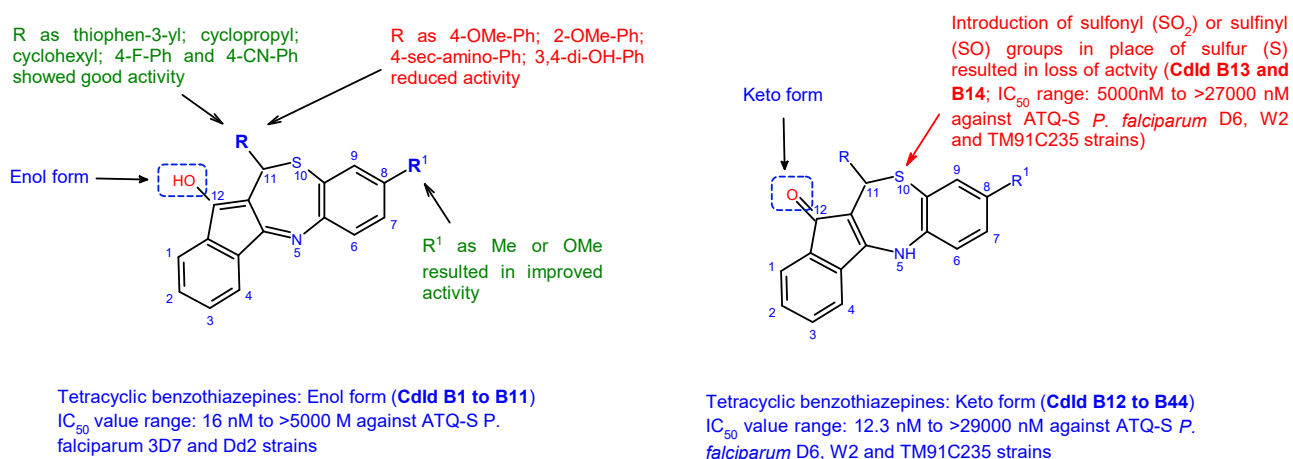


Figure 3.6: General structure for tetracyclic benzothiazepines (Scaffold B) indicating inhibitory ranges of side groups (Dong et al., 2011; Gutteridge et al., 2023).

SAR studies revealed that presence of methoxy group as R¹ resulted in compounds (**Cdd B-3 and B-23**) with promising antimalarial activity with IC₅₀ values ranging from 16 nM to 46.36 nM against ATQ-S *P. falciparum* strains 3D7, Dd2, D6, W2 and TM91C235. This could be due to the electron-donating nature of the methoxy group that increases the electron density of the aromatic system, thus increasing the nucleophilicity of the ring. The presence of the methoxy group also seemed to contribute towards stabilising the aromaticity in the system. In comparison, the presence of sulfonyl or sulfinyl group at C-10 position resulted in loss in activity with IC₅₀ values increasing to >5000 nM.

This could be due to both sulfonyl (**Cd1b B-13**) and sulfinyl (**Cd1b B-14**) being strong electron-withdrawing groups compared to sulphides, which resulted in reduced electron density in the aromatic system, thus reducing the nucleophilicity of the ring and destabilising the aromaticity of the system. The presence of a pyridin-4-yl (**Cd1d B-42**) and pyridin-3-yl (**Cd1d B-44**) rings as R (at C-11) resulted in reduced or loss of antimalarial activity (IC_{50} value range 2767 nM to >29000 nM).

3.3.3 Phthalimides

The phthalimide scaffold was designed to be used in many treatments, including antituberculosis as a cyt *bc*₁ complex inhibitor, anti-inflammatory compound developed as a phosphodiesterase-4 inhibitor (Barbosa et al., 2012) and as an antimalarial as a plasmepsin II and IV inhibitors in malaria (Singh et al., 2015). Phthalimide-containing compounds have been reported to have a potent antimycobacterial activity targeting the *bc*₁ complex of *M. tuberculosis* (van der Westhuyzen et al., 2015). Twelve phthalides (**Cd1d C-1 to C-12**) (**Figure 3.7**) were collated for their inhibitory activities against *P. falciparum* with IC_{50} values ranging from 4.2 μ M to >10 μ M (3D7 strain) (Okada-Junior et al., 2018). The most potent compound of the series, **Cd1d C-6** (4-amino-2-(4-methoxyphenyl)-1H-isoindole-1,3(2H)-dione) with a methoxy side group, inhibited ATQ-S *P. falciparum* 3D7 and K1 strains with IC_{50} values of 4200 nM and 4300 nM, respectively, along with inhibiting cyt *bc*₁ enzymatic activity by 74% at 70 μ M. This was significantly less active than atovaquone with an IC_{50} value of 3.4 nM for cyt *bc*₁ enzymatic activity (Okada-Junior et al., 2018). **Cd1d C-12** (**Figure 3.7**) was further less active with an IC_{50} value of 6800 nM against 3D7 strain. Whilst the rest of the phthalimide compounds were inactive with IC_{50} values >10 μ M. Compounds where R¹ was either hydrogen, nitro-, trifluoromethyl-, chloro- or methyl- groups displayed poor inhibitory activity (3% to 23% at 10 μ M) (Okada-Junior et al., 2018). The phthalimide derivatives collated, did not display any promising *in vitro* activity to warrant any further investigation.

Most potent compounds where $R^1 =$ OMe and $R^2 =$ H or $-\text{CH}_2\text{Ph}$; IC_{50} 4.2 μM and 6.8 μM (*Pf* 3D7)

Inactive when $R^1 = \text{NO}_2$, CF_3 , Cl, H, CH_3 and $R^2 =$ H or $-\text{CH}_2\text{Ph}$ ($\text{IC}_{50} > 10 \mu\text{M}$)

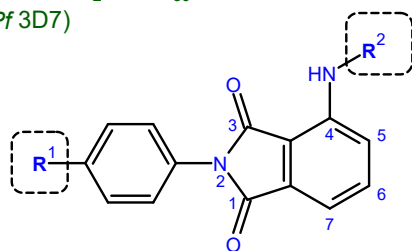
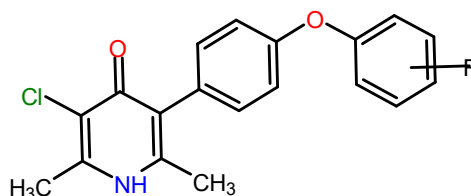
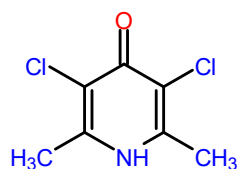


Figure 3.7: General structure for phthalimide (Scaffold C; **Cdd** **C-1** to **C-12**) displaying poor antimalarial activity as potential *Plasmodium* cytochrome *bc*₁ complex inhibitors (Okada-Junior et al., 2018).

3.3.4 Pyridine derivatives

In an attempt to explore different chemical inhibitors of parasite mitochondrial respiration as potential *Plasmodium* drugs, pyridine-based compounds related to clopidol (**Figure 3.8 (8a)**), an anticoccidial drug was identified (Fry and Williams, 1984). Clopidol has documented activity against *Plasmodium falciparum* chloroquine-sensitive strains (Fry and Williams, 1984; Markley et al., 1972; Yeates et al., 2008), as well as ATQ-R strain (US Patent US5053418, 1991). Compounds structurally related to clopidol, such as **Cdd** **D16** (GW308678; **Figure 3.8 (8b.1)**) and **Cdd** **D19** (GW844520; **Figure 3.8 (8b.2)**) bind to the *Qi* site of cyt *bc*₁, which is different from the atovaquone *Qo* binding site, and explains the ability of these compounds to overcome atovaquone resistance (Bueno et al., 2012; Capper et al., 2015). According to the collated data, GW844520 demonstrated promising activity against ATQ-R strain FCR3, with IC_{50} of 1 nM (Yeates et al., 2008). This along with favourable pharmacokinetic properties, initiated pre-clinical investigations; however, it displayed unexpected cardiotoxicity, which terminated further development (Flores-Sumoza et al., 2018).



8a; Clopidol

IC₅₀ = 20,000 nM (*Pf* T9-96)

IC₅₀ = 3370 nM (*Pf* FCR3-A)

IC₅₀ = 9730 nM (*Pf* W2)

(8b.1) **Cdld D16** or GW308678

R = 3-CF₃

IC₅₀ = 30 nM (*Pf* T9-96)

IC₅₀ = 2.5 nM (*Pf* FCR3A)

IC₅₀ = 5 nM (*Pf* 3D7A)

(8b.2) **Cdld D19** or GW844520

R = 4-OCF₃

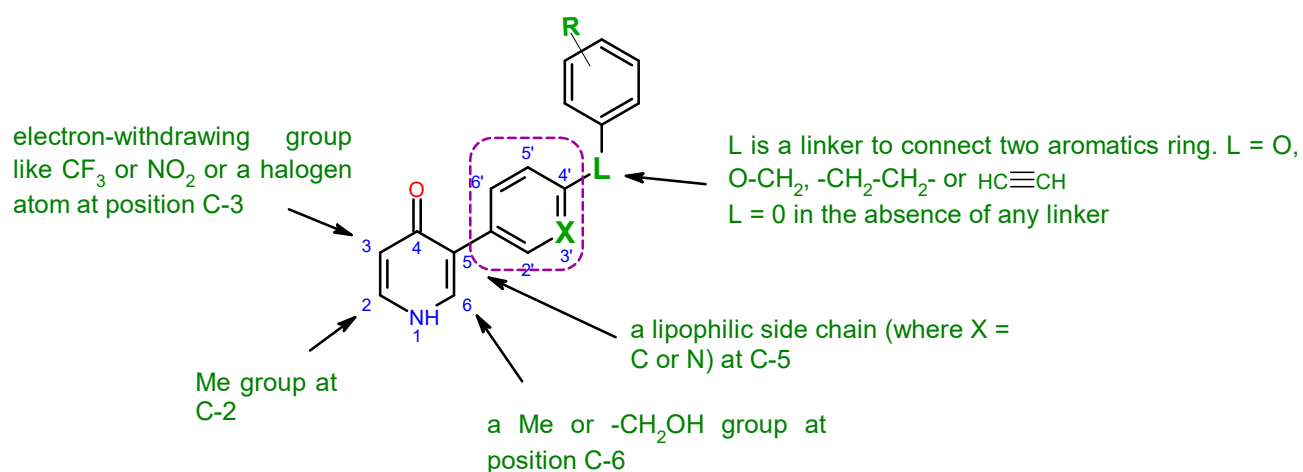
IC₅₀ = 30 nM (*Pf* T9-96)

IC₅₀ = 1 nM (*Pf* FCR3A)

IC₅₀ = 5 nM (*Pf* 3D7A)

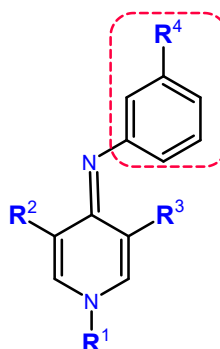
Figure 3.8: Chemical structures and antimalarial activities of clopidol (**8a**), GW308678 (**8b.1**) and GW844520 (**8b.2**) (Yeates et al., 2008).

Overall, a total of 118 unique pyridine-based compounds and respective *in vitro* antimalarial activity against various *P. falciparum* strains were collated (Bueno et al., 2011, 2012, 2018; Flores-Sumozá et al., 2018; Rodrigues et al., 2009, 2010, 2011; Yeates et al., 2008). For a better understanding, the collated compounds were categorised into three substructures depending on the structural features and SAR was studied (**Figures 3.9 (9a and 9b)**). Data analysis indicated that the 4(1H)-pyridones (**Figure 3.9 (9a)**; 98 compounds including **Cdld D1 to D56** and **E1 to E42**) were more active (IC₅₀ values ranged from 1 nM to 11 µM) compared to (1H-pyridin-4-ylidene)amines (**Figure 3.9 (9b)**; **Cdld F1 to F20** with IC₅₀ values ranging from 940 nM to >50 µM (Rodrigues et al., 2009)).



9a
4(1H)-pyridones (**Cdld D1 to D56 and E1 to E42**):
Compounds that exhibited good inhibitory activities
had the above structural specifications.

Two most potent compound of the series (**Cdld F12 and F13**, where $\text{R}^1 = \text{C}_2\text{H}_5$, $\text{R}^2 = \text{H}$ or Cl , $\text{R}^3 = \text{Cl}$, and $\text{R}^4 = -\text{CH}_2-\text{CH}_2-\text{C}_6\text{H}_4(4-\text{OCF}_3)$ with $\text{IC}_{50} \sim 1 \mu\text{M}$ (W2) and $1.7 \mu\text{M}$ (FCR3) (Rodrigues et al., 2009).



9b
N-phenylpyridin-4(1H)-imines (**Cdld F1 to F20**):
Compounds are less active compared to
4(1H)-pyridones.

Figure 3.9: Pyridine scaffolds (9a and 9b) (Bueno et al., 2011, 2012, 2018; Flores-Sumoza et al., 2018; Rodrigues et al., 2009, 2010, 2011; Yeates et al., 2008).

From the SAR analysis, common key takeaways were identified. The 5-phenyl-4(1H)-pyridones (**Cdld D1 to D56**) compounds with a substituted phenyl ring (**Figure 3.9 (9a)**, where X = C) as the lipophilic side chain, exhibited IC_{50} values in the range from 2 nM to 1300 nM, against the 3D7A (ATV-S) strain. Whilst the 5-pyridinyl-4(1H)-pyridones (**Cdld E1 to E42**) compounds with substituted pyridyl ring (**Figure 3.9 (9a)**, where X = N) as the lipophilic side chain displayed antimalarial activity with IC_{50}

values in the range from 2 nM to 472 nM. The least active of the group were the 1H-pyridin-4-ylidene)amines (**Figure 3.9 (9b)**; **CdId F1 to F20**).

From the SAR analysis, in general, it was observed that 4(1H)-pyridones (**CdIds D and E**) with a methyl group at position C-2, an electron-withdrawing group (CF₃ or NO₂) or a halogen atom (Cl or Br) at position C-3, a lipophilic side chain (with electron-withdrawing substituents) at position C-5, and a methyl or a hydroxymethyl group at position C-6 showed improved antimalarial activities compared to other pyridones. For the FCR3A (ATV-R) strain, these **CdIds D16, D19 and D38** displayed IC₅₀ values in the range from 1 nM to 6 nM. Notably, the presence of the –CH₂OH group at position-6 (**CdId D38 and D39**) was favourable for compounds to exhibit excellent anti-plasmodial activity (IC₅₀ value: 2 nM and 3 nM, respectively, against *Pf* 3D7A). In contrast, compounds (**CdId D33 and D34**) where this –CH₂OH group was present at position-2 displayed reduced activity (IC₅₀ values: 130 nM and 120 nM, respectively, against *Pf* 3D7A) (Bueno et al., 2011, 2012).

3.3.5 β -Methoxyacrylates and α -Methoxyiminoacetates

β -Methoxyacrylates are known to inhibit cytochrome c reductase and have been used in agrochemical industry to prepare pesticides due to their potential to inhibit the mitochondrial respiration process of some insects and fungal plant pathogens (Alzeer et al., 2000; Luo et al., 2022). These compounds were explored for their antimalarial activities to inhibit the mitochondrial electron transport chain, specifically the cyt *bc*₁ complex. The key structural features responsible for its potency and selectivity can be identified by analysing the SAR studies of 27 unique methoxyacrylate-based compounds collated from the literature (Alzeer et al., 2000; Rodrigues et al., 2010). The compounds were defined by three parts in the molecule namely, (A) the phenyl ring A with either β -methoxyacrylate (where, Z = C) or α -methoxyimino acetate (where, Z = N) substitution; (B) the mono- or di-substituted phenyl ring B; and (C) the linker 'L' that connects the two aromatic rings, A and B.

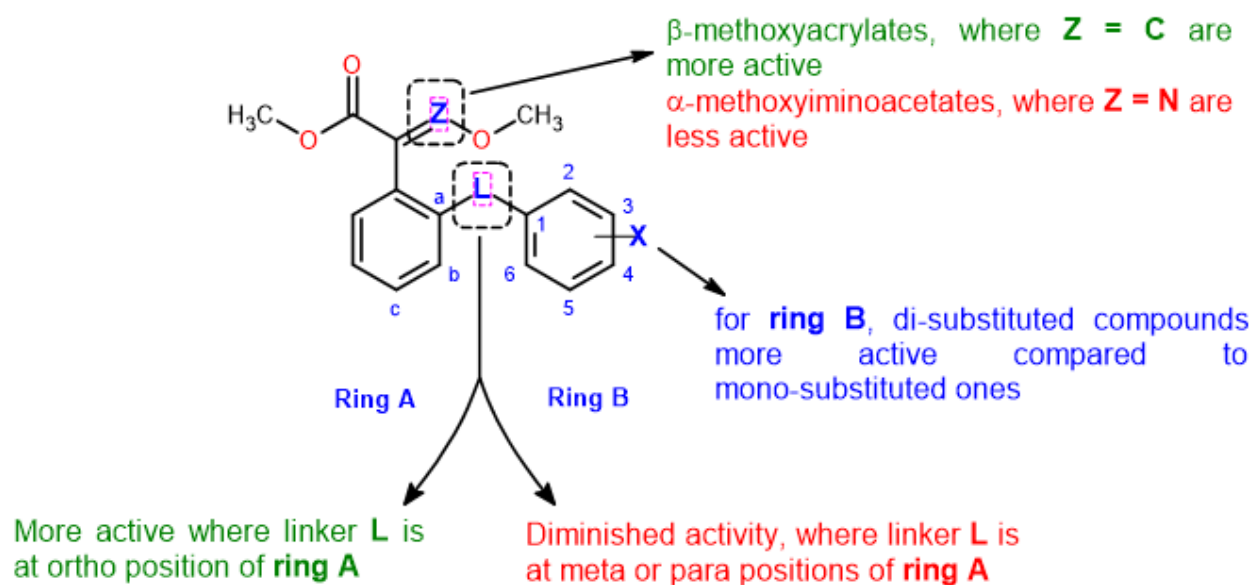


Figure 3.10: Methoxyacrylate (Scaffold G; **CdId G1 to G27**) (Alzeer et al., 2000).

Specific SAR features were identified upon analysis of these **CdId G1 to G27** (**Figure 3.10**); where compounds with α -methoxyacrylate group (**CdId G1 to G21**) on ring A were more active (IC_{50} range of 0.08 nM to >10 μ M for NF54 (ATV-S) and 0.14 nM to >10 μ M for K1 (ATV-S)) compared to β -methoxyimino acetates (**CdId G22 to G27**; IC_{50} range varied from 1.03 nM to >3.5 μ M for NF54 (ATV-S) and 3.6 nM to >5.9 μ M for K1 (ATV-R)).

Secondly, amongst the linkers 'L' tested, the best potency was exhibited by compounds where (1E,3E)-buta-1,3-dien-1-yl (-CH=CH-CH=CH-) was used as the linker connected to ring A at its *ortho* position (IC_{50} range: 0.08 nM to 20.1 nM against *Pf*NF54 and 0.14 nM to 78.6 nM against *Pf*K1). On the other hand, compounds with linkers like methyl sulphanyl (-CH₂-S) (**CdIds G1 and G3**), ethene (**CdId G2**), ethylidene(methoxy)amine (**CdId G4**), n-butane (**CdId G6**), furan (**CdId G7**), but-1-en-3-yne (**CdId G23**), buta-1,3-diyne (**CdIds G24, G25**) and thiophene (**CdId G26**) were less active (IC_{50} range: 0.42 nM to >3.5 μ M against *Pf*NF54 and 1.59 nM to >5.9 μ M against *Pf*K1). The least activity (IC_{50} range: nM) was shown by compounds where the linker was attached to the *meta* or *para* position on ring A (**CdIds G20 and G21**), which seemed to have diminished their activities.

Further, amongst the α -methoxyacrylates with (1E,3E)-buta-1,3-dien-1-yl as a linker at the *ortho* position, the highest potency was shown by compounds with di-substitution at positions 2 and 3 or 4 on ring B (**CdIds G15 to G19**). Amongst the mono substitution at ring B, compounds which were substituted with an electron withdrawing group either at position 2 (**CdIds G5, G9, and G10**) or 4

(**Cdd G14**) showed better anti-plasmodial activities compared to the ones substituted at position 3 (**Cdlds G11 to G13**) (**Figure 3.10**).

Amongst β -methoxyiminoacetates, compounds with Z-configuration (**Cdlds G22, G23, G24, G26 and G27**) showed better activities compared to the one with E-configuration (**Cdd G25**) (**Figure 3.11**). From various linkers, buta-1,3-dienyl provided the best results (**Cdd G22**).

Z-configuration is more active (**Cdlds G22, G23, G24, G26 and G27**)
 IC_{50} range : 1.03 nM to 56.51 nM (*Pf* NF54)
 IC_{50} range: 3.6 nM to 145 nM (*Pf* K1)

E-configuration is less active (**Cdd G25**)
 IC_{50} = 3599 nM (*Pf* NF54)
 IC_{50} = 5906 nM (*Pf* K1)

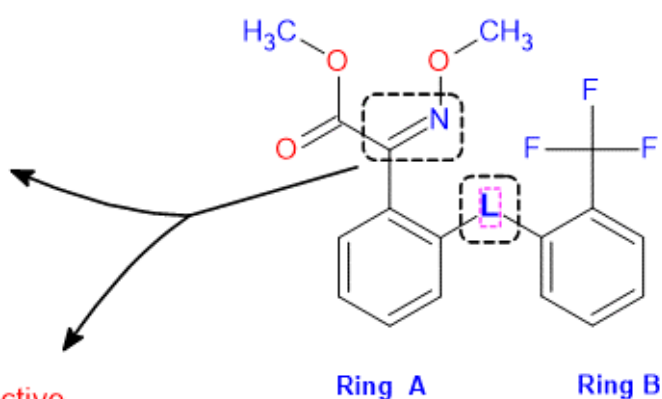


Figure 3.11: The influence of the Z- to E-configuration of the β -methoxyiminoacetates on the antimalarial activity (Alzeer et al., 2000).

3.3.6 Acridones and acridines

Acridones, also known as acridin-9(10H)-ones act as a structural and functional surrogate of the acridone framework of the antimalarial drug WR243251 (**figure 3.12**) (Valdés, 2011). Compounds (S)-WR 249685 and racemic floxacrine are known cyt bc_1 complex inhibitors with cross-resistance with atovaquone in bc_1 complex mutation (Rodrigues et al., 2010).

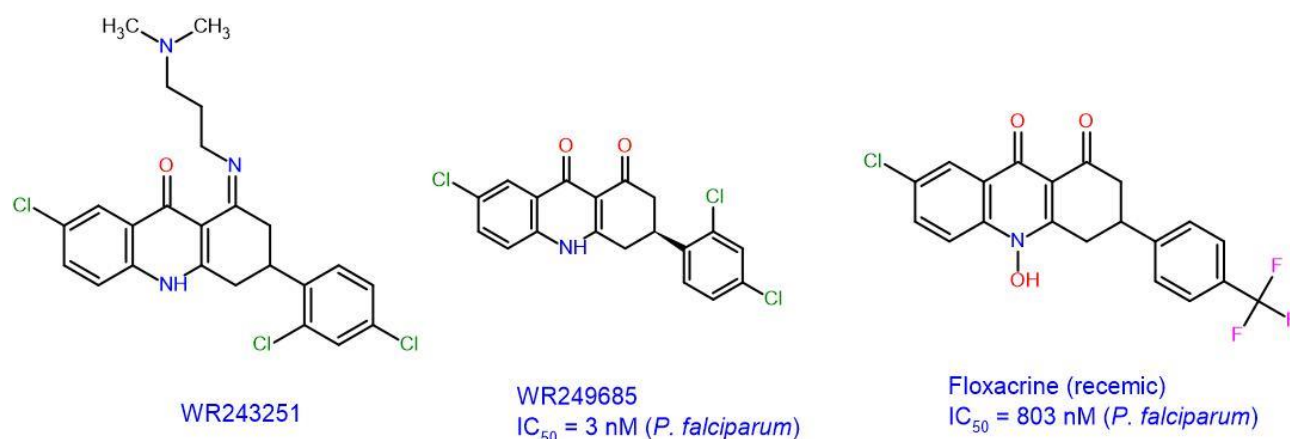
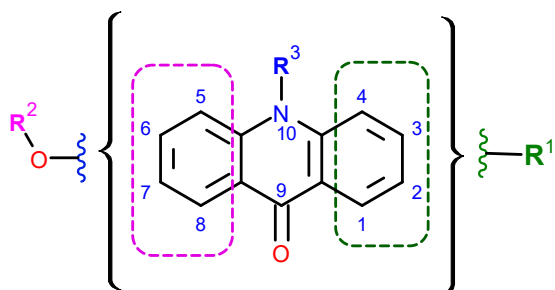


Figure 3.12: Chemical structures of **WR243251**, **WR249685** and **Floxacrine** (Carrasco et al., 2013).

According to the data collated, these compounds were found to be more active compared to atovaquone when tested for antimalarial activity against Dd2 (IC_{50} values ranging from 0.0008 nM to 50 μM) and D6 strains (IC_{50} values ranging from 0.0015 nM to 50 μM) of *P. falciparum* parasite. Atovaquone's activity was $IC_{50} = 0.3 \text{ nM}$ and 0.5 nM against D6 and Dd2 strains, respectively (Rodrigues et al., 2010; Winter et al., 2008).



Most active when $R^1 = \text{Cl}$, $R^2 =$ 5,6,6,6-tetrafluoro-5-(trifluoromethyl)hexyl, and $R^3 = \text{H}$.

Presence of terminal CF_3 group in the alkyl chain forming R^2 in all active compounds.

When $R^1 = 3\text{-NH}_2$, $R^2 = 6,6,6\text{-trifluorohexyl}$, and $R^3 = \text{H}$, with $IC_{50} = 78 \text{ nM}$ (against atovaquone resistance strain TM90-C2B).

Best activity when $-\text{OR}^2$ group is at C-6.

Diminished activity when $-\text{OR}^2$ group is at C-7 or C-4.

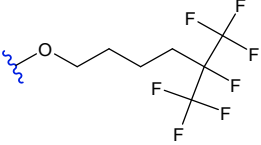
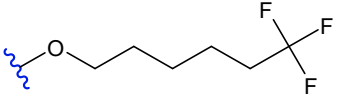
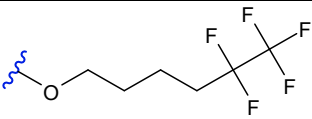
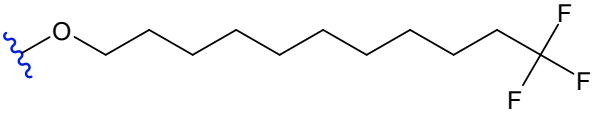
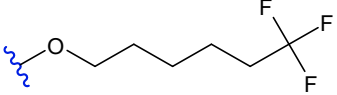
$R^1 = \text{H}$ or 2-NH_2 and $R^3 = \text{H}$ or O or CH_3 further reduced the activity

Figure 3.13: General structure for acridones (Scaffold H; **CdId H1** to **H34**) (Rodrigues et al., 2010).

Some general SAR features that were identified in this scaffold are outlined below:

- Acridones containing a long alkyl side chain (six or more carbon atoms) with a terminal CF₃ group as R² and 3-Cl or 3-NH₂ groups as R¹ exhibited higher antimalarial activities compared to other compounds of the series, for example, **Cdlds H1 to H6**.
- Compounds where the terminal CF₃ group of the alkyl side chain was replaced by Cl (**H15, H20**), Br (**H17, H22**), OH (**H11**) or a secondary amino group N(CH₃)₂ (**H21**), seemed to have diminished activity.
- R² with an alkyl chain containing either less than six carbon atoms (**Cdld H7, H9, H10**) or more than seven carbon atoms (**Cdld H4 and H6**) also displayed diminished activity.
- Similarly, compounds where chlorine at R¹ was replaced by NO₂ group (**Cdld H13**), showed a reduced activity compared to **Cdld H5**.
- Compounds where carbonyl oxygen of acridone at position C9 was replaced by nitrogen to give 9-amino acridine (**Cdld H16 and H12**) seemed to exhibit reduced activity compared to other compounds, indicating that the presence of carbonyl oxygen aids in improved activity.
- The presence of a ring N at position 10 is also essential. When replaced by oxygen resulting in xanthone (**Cdld H33**), the compound was found to have significantly reduced activities against D6 (IC₅₀: 16 µM) and Dd2 (IC₅₀: 18 µM) strains, respectively.
- Alkylation of the ring N with a methyl group at R³ resulted in great drop in activity for **Cdld H31** (IC₅₀ 4 µM for D6 and 3.5 µM for Dd2).
- Comparing **Cdld H8, H18 and H27**, the presence of OR² group at position C6 resulted in better activity compared to its presence at either C4 or C7 position.

Table 3.1: SAR data for compounds arranged in increasing order of their IC₅₀s and position of the -OR² group (Rodrigues et al., 2010).

CdId	R ¹	OR ² @ position C6	R ³	IC ₅₀ nM (Pf D6)	IC ₅₀ nM (Pf Dd2)
H1	3-Cl		H	0.0015	0.0008
H2	3-NH ₂		H	0.018	0.025
H3	3-Cl		H	0.02	0.02
H4	3-Cl		H	0.023	0.025
H5	3-Cl		H	0.06	0.07

3.3.7 Quinolones

Quinolone compounds, characterised by their bicyclic aromatic structure containing a ketone and a reduced aromatic ring with nitrogen, have garnered significant attention as potential inhibitors of the *Plasmodium* cyt *bc*₁ complex. This class of compounds, known for their diverse pharmacological activities, including anticancer, antibacterial and antiviral effects, demonstrates promise in disrupting the energy metabolism and survival of the malaria parasite (Dhiman et al., 2019; Sharma et al., 2022).

Quinolone compounds exert their inhibitory effects on the *Plasmodium* cyt *bc*₁ complex by interfering with the electron transfer process (Oettmeier et al., 1994). Through specific interactions with the ubiquinone-binding site within the complex, these compounds disrupt the flow of electrons, leading to a cascade of events that perturb the parasite's energy metabolism and trigger oxidative stress-induced damage (Nilsen et al., 2014; Stickles et al., 2015a, 2016,). Another promising class of compounds known as endochin-like quinolones (ELQs), have also demonstrated potential as novel and effective antimalarial compounds (Cross et al., 2010; McConnell et al., 2018;

Nilsen et al., 2014; Winter et al., 2011, 2008). Studies have demonstrated their promising antimalarial activity, even against drug-resistant *Plasmodium* strains. By shedding light on the promising attributes of ELQs, we contribute to the collective efforts to develop innovative antimalarial therapies.

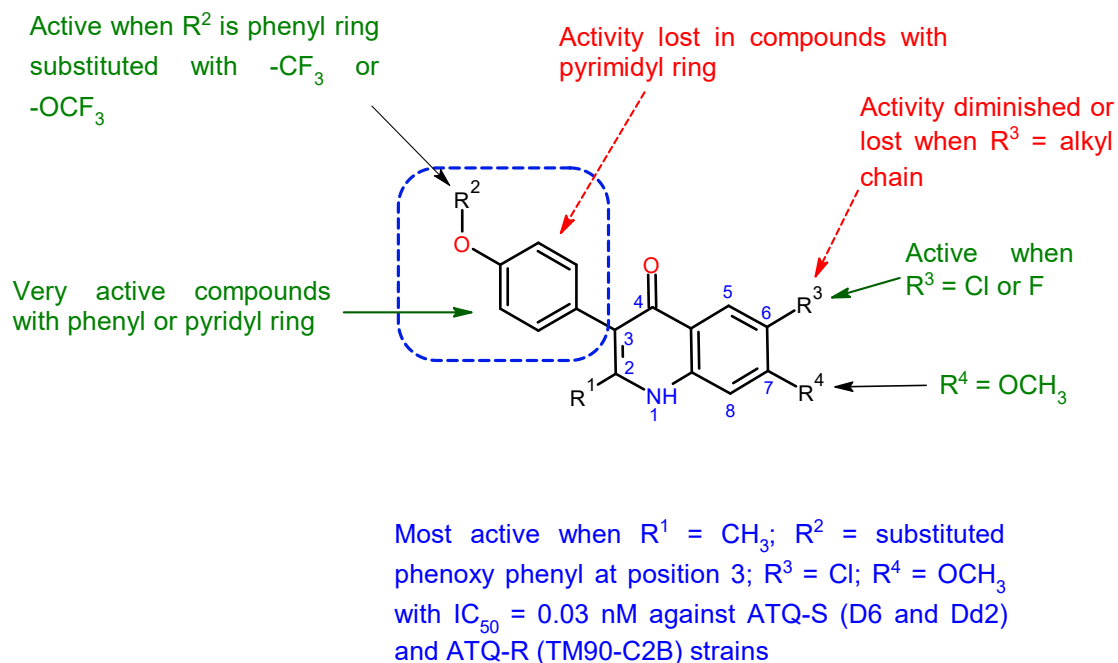


Figure 3.14: SAR for Quinolone (Scaffold I) (Nilsen et al., 2014; McConnell et al., 2018; Biagini et al., 2012).

SAR studies offer valuable insights into the fundamental molecular interactions that drive inhibitory activity, helping to guide the design of more potent and optimised antimalarial agents (McConnell et al., 2018; Stickles et al., 2015b; Vallieres et al., 2012).

A total of 257 distinct quinolone compounds have been studied here exhibiting antimalarial activities ranging from $\text{EC}_{50}/\text{IC}_{50}$ 0.03 nM to $>62 \mu\text{M}$ against various *P. falciparum* strains. Amongst the quinolones reviewed, the most active compounds were **Cdlds I-161** and **I-162** with EC_{50} s in the sub-nanomolar range against both, ATQ-S (D6 and Dd2) and ATQ-R (TM90-C2B) strains (Nilsen et al., 2014).

Some of the key features of SAR observed in general are outlined below:

- Compounds with methyl at position-2, phenoxy-phenyl (**ELQ300**, **Cdld I-153**) or (phenoxy)pyridyl (**Cdld I-170**) groups at position-3, Cl at position-6 and methoxy at position-7 displayed excellent activity against both ATQ-S and ATQ-R strains.

- As highlighted in the figure above, phenoxy group ($-OR^2$) with either $-CF_3$ or $-OCF_3$ (**CdIds I-161, I-162**) seemed to provide improved activity. Substitution of this $-CF_3$ or $-OCF_3$ with Cl or F (**CdIds I-163, I-164**) resulted in diminished activity.
- Replacement of Cl with F (**CdIds I-158, I-160, I-172**) as R^3 group affected the activity by increasing the IC_{50} values slightly. However, compound I-160 displayed good activity against both, the sensitive and the resistant strains. Similarly, replacing the phenoxy-phenyl or phenoxy-pyridyl group at position-3 with an alkyl chain led to reduced activity, for e.g. **CdIds I-69 and I-70**.
- Removal of either Cl at position-6 (**CdId I-157**) or $-OMe$ group at position-7 (**CdId I-156**) or both (**CdIds I-155 and I-142**) also led to compounds with slightly reduced activity compared to ELQ300 (**CdId I-153**) where both groups are present.
- Substitution of phenoxy-phenyl with either pyridinyloxy-phenyl (**CdId I-173**) or bi-phenyl (**CdIds I-194, I-199**) also resulted in reduced activity of compounds.
- Presence of unsubstituted phenyl (**CdId I-64**) or para-trifluoromethoxy phenyl at position-3 (**CdId I-201**) also resulted in compounds with diminished activities, suggesting that phenoxy-phenyl or phenoxy-pyridinyl groups contributed towards better anti-plasmodial activity of compounds.
- All ELQs showed better activities against *Pf* D6 and Dd2 strains than the TM90-C2B strain.

Table 3.2: SAR data for selective quinolone compounds) (Nilsen et al., 2014; McConnell et al., 2018; Biagini et al., 2012).

CdId	R1	R2	R3	R4	D6 (IC_{50} nM)	Dd2 (IC_{50} nM)	TM90-C2B (IC_{50} nM)
I-161	Me	(m- OCF_3)-phenoxy phenyl	Cl	OMe	0.03	0.03	<0.03
I-162	Me	(p- CF_3)-phenoxy phenyl	Cl	OMe	0.3	0.3	0.05
I-163	Me	(4-Cl)-phenoxy phenyl	Cl	OMe	1.9	1.8	1.2
I-164	Me	(4-F)-phenoxy phenyl	Cl	OMe	0.8	3.8	-
I-153 (ELQ-300)	Me	(p- OCF_3)-phenoxy phenyl	Cl	OMe	0.9 to 3.06	2.5 to 6.6	1 to 4.62
I-160	Me	(p- OCF_3)-phenoxy phenyl	F	OMe	1.4	1.4	0.5

In conclusion, quinolone compounds present a promising avenue for the design of *Plasmodium* cyt *bc*₁ inhibitors, offering new possibilities for antimalarial drug development. Their diverse pharmacological activities, coupled with their specific interaction with the cyt *bc*₁ complex, make quinolone compounds attractive candidates for further exploration and optimisation. The elucidation of their SAR and mode of action opens avenues for designing potent and selective antimalarial agents to combat this devastating disease.

3.3.8 Chalcones

Chalcones have emerged as strong contenders as antimalarial lead compounds. These ketone compounds have two aromatic rings, A and B, connected via a three-carbon α,β -unsaturated carbonyl group. Licochalcone A (**Figure 3.15**) inhibits complex II (succinate ubiquinone reductase, SQR) and complex III (also known as *bc*₁ complex or ubiquinol-cytochrome c reductase) of *P. falciparum* mitochondrial electron-transport chain (Chen et al., 1994). It inhibits the *bc*₁ complex at a very low concentration, making it a promising scaffold for developing novel antimalarial drugs (Mi-Ichi et al., 2005).

This study analysed eight chalcone compounds with antimalarial activities against *P. falciparum* W2 and D6 strains displaying IC₅₀ values ranging from 0.23 nM to 3.2 nM (*Pf* W2 strain) and 0.19 nM to 3.8 nM (*Pf* D6 strain) (Rodrigues et al., 2010).

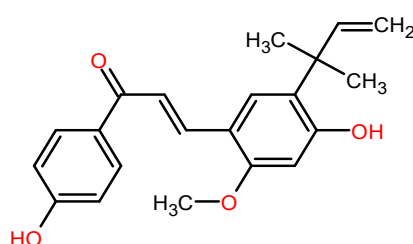
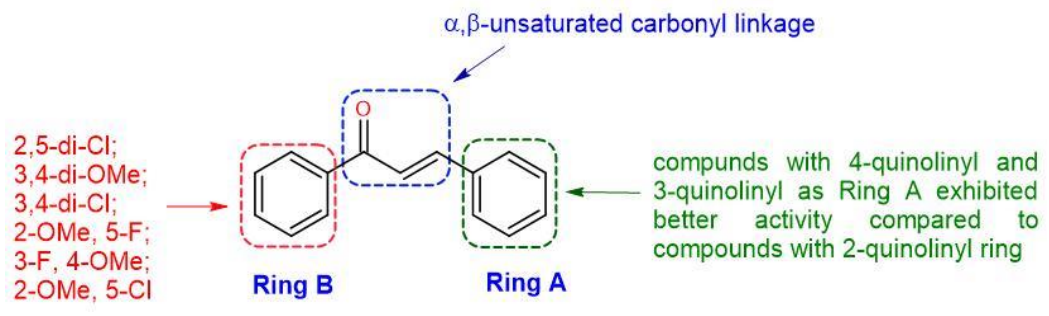


Figure 3.15: Chemical structure of Licochalcone A (Mi-Ichi et al., 2005).

Table 3.3: SAR data for chalcone compounds (Rodrigues et al., 2010).

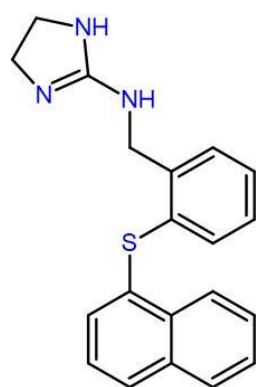
				
CdId	Ring B	Ring A	IC ₅₀ nM (<i>Pf</i> W2)	IC ₅₀ nM (<i>Pf</i> D6)
J-1	3,4-di-OMe phenyl	quinolin-4-yl	0.5	0.88
J-3	2,5-di-Cl phenyl	quinolin-4-yl	0.23	0.19
J-4	3,4-di-Cl phenyl	quinolin-4-yl	0.8	1.7
J-7	3-F, 4-OMe phenyl	quinolin-4-yl	3.2	0.93
J-2	2,5-di-Cl phenyl	quinolin-3-yl	1.7	0.62
J-6	2-OMe, 5-F phenyl	quinolin-3-yl	1.1	0.46
J-8	2-OMe, 5-Cl phenyl	quinolin-3-yl	0.59	0.95
J-5	2-OMe, 5-F phenyl	quinolin-2-yl	0.67	3.8

From the above-collated table, we observed that compounds where Ring A is a quinolin-4-yl moiety exhibited better antimalarial activity compared to compounds where Ring A is either quinolin-3-yl or quinolin-2-yl. Amongst the quinolin-4-yl compounds, the best activity was shown by compounds where Ring B is 2,5-di-chloro-phenyl. However, the presence of methoxy and fluoro groups as di-substituents in Ring B (**CdId J-7**) seem to diminish the activity of compounds.

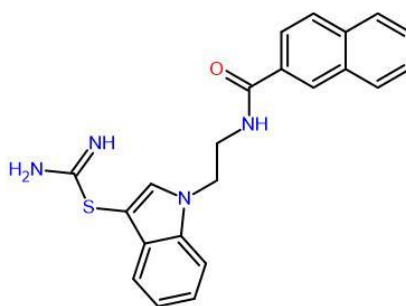
Since the number of collated chalcones was relatively low, it limited our ability to draw any definitive conclusions regarding their SAR and overall antimalarial potential. While preliminary observations could be made, the small dataset restricted the comprehensive trend analysis. To gain a clearer understanding of the activity profile and optimization potential of these scaffolds, a larger collection of structurally related compounds is needed. Expanding the dataset with additional analogues will allow for a more robust SAR evaluation and better insights into their efficacy, selectivity, and potential against resistant *Plasmodium* strains.

3.9 Miscellaneous compounds

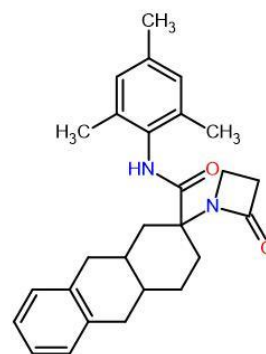
In this study, 46 novel inhibitors were reportedly screened for their antimalarial activity targeting cyt *bc*₁ complex. Since these compounds did not structurally fit into any scaffolds described above, they were captured as miscellaneous compounds and categorised under scaffold K. The compounds were mainly tested against the *P. falciparum* W2 strain and few compounds against the FCR3 strain. Most of the compounds were found to be inactive (IC_{50} s >10 μ M), with a few identified as potent molecules. The best activity was shown by compound **Specs AE-641 00605023 (Cdid K-41)** with an IC_{50} value of 0.43 μ M against the FCR3 strain. For the W2 strain, the best activity was demonstrated by **Life Chemicals F1386-0209 (Cdid K-40)** with an IC_{50} value of 1.58 μ M (Carrasco et al., 2013; Rodrigues et al., 2011).



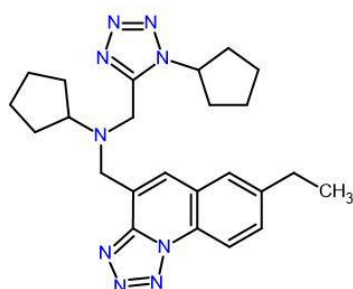
Specs AE-641 00605023
 IC_{50} = 0.43 μ M (FCR3)
 IC_{50} = 8.39 μ M (W2)



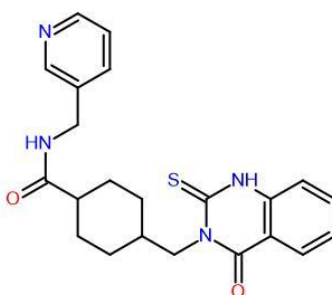
Life Chemicals F1386-0209
 IC_{50} = 2.71 μ M (FCR3)
 IC_{50} = 1.58 μ M (W2)



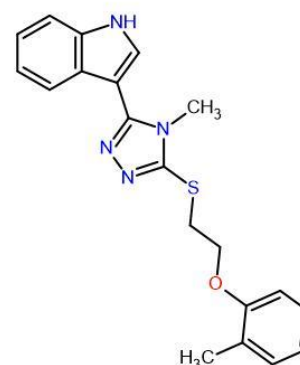
Toslab 802803
 IC_{50} = 2.81 μ M (FCR3)
 IC_{50} = 2.88 μ M (W2)



Asinex ASN 06130599
 IC_{50} >10 μ M (FCR3)
 IC_{50} = 9.16 μ M (W2)



ChemBridge 7758134
 IC_{50} = 1.97 +/- 0.9 μ M (W2)



ChemBridge 7969627
 IC_{50} = 6.69 +/- 2.1 μ M (W2)

Figure 3.16: Selective miscellaneous compounds (Scaffold K) (Carrasco et al., 2013; Rodrigues et al., 2011).

3.4 Conclusion

Certain chemical scaffolds exhibited a higher proportion of active compounds, making them strong candidates for further optimization. Scaffolds effective against resistant strains are particularly valuable for combating ATQ-R malaria. This analysis provides critical insights into structure-activity relationships and helps prioritize scaffolds for preclinical development.

Among the 616 collated compounds, only 135 demonstrated activities against ATQ-R strains, and 22 inhibited the *Plasmodium* cyt *bc*₁ enzyme, highlighting a significant gap in research. Quinolones (scaffold I) emerged as the most explored and potent class, with 196 out of 257 compounds active against Plasmodium, including 130 effective against resistant strains and 11 inhibiting the cyt *bc*₁ enzyme. Tetracyclic benzothiazepines (scaffold B) showed promise, with 31 out of 44 compounds active against *P. falciparum*, though none were tested against resistant strains; 8 inhibited the cyt *bc*₁ enzyme. Other scaffolds exhibited limited activity against resistant strains:

- Naphthoquinones (scaffold A): 22 out of 70 were active against *P. falciparum*, but none were tested against resistant strains or cyt *bc*₁ inhibition.
- Pyridines (118 compounds, scaffolds D, E, F): 82 were active, but only three (CdIds D-16, D-19, D-38) showed potency against the FCR3 strain and cyt *bc*₁ enzyme.
- Acridones-acridine (Scaffold H): 28 out of 34 were active, but only one (CdId H2) was effective against resistant *P. falciparum* (IC₅₀ = 78 nM).
- Chalcones (Scaffold J): The least explored, with eight compounds showing high potency against sensitive strains, but none tested against resistant strains or enzyme inhibition.
- Methoxyacrylates (Scaffold G): 25 out of 27 were highly active against sensitive strains, but no data was available for resistant strains or cyt *bc*₁ inhibition.
- Miscellaneous (Scaffold K): Most had IC₅₀ > 1 μM, with CdId K-41 being the only compound reported active against the resistant FCR3 strain.

The lack of extensive testing against ATQ-R strains and enzyme inhibition underscores the urgent need for more studies in this area. Identifying and optimizing lead compounds with antimalarial activity, favourable pharmacokinetics, and drug-likeness properties is essential to addressing drug resistance.

In conclusion, compiling data from existing literature is critical for advancing antimalarial drug discovery. This approach streamlines research by avoiding redundant efforts, refocusing on

promising leads, and potentially repurposing previously disregarded compounds. A well-organized database serves as a valuable resource for interdisciplinary collaboration, accelerating the identification of novel therapeutic agents to combat drug-resistant malaria and reduce disease burden globally.

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SUPPLEMENTARY INFORMATION

Table 3.4: Activities of atovaquone against cyt *bc*₁ enzyme and different strains of *P. falciparum*.

<i>Plasmodium falciparum</i> strains	Antimalarial activity IC ₅₀ values (nM)	References
Cyt <i>bc</i> ₁ enzyme inhibition potential		
<i>Homo sapiens</i>	350 ± 12	(Dong et al., 2011)
<i>P. falciparum</i>	0.2 ± 0.03	
Anti-plasmodial activity against various <i>P. falciparum</i> strain		
D6	0.1 to 0.3	(Nilsen et al., 2014, 2013; Winter et al., 2008)
D1	0.7	(Stickles et al., 2015a)
Dd2	0.1 to 1	(Dong et al., 2011; Nilsen et al., 2014, 2013; Stickles et al., 2015a; Winter et al., 2008)
Dd2 EndR2	2.1	(Nilsen et al., 2013)
Dd2 EndR1	5.13	(Nilsen et al., 2013)
Dd2 EndR3	8.26	(Nilsen et al., 2013)
FCR3-A	1360	(Yeates et al., 2008)
FCR3	1890	(Rodrigues et al., 2009)
K1	1.12	(Alzeer et al., 2000)
NF54	0.3	(Alzeer et al., 2000)
T9-96	3	(Bueno et al., 2012; Rodrigues et al., 2011, 2010; Yeates et al., 2008)
W2	0.3; 0.54; 1.2	(Cross et al., 2010; Nilsen et al., 2013; Rodrigues et al., 2009)
TM90C2B	>170 to 12000	(Biagini et al., 2012; Cross et al., 2010; Nilsen et al., 2014, 2013; Stickles et al., 2015a; Winter et al., 2008)
TM93-C1088	>2500	(Nilsen et al., 2014)
3D7	0.9 to 1	(Biagini et al., 2012; Dong et al., 2011)
3D7A	0.2 to 0.27	(Bueno et al., 2012; Yeates et al., 2008)
3D7 luc	0.36	(Nilsen et al., 2013)
A6	>170	(Nilsen et al., 2013)
ARC08-0063	0.17	(Nilsen et al., 2013)
PL08-009	0.17	(Nilsen et al., 2013)
PL08-025	2.18	(Nilsen et al., 2013)
Nigerian	0.75	(El Hage et al., 2009)
TM91C235	1.2	(Hughes et al., 2011)
TM902CB	12000	(Biagini et al., 2012)

Chapter 4

Analysis of Collated Compounds for Activity Against Resistant Strains: Identification, Evaluation, and *In Silico* Insights

Drug resistance remains a significant challenge in antimalarial therapy, necessitating the identification of novel compounds effective against resistant *Plasmodium* strains. This chapter focuses on the analysis of previously collated compounds (discussed in Chapter 3), specifically those exhibiting activity against ATQ-R strains. By systematically evaluating these compounds, this study aimed to identify promising candidates for further optimization. *In silico* approaches, including molecular docking, MMGBSA rescoring, MD simulations, and ADMET predictions, were employed to assess their binding potential, stability, and drug-likeness properties. The findings provide valuable insights into structure-activity relationships, aiding in the prioritization of scaffolds for preclinical development and the design of more effective antimalarial therapies.

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Conceptualization	✓		✓
Literature search	✓		
Database compilation and data analysis	✓		
Writing – preparation of original draft	✓		
Writing – review and editing	✓	✓	✓
Supervision		✓	✓

Collation and Computational Analysis of Antimalarial Compounds Targeting Atovaquone-Resistant *Plasmodium falciparum*

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Abstract

Malaria continues to pose a major global health burden, highlighting the urgent need for new therapeutic strategies. The *Plasmodium* cytochrome *bc*₁ complex, a critical component of the parasite's mitochondrial electron transport chain, represents a well-validated drug target. In this study, a diverse set of previously reported antimalarial compounds was systematically collated and categorised into various chemical scaffolds, with emphasis on their activity against atovaquone-resistant strains. Computational approaches, including molecular docking, molecular dynamics (MD) simulations, MM-GBSA binding free energy calculations, and *in silico* predictions of drug-likeness and ADMET properties, were employed to identify promising candidates. Structure–activity relationship (SAR) analysis was used to evaluate key features influencing potency. The results highlight potential lead compounds with favourable pharmacokinetic profiles and suggest their suitability for further structural optimization and experimental validation in the pursuit of effective treatments targeting drug-resistant malaria.

Keywords: malaria, *Plasmodium falciparum*, mitochondria, cytochrome *bc*₁ complex, atovaquone, resistance, quinolones

4.1 Introduction

Malaria is a severe protozoal disease prevalent in 83 malaria-endemic countries resulting in 263 million malaria cases and 597,000 deaths in the year 2023, with WHO African countries, namely Nigeria (26%), the Democratic Republic of Congo (13%), Uganda (5%), Ethiopia (4%) and Mozambique (4%), collectively contributing to more than 50% of this disease burden (WHO, 2025). One of the major reasons for the increase in malaria cases is the development of resistance to the currently available chemotherapy and insecticides. Hence, there is an urgent need to explore novel compounds that are effective against resistant strains and also have a clear understanding of the pathway that are approved drug targets.

The *Plasmodium* cytochrome *bc*₁ (cyt *bc*₁) complex is a vital component of the parasite's mitochondrial electron transport chain (mtETC), playing a key role in the regeneration of ubiquinone that acts as an electron acceptor for dihydroorotate dehydrogenase, which is essential for biosynthesis of pyrimidine (Gutteridge, Dave and Richards, 1979; Painter et al., 2007). It catalyzes electron transfer from ubiquinol to cytochrome *c*, generating a proton gradient essential for mitochondrial function and parasite survival (Vaidya and Mather, 2005, 2009). Inhibition of this complex disrupts energy metabolism, leading to parasite death, making it an attractive target for antimalarial drug development (Fisher, Meunier and Biagini, 2020; Esser and Xia, 2024).

Atovaquone (Figure 4.1) is a widely used antimalarial drug that targets the cyt *bc*₁ complex by binding to the ubiquinol oxidation site of cytochrome *b*, thereby inhibiting electron transport and ATP synthesis (Srivastava, Rottenberg and Vaidya, 1997; Baggish and Hill, 2002; Osei-Akoto, Orton and Owusu-Ofori, 2005; Nixon, Moss, et al., 2013a). However, resistance to atovaquone has emerged due to point mutations in the cytochrome *b* gene, particularly at position 268 (Y268S, Y268N, and Y268C), which reduce drug binding leading to treatment failure (Korsinczky et al., 2000; Vaidya and Mather, 2000; Birth, Kao and Hunte, 2014).

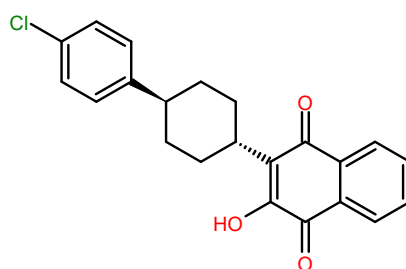


Figure 4.1. Structure of atovaquone (Nixon et al., 2013a)

Though several inhibitors targeting the *Plasmodium* cyt *bc*₁ complex have been developed to effectively disrupt the mtETC (Biagini et al., 2008; Rodrigues, Lopes and Moreira, 2010; Bueno et al., 2012; Capper et al., 2015; David Hong et al., 2018; McConnell et al., 2018), there are a limited number of compounds that effectively target ATQ-R strains (Winter et al., 2011; Biagini et al., 2012; Nixon, Pidathala, et al., 2013b; Stickles et al., 2016). This highlights the need for continued efforts to identify and optimise novel inhibitors targeting cyt *bc*₁ with improved potency against ATQ-R *Plasmodium* strains. To fulfil this aim, the study focused on the collation of chemical and biological data of previously published compounds from the literature. To complement this initial data, *in silico* methods including molecular modelling and assessment of pharmacokinetic and drug-likeness were conducted to identify lead compounds that can be further explored and optimised to be developed to target ATQ-R malaria strains.

4.2 Methods

The present study was done in two parts (i) data collation and evaluation of SAR and (ii) *in silico* studies to evaluate the binding modes, drug-likeness, pharmacokinetics and toxicity profiles.

4.2.1 Data Collation and SAR study

The data from the published literature was collated by manual curation of information on chemical compounds and respective biological activities from various accredited published research literature (including Elsevier, Future Science Ltd, ACS Publications, Wiley-VCH Verlag GmbH & Co., The Royal Society of Chemistry, Springer Science) searched using academic databases such as PubMed and Google Scholar. Articles were searched using combination of keywords that included malaria, electron transport chain, cytochrome *bc*₁ complex, complex III, cytochrome *bc*₁ inhibitors, *Plasmodium falciparum*, resistance, ELQs, endochin-like quinolones, beta-methoxyacrylates, atovaquone, naphthoquinones, quinolones, 4-pyridones, (1H-pyridin-4-ylidene)amines, chalcones, acridones, and tetracyclic benzothiazepines. Chemical structures were drawn and organised into tables using Instant JChem software (version 22.19.0, Chemaxon). The captured data were saved as CSV files containing information on the chemical structures of compounds in the form of Simplified Molecular Input Line Entry System (SMILES) notations, their molecular formulas, molecular weights, various ATQ-R *P. falciparum* strains, biological activities (IC₅₀/EC₅₀), and citation of the articles that included PubMed ids (PMIDs), digital object identifiers (DOIs), and year of publications. For analysis,

only compounds with IC₅₀ or EC₅₀ values ≤ 1 μ M were considered as potential candidates. A SAR study was undertaken to explain the molecular differences between activities of different compounds.

4.2.2 *In silico* studies

4.2.2.1 Molecular modelling

3D structures of ligands were prepared using LigPrep program of Schrödinger Maestro software and OPLS4 force field, with protonation states being predicted using Epik with a target pH of 7.0 ± 2.0 (Schrödinger Release 2023-2: Epik) (Shelley et al., 2007). The protein structure (PDB 3CX5) was prepared using protein preparation wizard from Schrödinger (Schrödinger Release 2023-2: Protein Preparation Workflow) (Solmaz and Hunte, 2008). All extra chains and ligands were deleted, keeping only the chains C and P involved in the binding pocket. To build the mutant model, tyrosine at 279th position (equivalent to 268th position of *Plasmodium* parasite) in the wild type (WT) model was replaced by serine to account for Y279S mutation (equivalent to Y268S in *P. falciparum*) (Song et al., 2015; Chebon-Bore et al., 2021; Jacobsen, Husen and Solov'yov, 2021). The protein was later pre-processed and minimised to relieve unfavourable constraints.

Molecular docking was performed using Glide extra precision (XP) scoring function and ligands structures were treated as flexible, which allowed for sampling of ring conformations (including input ring conformation) and nitrogen inversion, to obtain 5 poses per ligand (Friesner et al., 2004, 2006). Prime MM-GBSA (Molecular Mechanics/Generalized Born Model and Solvent Accessibility) v3.000 program was used to estimate the binding free energy (or ΔG) for each docked pose complex with the VSGB solvation model and OPLS4 force field (Li et al., 2011; Mulakala and Viswanadhan, 2013; Genheden and Ryde, 2015).

The complexes with the best binding pose score (most negative ΔG value) were submitted for a 100 ns MD simulations using Desmond program found within the Schrödinger software suite, and the OPLS4 force field (Abdullah et al., 2023). Water molecules present in the binding pocket from the docking procedure were retained, and the system was solvated using the TIP4P water model. An orthorhombic simulation box was set up with boundaries 10 Å away from the protein, containing more than 130,000 atoms. Rest of the settings were kept on default and MD simulation was done for 100 ns with NPyT conditions (300 K, 1.103 bar and 2000 bar.Å). The MD simulations were

analysed using the simulation interaction diagram tool within the Schrödinger Suite (Schrödinger Release 2023-2: Desmond Molecular Dynamics System).

4.2.2.2 ADMET and Druglikess

Absorption, distribution, metabolism, elimination and toxicity (ADMET) properties, including the molecular properties that are incorporated in Lipinski's rule of five (Ro5), gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, along with number of rotatable bonds (RB), topological polar surface area (TPSA), and bioavailability were calculated using SwissADME program (Daina, Michielin and Zoete, 2017; SwissADME, 2017). ProTox-3.0 tool was used for predicting the potential toxicity of chemical compounds, where acute toxicity (rodent) as LD₅₀ in mg/kg body weight, organ toxicity and toxological endpoints were predicted (Banerjee et al., 2024; ProTox-3.0).

4.3. Results and Discussion

4.3.1 Data collation and SAR study

From data collation, it was observed that several classes of compounds have been designed and evaluated for their antimalarial activity against *Plasmodium* cyt *bc*₁ complex. However, only a limited number of compounds have been assessed for their effectiveness against the ATQ-R strains (**Table 4.1**).

Table 4.1: Summary of total number of compounds collated and number of active compounds from different chemical scaffolds where the IC₅₀ value is ≤1 μM evaluated as potential *P. falciparum* cyt bc₁ inhibitors.

Chemical scaffolds	# Total compounds collated	# Compounds active against <i>Plasmodium</i> strains	# Compounds active against ATQ-R <i>Plasmodium</i> strains	# Compounds active against <i>P. falciparum</i> cyt bc ₁ enzyme
Quinolones (scaffold I)	257	196	130	11
Pyridines (scaffold D, E, F)	118	82	3	3
Acridines-acridones (scaffold H)	34	28	1	-
Miscellaneous (scaffold K)	46	1	1	-
Known drugs	15	10	4	2
Tetracyclic benzothiazepines (scaffold B)	44	31	-	8
Naphthoquinones (scaffold A)	70	22	-	-
Methoxyacrylates (scaffold G)	27	25	-	-
Chalcones (scaffold J)	8	8	-	-
Phthalimides (scaffold C)	12	-	-	-
Total compounds	631	403	139	24

A total of 30 published articles (from the year 2000 to 2023) were identified that investigated *Plasmodium* cyt bc₁ complex as the target (Table 4.1). From these, a total of **616** distinct structures and **15** known drugs, spanning 11 scaffolds including naphthoquinones, tetracyclic benzothiazepines, phthalimides, pyridine-based inhibitors, β-methoxyacrylates and α-methoxyimino acetates, acridones and acridines, quinolones, chalcones, and a group of miscellaneous compounds were collated. From the total of **631** compounds, only **139** compounds (including four known drugs) were found to be active against at least one of the ATQ-R strains with **24** evaluated directly on the *P. falciparum* cyt bc₁ enzyme.

Amongst the active compounds identified, quinolones (scaffold I) emerged as the most extensively studied and potent, with **130** compounds reported to be active against ATQ-R strains (IC₅₀ values ranging from <0.03 nM to 824 nM), and 11 compounds showing significant inhibition of the *Plasmodium* cyt bc₁ enzyme (IC₅₀ range: 0.56 nM to 500 nM). In contrast, out of 118 pyridine-based compounds (grouped under scaffolds D, E and F all together), only 23 compounds were tested against the ATQ-R strain, and just three compounds (**CdIds D-16, D-19 and D-38**) demonstrated notable activity against the ATQ-R FCR3 strain with IC₅₀ values <1 μM (Supplementary data in excelsheet). These three compounds also displayed strong inhibitory activity and selectivity toward the *P. falciparum* cyt bc₁ enzyme over the human counterpart (Bueno et al., 2012). Within 34

compounds belonging to acridones-acridines (scaffold H), only two compounds were reported for activity against the ATQ-R *P. falciparum* TM90-C2B strain. Of these, only **Cdid H2** or 3-amino-6-[(6,6,6-trifluorohexyl)oxy]acridin-9(10H)-one) showed activity with an IC₅₀ value of 78 nM, with no available data on enzyme inhibition (Winter et al., 2008). From the miscellaneous compounds (scaffold K), only **Cdid K-41** was reported to be active against the ATQ-R FCR3 strain (IC₅₀ = 430 nM) (Carrasco et al., 2013).

Amongst other scaffolds that included naphthoquinones, tetracyclic benzothiazepines, phthalimides, methoxyacrylates, and chalcones, none of the compounds were reported to be tested against any of the ATQ-R strains such that analysis of biological efficiency of these compounds against resistance was not actioned. Furthermore, no data for *Plasmodium* cyt *bc*₁ enzyme inhibition potency of these compounds was reported. However, eight of the tetracyclic benzothiazepines (scaffold B) demonstrated notable potency and selectivity in inhibiting the *P. falciparum* cyt *bc*₁ enzyme, with significantly lower activity against human counterpart, indicating strong target specificity (Dong et al., 2011), and a single phthalimide (scaffold C) (**Cdid C6** or 4-amino-2-(4-methoxyphenyl)-1H-isindole-1,3(2H)-dione) was reported to inhibit *P. falciparum* cyt *bc*₁ enzyme activity upto 74% at 70 µM (Okada-Junior et al., 2018).

Amongst the known drugs, artemisinin (IC₅₀ = 2.1 nM), antimycin A (IC₅₀ = 39 nM), chloroquine (IC₅₀ = 91 - 134 nM), and myxothiazol A (IC₅₀ = 320 nM) were reported to be active against the ATQ-R *P. falciparum* TM90-C2B strain (Winter et al., 2008; Stickles et al., 2015). Chloroquine was additionally reported to be active against Tm93-C1088, TM902CB, A6, and FCR3 strains (Rodrigues et al., 2009; Biagini et al., 2012; Nilsen et al., 2013, 2014).

None of the collated compounds were found to active against the *P. falciparum* A6 strain except chloroquine (Biagini et al., 2012). WR249685 (IC₅₀ = 3 nM) and atovaquone (IC₅₀ range: 0.2 nM to 3.4 nM) displayed activity against the parasite enzyme (Dong et al., 2011; Okada-Junior et al., 2018).

Overall, from a total of **616** distinct compounds (excluding known drugs), only **135** were found to display potency against at least one of the ATQ-R strains and 22 were reported to inhibit *Plasmodium* cyt *bc*₁ enzyme activity. With so few compounds reported for activity against ATQ-R strains and *Plasmodium* cyt *bc*₁ enzyme inhibition, there is a significant gap. It is crucial to test more compounds for their effectiveness against ATQ-R strains, optimise potent candidates, and evaluate their pharmacokinetics and drug-likeness properties to identify lead structures.

4.3.2 SAR analysis of scaffolds:

Further analysis of four scaffolds (quinolones, pyridines, acridones and miscellaneous) displaying activity against ATQ-R strains, identified some intriguing SAR features outlined in the figures below for each scaffold.

4.3.2.1 Quinolones (scaffold I): This scaffold demonstrated the highest activity against ATQ-R strains and thus were selected to be studied further for their SAR (**Table 4.2**).

Table 4.2: The SAR of the top-most active quinolones against ATQ-S D6 and Dd2 and ATQ-R TM90-C2B *P. falciparum* strains with reported *in vitro* nanomolar activity (Nilsen et al., 2014).

Cdd	Compound structure				Antimalarial activity (IC ₅₀ value: nM)		
	R1	R2	R3	R4	D6	Dd2	TM90-C2B
I-161	Me	(3-OCF ₃)-phenyl	Cl	OMe	0.03	0.03	<0.03
I-162	Me	(4-CF ₃)-phenyl	Cl	OMe	0.3	0.3	0.05
I-165	Me	(3-OCF ₃),4-F-phenyl	Cl	OMe	0.4	0.5	0.3
I-167	Me	(4-OCF ₃)-phenyl with X ₂ = N	Cl	OMe	0.3	0.2	0.4
I-168	Me	(4-OCF ₃)-phenyl with X ₁ = N	Cl	OMe	0.4	0.5	0.2
Atovaquone					0.1 to 0.34	0.1 to 0.5	>2500

4.3.2.2 Pyridones (Scaffold D)

From the SAR analysis, in general, it was observed that 4(1H)-pyridones (scaffold D) with a methyl group at position C-2, an electron-withdrawing group (CF₃ or NO₂) or a halogen atom (Cl or Br) at position C-3, a lipophilic side chain (with electron-withdrawing substituents) at position C-5, and a methyl or a hydroxymethyl group at position C-6 showed improved antimalarial activities compared to other pyridines (**Figure 4.2**). Compounds **Cdlds D-16**, **D-19** and **D-38** displayed activity against ATQ-R FCR3A strain (IC₅₀ range: 1 nM to 6 nM) and *bc*₁ enzyme (IC₅₀ range: 2 nM to 7 nM) (Yeates et al., 2008; Bueno et al., 2012).

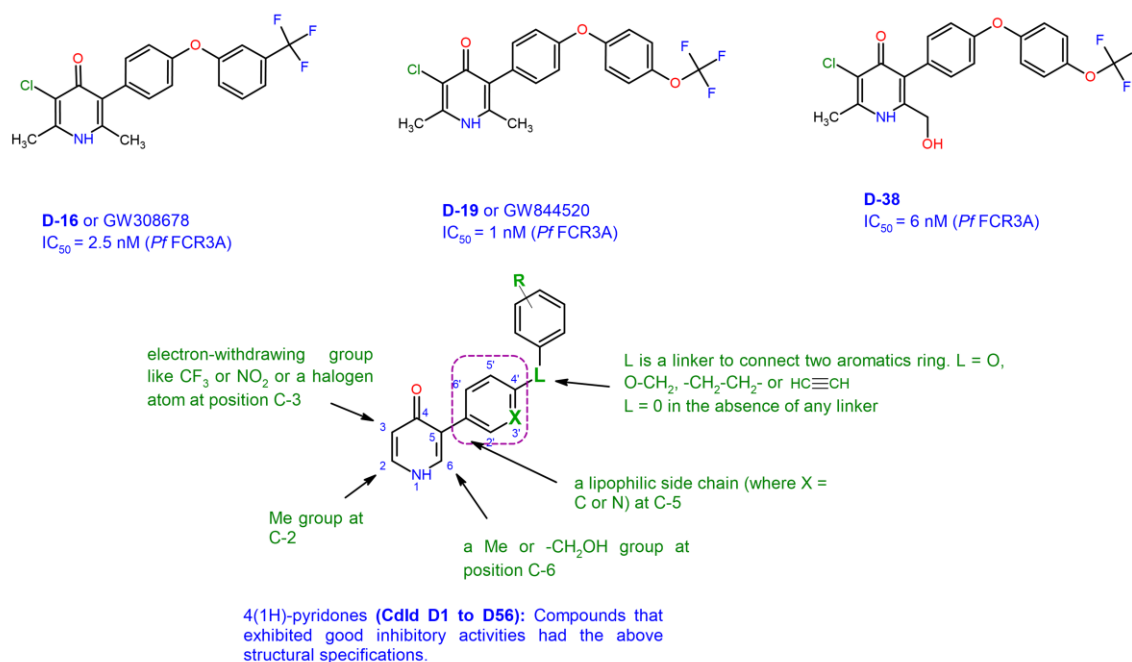


Figure 4.2: SAR analysis of most active pyridines against ATQ-R FCR3 *P. falciparum* (Yeates et al., 2008; Bueno et al., 2012).

4.3.2.3 Acridones (Scaffold H)

Cdld H-2 (3-amino-6-[(6,6,6-trifluorohexyl)oxy]-9,10-dihydroacridin-9-one) showed great potency amongst acridones against the ATQ-R TM90-C2B strain when compared to known drugs artemisinin, chloroquine and atovaquone, and thus can be an interesting compound to be explored further for its potential use against the resistant strains.

Table 4.3: The SAR features for the **acridone scaffold**, namely **Cdd H-2** (3-amino-6-[(6,6,6-trifluorohexyl)oxy]-9,10-dihydroacridin-9-one), in comparison to **Cdd H-19** (6-chloro-2-methoxyacridin-9(10H)-one) and known drugs artemisinin, chloroquine and atovaquone (Winter et al., 2006, 2008; Rodrigues, Lopes and Moreira, 2010).

For Cdd H2, OR² @ position C6 R¹ : 3-NH₂ R³ : H For Cdd H19, OR² @ position C7 R¹ : 3-Cl			
Antimalarial activity (IC ₅₀ value: nM)			
Cdd	<i>P. falciparum</i> D6	<i>P. falciparum</i> Dd2	<i>P. falciparum</i> TM90-C2B
H2	0.02	0.02	78
H19	45	65	>2500
Artemisinin	0.81	0.6	2.1
Chloroquine	6.4	113	134
Atovaquone	0.3	0.5	5090

4.3.2.4 Miscellaneous (Scaffold K)

From the miscellaneous category, the best activity was shown by compound **Specs AE-641 00605023 (Cdd K-41)** with an IC₅₀ value of 0.43 μM against the ATQ-R FCR3 strain (Carrasco et al., 2013).

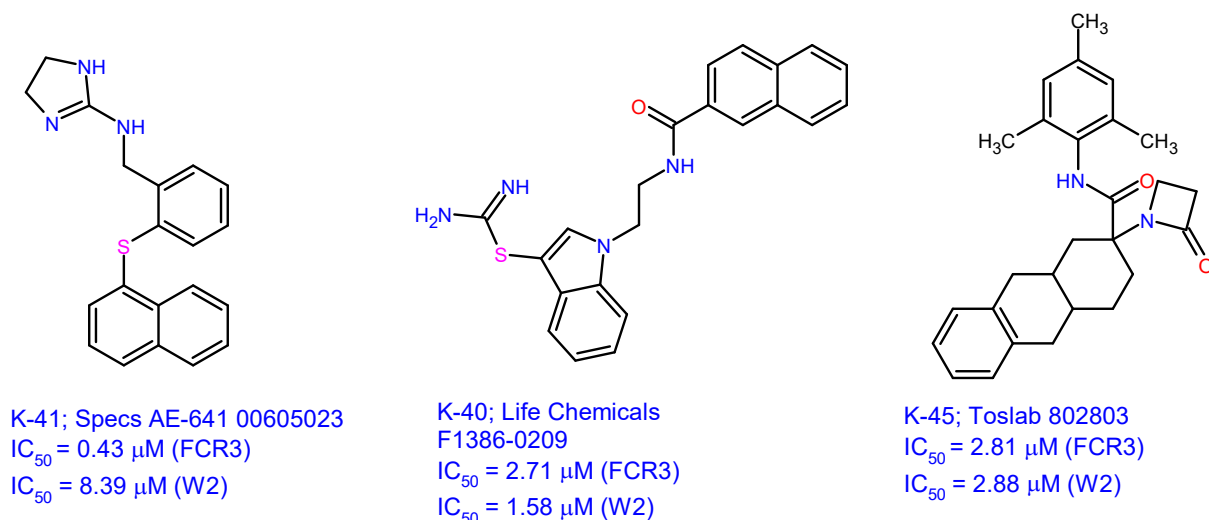


Figure 4.3: Structures of active compounds from miscellaneous compounds (Carrasco et al., 2013).

From the analysis of all the four scaffolds, it was observed that except **K-41**, all of the most active compounds contained either tri-fluoro methyl or tri-fluoro methoxy group within their molecular structures, suggesting that the fluoro group contributed towards enhancing the activity of compounds, specifically against resistant strains.

Docking and MD Simulation Insights

The docking scores for all 135 compounds (excluding four known drugs) against the Y279S mutant model ranged from -12.51 to 0.41 kcal/mol, whereas the MM-GBSA ΔG binding score ranged from -64.82 to 7.65 kcal/mol. According to the docking scores and MM-GBSA calculations, compound **I-124** (IC_{50} = 475 nM against TM90-C2B strain) was ranked highest of all the compounds with the docking score = -12.514 kcal/mol and MM-GBSA = -64.82 kcal/mol. However, visual inspection of compound I-124 showed that it did not form any H-bonds with the key residues His¹⁸¹ or Glu²⁷² which are essential for maintaining interactions within the binding pocket and ensuring inhibitory function.

Atovaquone is a known cyt *bc*₁ complex inhibitor and interacts with His¹⁸¹ via H-bonding and with Glu²⁷² through a water bridge (in the WT model). Interactions with these two residues are crucial for maintaining its position in the binding pocket and ensuring its inhibitory function (Kessl et al., 2003; Birth, Kao and Hunte, 2014; Song et al., 2015). When the bulky residue Tyr²⁷⁹ was replaced by the smaller Ser²⁷⁹ (in the Y279S mutant model), the essential interaction with His¹⁸¹ was lost, causing atovaquone to shift away from these residues, ultimately leading to a loss of activity in the mutant strain, and hence interaction with His¹⁸¹ and Glu²⁷² are of key importance (Carrasco et al., 2013; Akhoun et al., 2014).

Additionally, compound I-124 seemed oversized and too flexible (#RB = 13) to bind properly with the binding pocket residues. This was supported by the pharmacokinetic prediction results which suggested that **I-124** violated 2 Lipinski's Ro5 with high MW (>500) and high logP value (MlogP>4.15). Further, it exhibited lowest bioavailability score of 0.17, low GI absorption and lacked permeability to BBB. Hence, this compound was excluded from further studies.

Next the attention was focussed on the top five active compounds belonging to four different scaffolds as highlighted in the previous section. On visual inspection of the docked poses, it was observed that **I-161**, **I-162** and **K-41** did not interact with key residues His¹⁸¹ and Glu²⁷². Compound

H-2 interacted with His¹⁸¹ via H-bond and with Glu²⁷² via water bridge and aromatic H-bond, whereas compound **D-38** was seen interacting only with His¹⁸¹ via H-bond.

Table 4.4: Comparison of docking scores, MM-GBSA ΔG_{Bind} and key interactions of five most active compounds collated against ATQ-R strain with atovaquone in the cyt *bc*₁ binding cavity of the Y279S mutant model

Ligand	Docking score (kcal/mol)	MM-GBSA ΔG_{Bind} (kcal/mol)	Key interactions
H-2	-10.22	-33.91	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge and aromatic H-bond)
K-41	-9.92	-43.66	No H-bonded interaction seen in the docked pose
D-38	-8.96	-42.46	His ¹⁸¹ (H-bond)
I-162	-8.81	-47.24	Leu ²⁷⁵ (aromatic H-bonds)
I-161	-8.16	-46.44	Ser ²⁷⁹ and Leu ²⁷⁵ (aromatic H-bonds)
Atovaquone	-8.30	-21.66	No interactions with His ¹⁸¹ , Glu ²⁷² and Ser ²⁷⁹

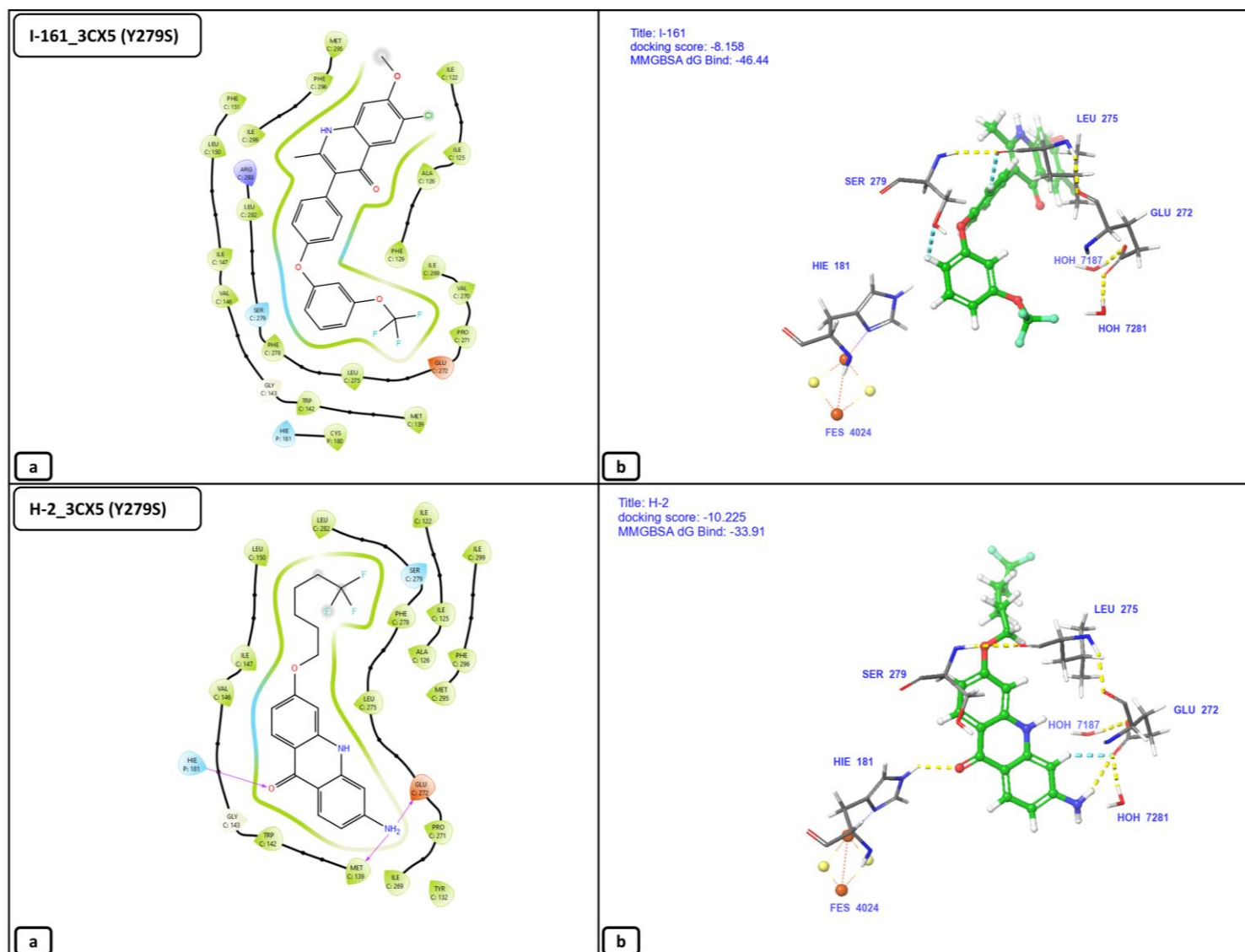


Figure 4.4: 2D and 3D representation of key interactions of two compounds, quinolone **I-161** and acridone **H-2** docked in 3CX5 (Y279S) mutant model

From the above observation, it became important to explore further and understand the binding modes which could explain the reason why **I-161** is the most active ($IC_{50} < 0.03$ nM) against TM90-C2B (carrying Y279S mutation) without any interaction with His¹⁸¹ and Glu²⁷² in the docked poses, and **H-2** despite a higher IC_{50} value of 78 nM, displayed interaction with the key residues in the docked poses. To understand this further, 100 ns MD simulation studies were performed with CdlDs **I-161** and **H-2** as the next step.

MD simulations

The MD simulation results for two compounds, **I-161** (quinolone) and **H-2** (acridone), which belong to different chemical scaffold and have demonstrated activity against *Pf*TM90-C2B strain harbouring the Y268S mutation (Korsinczky et al., 2000; Fisher et al., 2012; Nilsen et al., 2013, 2014; McConnell et al., 2018), were analysed to evaluate their binding trajectory and key ligand-protein interactions in the Y279S mutant model, where Tyr²⁷⁹ was substituted with Ser²⁷⁹.

Protein-Ligand Root Mean Square Deviation (RMSD)

RMSD determination helped examine the target protein's conformational changes when combined with ligand molecules for a 100 ns MD simulation. At the beginning of the simulation, there was a jump in the RMSD as the system equilibrated and reached a stationary state. This jump was higher for **I-161** compared to **H-2**, but subsequently the RMSD remained relatively stable until the end of the simulation (**Figure 4.5**). The core structure of the protein remained stable, as indicated by localised analysis of key domains in the protein root mean square fluctuation (RMSF) values (**Table 4.5**). The RMSD value for both ligands changed within a narrow range and remained lower than the RMSD of the protein. This indicated that both ligands remained securely within the binding pocket and did not diffuse away, aligning with effective binding interactions typically observed in stable protein-ligand complexes (Gohlke and Klebe, 2002).

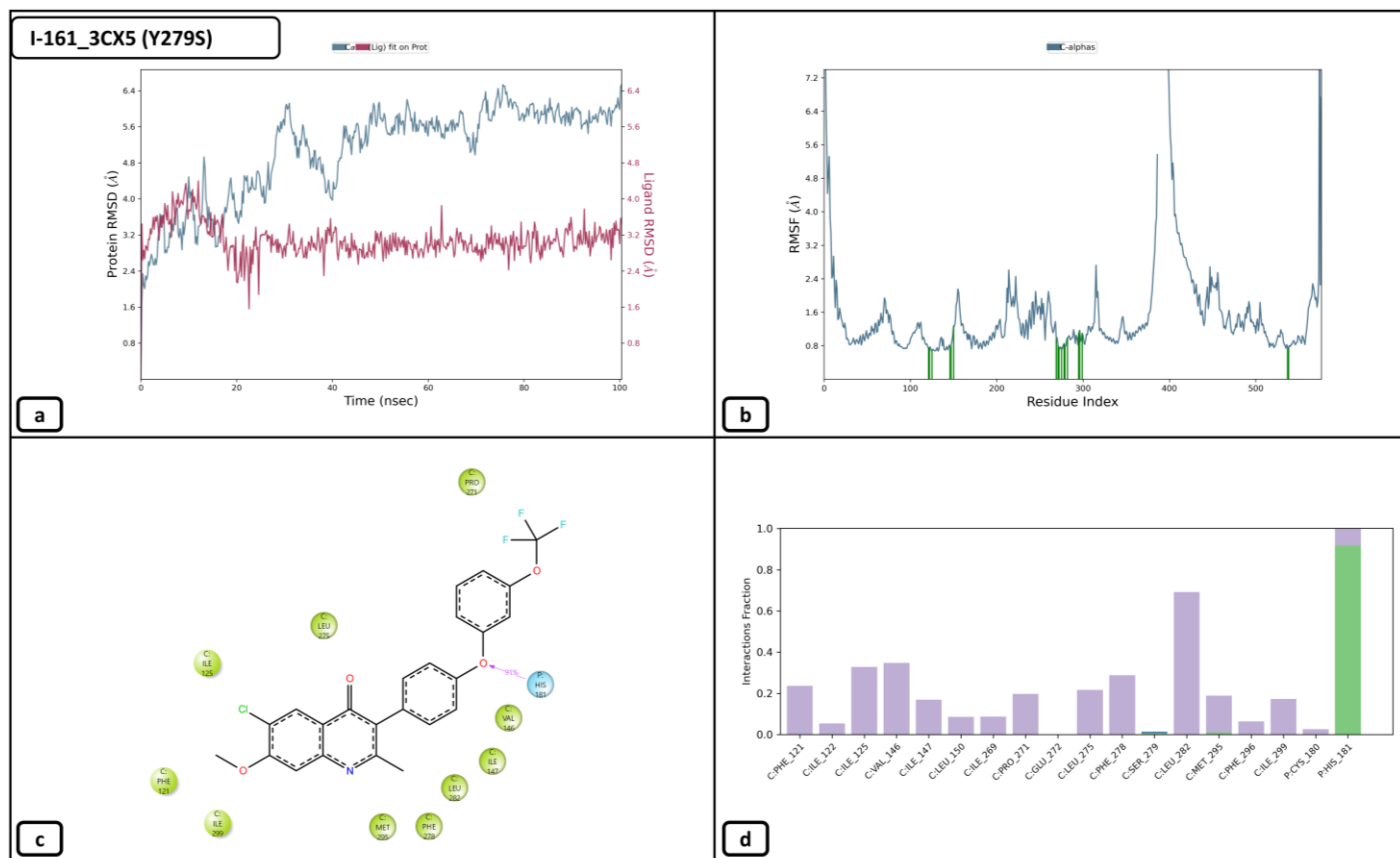


Figure 4.5a: The MD simulations of CdIds **I-161** in complex with **3CX5 (Y279S)** resistant-model. **(a)** RMSD plot: the blue curve represents the RMSD of the protein α atoms, providing insights into the backbone stability and overall conformational changes during the simulation. The red curve corresponds to the ligand RMSD, showing its movement relative to the protein binding site. A steady RMSD after 20 to 40 ns suggested system equilibration, indicating that both the protein and ligands had stabilised in their respective conformations. **(b)** Protein RMSF plot characterising local fluctuations along the protein chain, where green vertical bars show the protein residues interacting with the ligand, and peaks are the loop regions that fluctuate the most during simulation. **(c)** Simulation interaction diagram (SID) showing ligand atoms interacting with the protein residues for more than 10% of the simulation. **(d)** Protein-ligand contact histogram representing the frequency of the interactions categorised into: hydrogen bonds (green bars), hydrophobic bonds (purple bars), and water bridges (blue bars). X-axis represents the residue numbers and Y-axis represents the interaction fraction. Residues with higher interaction frequencies indicate key binding site contacts contributing to ligand stability.

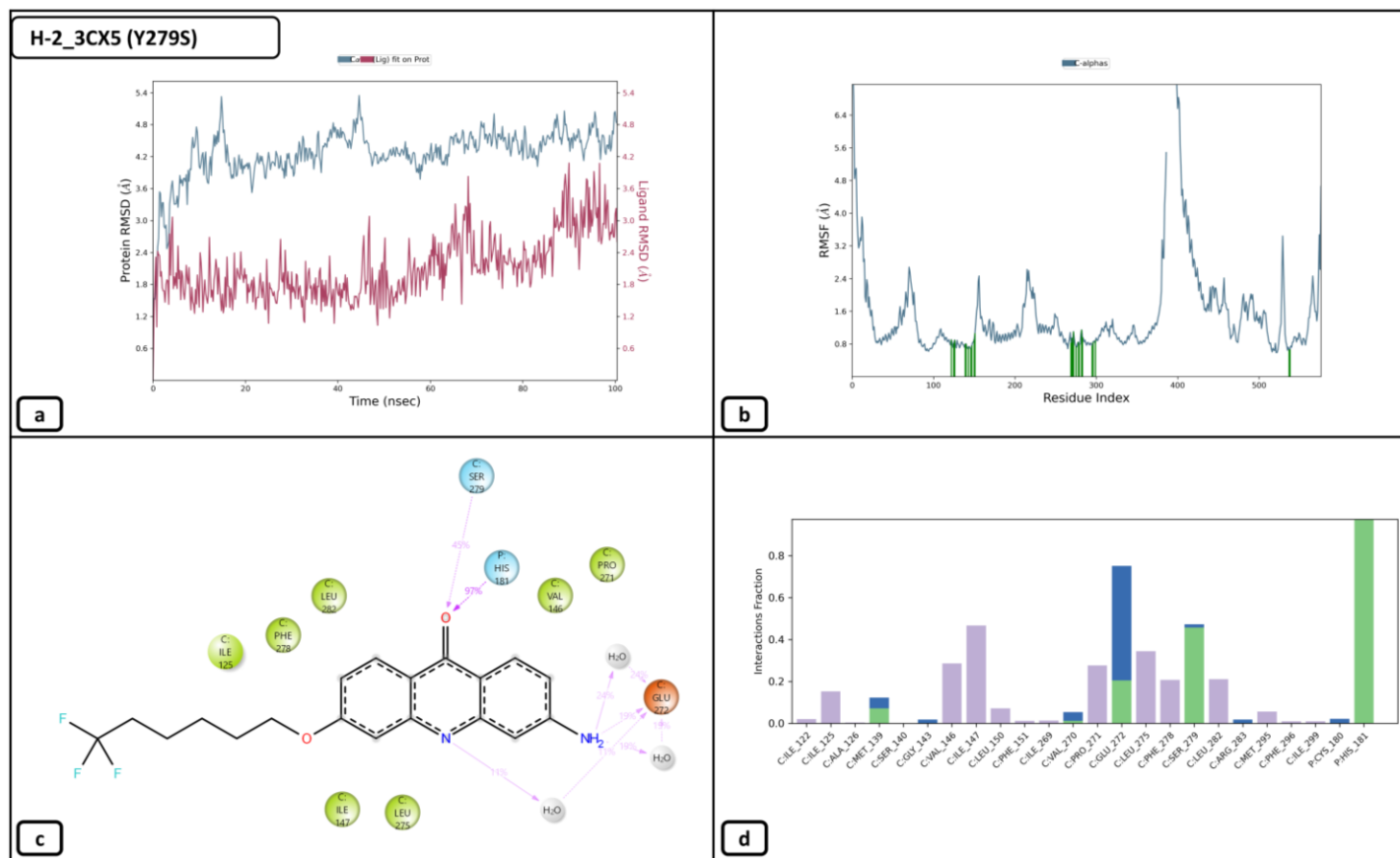


Figure 4.5b: The MD simulations of CdLds **H-2** in complex with **3CX5 (Y279S)** resistant-model. **(a)** RMSD plot: the blue curve represents the RMSD of the protein C α atoms, providing insights into the backbone stability and overall conformational changes during the simulation. The red curve corresponds to the ligand RMSD, showing its movement relative to the protein binding site. A steady RMSD after 20 to 40 ns suggested system equilibration, indicating that both the protein and ligands had stabilised in their respective conformations. **(b)** Protein RMSF plot characterising local fluctuations along the protein chain, where green vertical bars show the protein residues interacting with the ligand, and peaks are the loop regions that fluctuate the most during simulation. **(c)** Simulation interaction diagram (SID) showing ligand atoms interacting with the protein residues for more than 10% of the simulation. **(d)** Protein-ligand contact histogram representing the frequency of the interactions categorised into: hydrogen bonds (green bars), hydrophobic bonds (purple bars), and water bridges (blue bars). X-axis represents the residue numbers and Y-axis represents the interaction fraction. Residues with higher interaction frequencies indicate key binding site contacts contributing to ligand stability.

Table 4.5: RMSF values (Å) of key residues interacting with **I-161** and **H-2**.

Residue	Chain	C α -Protein RMSF (Å)	
		I-161	H-2
His ¹⁸¹	P	0.75	0.67
Glu ²⁷²	C	0.75	1.10
Ser ²⁷⁹	C	0.77	0.77

Based on the above table 4.5, it could be inferred that I-161 maintained interaction with key residues His¹⁸¹, Glu²⁷² and Ser²⁷⁹ with RMSF value well below 1 Å. On the other hand, for H-2, the RMSF value for Glu²⁷² was slightly higher. This could account for one of the reasons why H-2 displayed higher IC₅₀ value compared to I-161.

Ligand protein contacts

The ligand-protein interactions between cyt *bc*₁ and **I-161** and **H-2** (**Figure 4.5**), indicated that the residues with the highest interaction fractions was His¹⁸¹ for both compounds. I-161 interacted with His¹⁸¹ for 91% of the simulation time via H-bond. In addition to His¹⁸¹, I-161 displayed hydrophobic interactions with other residues, mainly with Leu²⁸². No significant interaction was observed with Glu²⁷² and Ser²⁷⁹.

On the other hand, H-2 interacted with His¹⁸¹ for 97% of the simulation time via H-bond formation between carbonyl oxygen and His¹⁸¹. Additionally, there was interaction with Ser²⁷⁹ for 45% of the simulation time via H-bond with the carbonyl oxygen of the ligand. Furthermore, the amino group also interacted with Glu²⁷² via water bridge.

On comparing the two ligands, compound H-2 displayed better and stable interactions with the key residues His¹⁸¹, Glu²⁷² and Ser²⁷⁹ in comparison to compound I-161 and thus can be explored further to be optimised to design and develop novel compounds with efficacy against ATQ-R strains.

4.3.3 ADMET and Drug-Likeness Evaluation

Several highly potent compounds displayed favourable pharmacokinetic properties, making them suitable for further optimization; whilst other active compounds exhibited poor solubility or toxicity risks, warranting structural modifications or elimination as a potential lead structure.

Of all the **135** distinct compounds, 14 compounds violated Lipinski's Ro5 (I-95, I-124, I-125, I-127, I-128, I-130, I-132, I-178, I-180, I-184, I-185, I-186, I-189, and I-198). Compounds **I-124**, **I-125** and **I-127** violated 2 rules with high molecular weights (MWs >500) and high logP values (MlogP >4.15). These compounds were also predicted to exhibit the lowest bioavailability score of 0.17, along with low GI absorption and non-permeant to BBB. The remaining compounds complied with Lipinski's Ro5 and exhibited a bioavailability score of 0.55. They were predicted to have moderate to low solubility, with consensus LogP values ranging from 1.9 to 6.37. Most of the compounds were predicted to have high GI absorption and a balanced number of rotatable bonds (RBs) for a stable binding at the protein's active site.

Table 4.6 highlights the varied physicochemical and pharmacokinetic properties of selected active compounds against ATQ-R strains. Quinolone **I-161** and **I-162** showed relatively high LogP values (~6.0) with low GI absorption; whereas acridone **H-2** exhibited a LogP within the acceptable range along with high GI absorption.

Table 4.6: Summary for ADME and drug-likeness results for some most active compounds.

Ligand id	PHYSICOCHEMICAL PROPERTIES						DRUGLIKENESS		PHARMACOKINETIC PROPERTIES	
	Lipinski Ro5				Veber)					
	MW	LogP	#HBA	#HBD	#RB	TPSA	Lipinski #violations	Bioavailability Score	GI absorption	BBB permeant
Criteria	≤ 500 g/mol	≤ 5	≤ 10	≤ 5	≤ 10	≤ 140 Å²	≤ 1 violation	≥ 0.55	High is favourable	No is favourable
I-161	475.84	5.92	7	1	6	60.55	0	0.55	Low	No
I-162	459.84	6.02	6	1	5	51.32	0	0.55	Low	No
D-16	393.79	5.47	5	1	4	42.09	0	0.55	Low	No
D-19	409.79	5.32	6	1	5	51.32	0	0.55	Low	No
D-38	425.79	4.52	7	2	6	71.55	0	0.55	High	No
H-2	364.36	4.33	5	2	7	68.11	0	0.55	High	No
K-41	333.45	3.73	1	2	5	61.72	0	0.55	High	Yes
Atovaquone	366.84	4.64	3	1	2	54.37	0	0.85	High	Yes

MW = molecular weight; LogP = log of octanol/water partition coefficient; #HBA = number of hydrogen bond acceptor(s); #HBD = number of hydrogen bond donor(s); #RB = number of rotatable bond(s); TPSA = topological polar surface area; Lipinski #violations = number of violations of Lipinski's rule; GI = gastrointestinal; BBB = blood-brain barrier

BOILED Egg (or Brain Or Intestinal Estimated) Plot

Drug-likeness evaluation showed that most compounds clustered in the yellow core region, indicating sufficient lipophilicity and polarity (TPSA <90 Å²) to penetrate the BBB, making them suitable candidates for drugs acting on the central nervous system (**Figure 4.6**). In contrast, some compounds were located in the white region, suggesting favourable oral bioavailability due to an optimal balance between lipophilicity and polarity, supporting efficient absorption across the intestinal barrier.

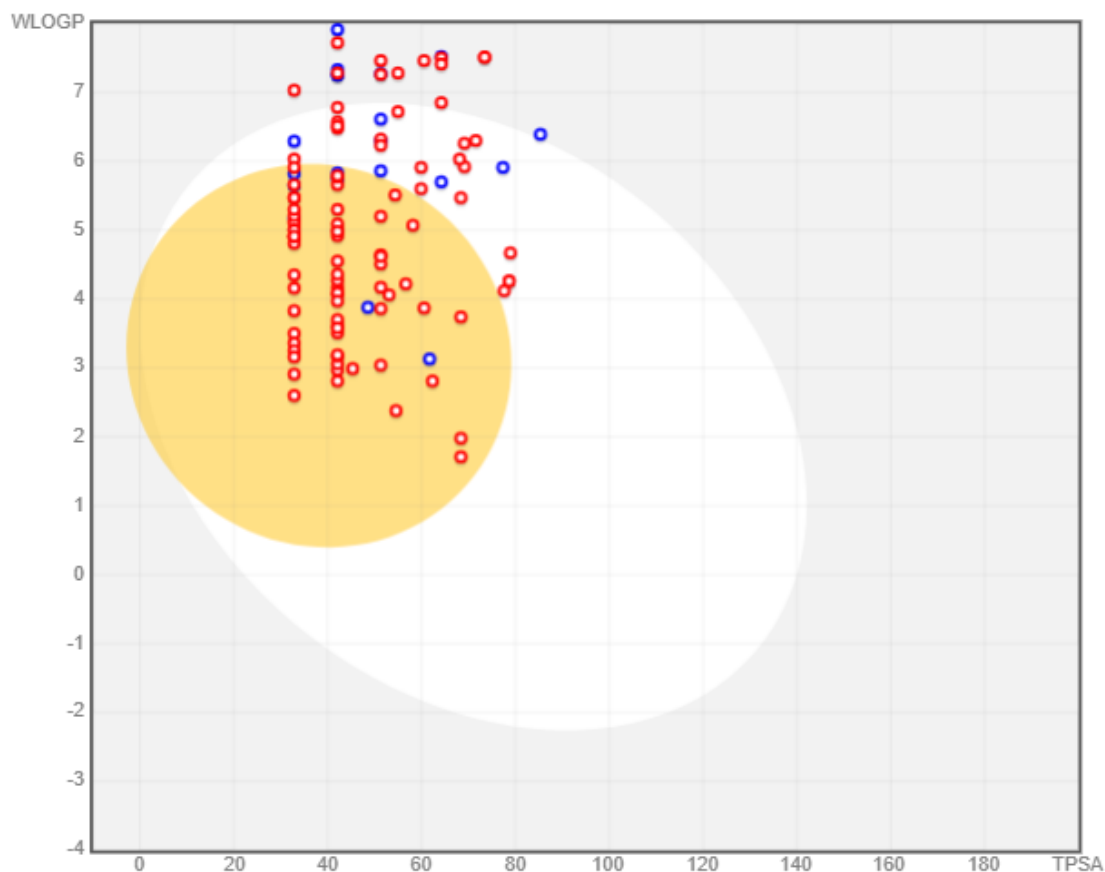


Figure 4.6: BOILED Egg (or Brain Or Intestinal Estimated) Plot of WlogP vs TPSA for 135 compounds. Yellow indicates BBB-permeant, white indicates human intestinal absorption (HIA) i.e. gastrointestinal permeant, blue indicates P-glycoprotein positive, red indicates P-glycoprotein negative.

Compounds with CdlDs **D-16, D-19, I-7, I-95, I-128, I-130, I-131, I-133, I-134, I-142, I-157, I-167, I-171, I-172, I-173, and I-189** were positioned outside the Egg region exhibiting poor pharmacokinetics for oral absorption or BBB permeability. It signified that all these compounds

were highly lipophilic with elevated WLogP values, and are therefore prone to issues such as low solubility or high efflux by P-gp. Cdlids **I-197**, **I-171** and **I-95** were observed to be located at the borderline of the white part of the Egg. In comparison, compounds **I-180**, **I-198**, **I-194**, **I-116**, **I-163**, **I-158**, **K-41**, **I-119**, and **I-129** though inside the egg regions were marked as a P-gp substrate with a blue dot, suggesting possible active efflux by P-glycoprotein, which could limit their GI absorption or BBB penetration.

Among the top selected compounds, **Cdlids I-161**, **I-162**, **D-16** and **D-19** were positioned outside the egg in the grey region, suggesting unfavourable physicochemical properties for GI absorption due to high WLogP values. In contrast, compounds with **Cdlids H-2** and **D-38** were located in the white region, indicating a balance of lipophilicity and polarity that supports good oral absorption but not BBB penetration. **Cdld K-41** was mapped in the core yellow region, signifying potent BBB penetration; however, it was also predicted to be P-glycoprotein substrate. This implied that despite its physicochemical suitability for CNS entry, active efflux by P-gp could limit its actual brain availability.

Toxicity

Both compounds **I-161** and **I-162** were predicted to be very safe and non-toxic with $LD_{50} \geq 4000$ mg/kg, toxicity class 5, non-carcinogenic, non-mutagenic and non-cytotoxic. However, these compounds were flagged as hepatotoxic and neurotoxic along with potential immunotoxicity. Compounds **D-16** ($LD_{50} = 500$ mg/kg), **D-19** ($LD_{50} = 518$ mg/kg), **D-38** ($LD_{50} = 500$ mg/kg), **H-2** ($LD_{50} = 1000$ mg/kg) and **K-41** ($LD_{50} = 347$ mg/kg) fell in the category of harmful compounds with toxicity class 4. Though compounds **D-19**, **D-38** and **K41** were predicted to be non-carcinogenic, non-mutagenic, non-immunotoxic and non-cytotoxic, they are predicted to be toxic due to low LD_{50} values (**Table 4.7**). These results are *in silico* predictions and have potential to be false positives or negatives, therefore experimental validation is suggested with further optimisation of the compounds with organ-specific toxicities.

Table 4.7: Summary of the toxicity profiles of the test compounds and atovaquone based on ProTox 3.0 predictions.

Compound ID	LD ₅₀ (mg/kg)	Toxicity Class	Organ Toxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
Atovaquone	2000	Class 4	Nephrotoxic, Respiratory toxic	Non-carcinogenic	Non-immunotoxic	Non-mutagenic	Non-cytotoxic
I-161	4000	Class 5	Hepatotoxic, Neurotoxic	Non-carcinogenic	Immunotoxic	Non-mutagenic	Non-cytotoxic
I-162	5000	Class 5	Hepatotoxic, Neurotoxic	Non-carcinogenic	Immunotoxic	Non-Mutagenic	Non-cytotoxic
D-16	500	Class 4	Hepatotoxic, Neurotoxic	Non-carcinogenic	Immunotoxic	Non-Mutagenic	Non-cytotoxic
D-19	518	Class 4	Hepatotoxic, Neurotoxic	Non-carcinogenic	Non-immunotoxic	Non-mutagenic	Non-cytotoxic
D-38	500	Class 4	Nephrotoxic, Respiratory toxic	Non-carcinogenic	Non-immunotoxic	Non-mutagenic	Non-cytotoxic
H-2	1000	Class 4	Neurotoxic, Respiratory toxic	Carcinogenic	Immunotoxic	Mutagenic	Non-cytotoxic
K-41	347	Class 4	Neurotoxic, Respiratory toxic	Non-carcinogenic	Non-immunotoxic	Non-mutagenic	Non-cytotoxic

Class 1: fatal if swallowed ($LD_{50} \leq 5$ mg/kg); **Class 2:** fatal if swallowed ($5 < LD_{50} \leq 50$ mg/kg); **Class 3:** toxic if swallowed ($50 < LD_{50} \leq 300$ mg/kg); **Class 4:** harmful if swallowed ($300 < LD_{50} \leq 2000$ mg/kg); **Class 5:** may be harmful if swallowed ($2000 < LD_{50} \leq 5000$ mg/kg); **Class 6:** non-toxic ($LD_{50} > 5000$ mg/kg)

4.4 Conclusions

This study evaluated a series of collated compounds that were reported to be active against ATQ-R strains through a combination of *in silico* approaches to identify potential antimalarial candidates. According to the literature, quinolone compounds I-161 and I-162, and acridone H-2 demonstrated the most promising potential as antimalarial agents against *Pf*TM90-C2B strain, thus warranting further exploration for the development of antimalarial drugs targeting ATQ-R strains. These results were further supported by *in silico* assessments, where compound I-161 and H-2 exhibited favourable interactions with key binding site residues His¹⁸¹ and Glu²⁷², compared to atovaquone which lost these important interactions in the Y279S resistant model. Though these compounds displayed favourable drug-likeness, H-2 was predicted to be more toxic compared to I-161, I-162 and atovaquone, suggesting that I-161 and I-162 may be more promising candidates for further optimisation and experimental evaluation. These findings emphasize the importance of *in silico* approaches in early-stage drug discovery.

The insights gained from this study provide a rational basis for further structural optimisation and development of novel compounds. Future studies should focus on expanding the compound libraries to improve SAR understanding, experimental validation of computationally identified lead compounds, and structural modifications to enhance bioavailability and reduce toxicity risks. This should be followed by validating their efficacy *in vivo* to accelerate the development of novel antimalarial therapies to fight against the emerging drug resistance.

Supplementary information: 2D and 3D ligand interaction diagrams for Cldds I-162, D-38 and K-41 against 3CX5 (Y279S) mutant models.

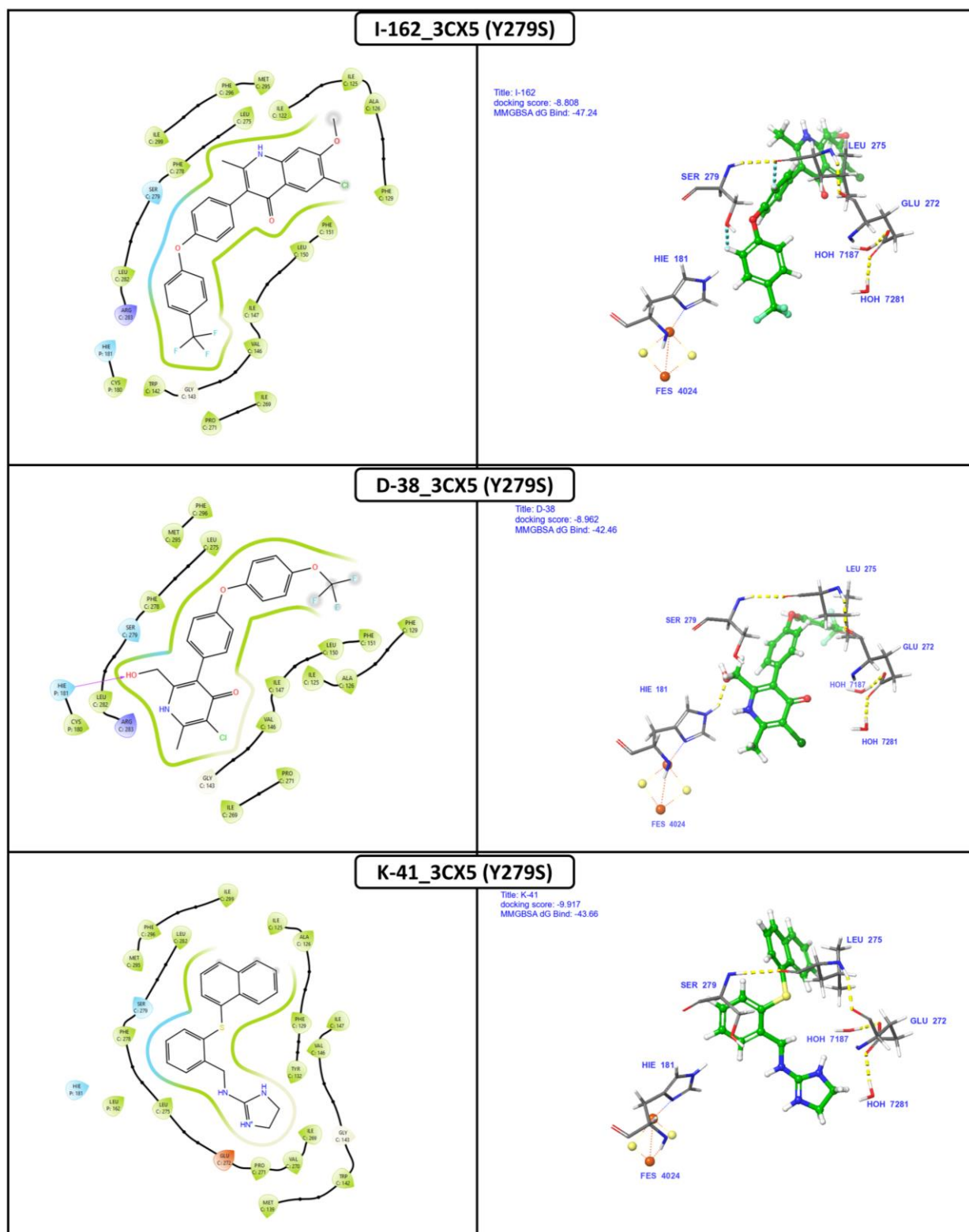


Figure 4.7: 2D and 3D ligand interaction diagrams for Cldds I-162, D-38 and K-41 against 3CX5 (Y279S) mutant models.

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Chapter 5

***In vitro* and *in silico* approaches to determine potency of 1,4-naphthoquinone-1,2,3-triazole hybrid compounds against atovaquone-resistant malaria**

This chapter is presented as a submitted manuscript discussing a series of 1,4-naphthoquinone-1,2,3-triazole hybrids as potential antimalarial compounds active against a atovaquone-resistant strain. The *in vitro* antimalarial activity was determined in atovaquone-resistant *Plasmodium falciparum* FCR3 strain and *in silico* studies studies were performed to determine the binding modes in a mutant model built using molecular modelling tools. Additionally, pharmacokinetic properties and drug-likeness potential were also predicted using *in silico* tools. The *in vitro* and *in silico* results were compared with that of atovaquone to determine whether the test hybrids exhibited superior performance, aiding in their potential advancement as antimalarial drug candidates.

Chapter type: Submitted manuscript

Journal: Experimental Parasitology

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Title of manuscript: Targeting Atovaquone-Resistant Malaria: *In vitro* and *In silico* Insights into 1,4-naphthoquinone-1,2,3-triazole Hybrid Molecules

Student's contributions:

Contributions	Priyanka Agarwal	David D. N'Da	C. Johan van der Westhuizen	Robyn L. van Zyl
Conceptualization	✓			✓
Methodology	✓			
Compounds provided by		✓		✓
Investigation	✓			
Data curation and data analysis	✓			
Writing – original draft	✓			
Writing – review and editing	✓	✓	✓	✓
Supervision			✓	✓
Funding acquisition				✓

A Dual *In vitro* and *In silico* Approach to Evaluate 1,4-Naphthoquinone-1,2,3-triazole Hybrids Against Atovaquone-Resistant Malaria

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Abstract

Malaria continues to pose a major global health burden affecting millions annually. Despite advancements in treatment, the emergence of drug-resistant *Plasmodium* strains has undermined current treatment strategies, including atovaquone. Atovaquone is a key mitochondrial inhibitor targeting the cytochrome *bc*₁ (cyt *bc*₁) complex, with resistance primarily driven by mutation in the cytochrome *b* gene. Moreover, atovaquone's reliance on a single target site and its pharmacokinetic limitations further underscore the urgent need for alternative drugs. To address these challenges, this dual *in vitro* and *in silico* study evaluated ten 1,4-naphthoquinone-1,2,3-triazole hybrids targeting atovaquone-resistant (FCR3) *P. falciparum*. Molecular modelling studies were performed on *Saccharomyces cerevisiae* (PDB ID 3CX5), involving the building of a mutant model to simulate the Y279S mutation (equivalent to Y268S mutation in *P. falciparum*), in order to rationalise the observed results. Additionally, pharmacokinetic properties and drug-likeness of these hybrids were predicted *in silico*. Hybrids **D12** and **D13** exhibited strong antiplasmodial activities, 61- and 52-fold, respectively, more than atovaquone. Molecular modelling studies indicated a strong correlation between *in silico* and *in vitro* activities by displaying binding interactions between the ligand and the mutant model. Structure-activity relationships (SAR) analysis identified key structural features essential for favourable binding interactions with target binding site residues. Furthermore, *in silico* evaluations of these hybrids suggested good oral bioavailability and high gastrointestinal absorption, with no significant risk of severe toxicity. Hybrids **D12** and **D13** exhibit potential as lead

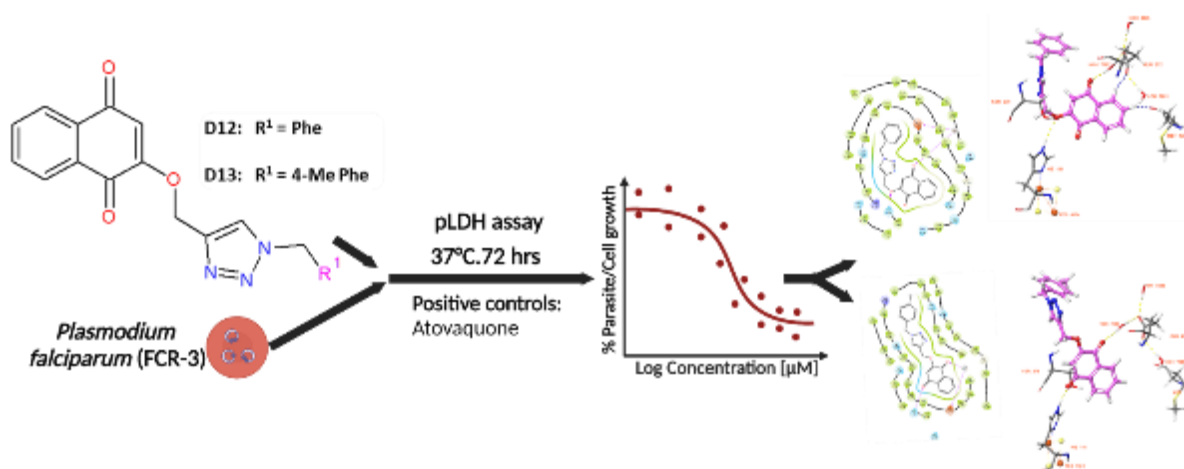
candidates, with their strong *in vitro* efficacy well-supported by *in silico* data, warranting further optimisation and development.

Keywords: malaria, *Plasmodium falciparum*, atovaquone, 1,4-naphthoquinone-1,2,3-triazole hybrids, cytochrome *bc*₁

Highlights of the article:

- 1,4-Naphthoquinone hybrids had sub-micromolar activity against atovaquone-resistant malaria
- PDB ID 3CX5 used for *in silico* studies and Y279S mutant model built using Schrödinger software suite
- Two most active hybrids had favourable interactions with key residues His¹⁸¹ and Glu²⁷² in target
- ADMET properties of hybrids with favourable drug-likeness and *in silico* toxicity profiles

Graphic Abstract:



5.1 Introduction

Malaria is a severe protozoal disease prevalent in 83 endemic countries with 263 million cases and 597,000 deaths reported in 2023. Over half of this burden is concentrated in WHO African regions (WHO, 2025). A major contributor to the rising incidences is the growing resistance of *Plasmodium falciparum* to current antimalarial drugs and insecticides. This highlights the urgent need for new therapies that are effective against resistant strains, have improved pharmacokinetic profile and are based on validated molecular targets.

One such pathway is the *Plasmodium* mitochondrial electron transport chain (PmtETC) containing five key enzymes: NADH:ubiquinone oxidoreductase (*Pf*NDH2), succinate dehydrogenase (Complex II or SDH), glycerol-3-phosphate dehydrogenase (G3PDH), malate:quinone oxidoreductase (MQO), and dihydroorotate dehydrogenase (DHODH) (Nixon et al., 2013b). The PmtETC regenerates ubiquinone, a crucial electron acceptor for DHODH, which is essential for pyrimidine biosynthesis (Gutteridge et al., 1979; Painter et al., 2007). Along with this, cytochrome *bc*₁ complex (cyt *bc*₁), cytochrome c, aa3 cytochrome c oxidase, and F1Fo-ATPase (complex V) are also components of the ETC (Fisher et al., 2014, 2020).

The cyt *bc*₁ complex (complex III; EC 1.10.2.2) plays an important role in mitochondrial respiration by oxidizing ubiquinol to ubiquinone and transferring electrons to maintain the mitochondrial membrane potential (Vaidya and Mather, 2005, 2009). This dimeric complex consists of up to 11 subunits in eukaryotes, with three core catalytic subunits: cytochrome *b*, cytochrome *c*₁ and Rieske iron-sulfur protein (Gardner et al., 2002; Hikosaka et al., 2015; Nixon et al., 2013b). The electron transfer process, known as Q-cycle, is coupled with proton translocation, generating an electrochemical gradient across the mitochondrial membrane (Mitchell, 1975).

Atovaquone, a hydroxynaphthoquinone analogue of ubiquinone, is used in combination with proguanil to treat and prevent *P. falciparum* malaria (Baggish and Hill, 2002; Nixon et al., 2013a; Osei-Akoto et al., 2005). It inhibits the *bc*₁ complex by competing with ubiquinol, disrupting the mitochondrial function (Biagini et al., 2006; Srivastava et al., 1997). However, the parasite has developed resistance, primarily due to point mutation at position 268 in cytochrome *b* (Y268S, Y268N and Y268C), along with other less common variants (Gil et al., 2003; Jacobsen et al., 2021; Kessl et al., 2007; Korsinczky et al., 2000; Peters et al., 2002; Plucinski et al., 2014; Schwartz et al., 2003; Schwöbel et al., 2003). Additionally, atovaquone is expensive, suffers from poor solubility, high lipophilicity (LogP ~5) and low bioavailability, requiring administration with fatty meals to enhance absorption (Nixon et al., 2014). Given these limitations, it is critical to identify new drug candidates targeting the cyt *bc*₁ complex that can overcome resistance and offer improved pharmacokinetics.

Hybrid molecules have emerged as promising candidates, designed to target multiple pathways in *P. falciparum*, enhancing potency and reducing the likelihood of resistance. Hybrids combining naphthoquinones and 1,2,3-triazoles have demonstrated strong antimalarial activity. Naphthoquinones target the *bc*₁ complex, while triazoles have shown efficacy against various parasites, including *P. falciparum* (Faidallah et al., 2016; Graciano et al., 2022; Prasad Raiguru et al., 2023). Triazole-naphthoquinone conjugates have exhibited some potent *in vitro* activity against

chloroquine-sensitive F32 Tanzania *P. falciparum* strain (IC₅₀: 0.8 μ M - >20 μ M) (Oramas-Royo et al., 2019), and lapachol-derived hybrids have shown promising docking interactions with PfDHODH and activity against chloroquine-resistant W2 *P. falciparum* strain (IC₅₀: 4.0 μ M - 19.4 μ M) (Soares, 2018), suggesting their potential as next-generation antimalarials.

This study investigates the *in vitro* efficacy of a series of 1,4-naphthoquinone-1,2,3-triazole hybrids (Erasmus et al., 2021) against an atovaquone-resistant *P. falciparum* strain. Molecular modeling was used to explore the binding behavior within the Y268S mutant model, the most common resistance-conferring variant. *In silico* analyses were also conducted to assess pharmacokinetic properties and drug-likeness.

Due to the lack of a *P. falciparum* bc₁ crystal structure, *S. cerevisiae* bc₁ complex (PDB 3CX5) was used as a model, given its high sequence identity (about 68%) with *Plasmodium* cytochrome *b* (Kessl et al., 2003, 2005) and similar sensitivity to atovaquone (IC₅₀: 3 - 4 nM) (Valli res et al., 2013). This makes yeast a suitable model for studying drug interactions and resistance mechanism targeting the bc₁ complex .

While *in silico* modelling using different species with high sequence similarity can aid hypothesis generation, it has limitations. A 68% sequence similarity does not guarantee functional equivalence; as critical differences may exist in binding or allosteric sites. Structural variations can influence ligand binding and enzyme activity, and species-specific metabolic pathways may alter the physiological response, requiring *in vitro* or *in vivo* validation.

Despite limitations, *in silico* approaches offer a cost-effective and rapid means to prioritize compounds for further testing. However, due to possible false positive or negatives, experimental validation remains essential.

This study adopted a dual approach, combining *in silico* and *in vitro* methods to evaluate the antimalarial potential of 1,4-naphthoquinone-1,2,3-triazole hybrids. The *in vitro* assays were conducted against the *P. falciparum* FCR3 strain, known for atovaquone-resistance (Carrasco et al., 2013; Rodrigues et al., 2009; Yeates et al., 2008). Molecular modeling used the yeast bc₁ complex (PDB 3CX5) to simulate binding to the Y279S mutant, analogous to the Y268S mutation in *P. falciparum*. Results from both approaches were compared to atovaquone to assess whether the hybrids demonstrated superior activity and pharmacokinetic properties, supporting their potential as next-generation antimalarial agents.

5.2 Materials and methods

5.2.1 1,4-Naphthoquinone-1,2,3-triazole hybrids

A series of ten 1,4-naphthoquinone-1,2,3-triazole hybrids previously synthesized and characterised by Erasmus et al. (2021) was investigated for antimalarial activity (**Table 5.1**).

5.2.2 *In vitro* anti-plasmodial activity

The FCR3 strain of *P. falciparum* was maintained continuously *in vitro* in supplemented RPMI-1640 culture media at 37°C and gassed with a mixture of 2% O₂, 5% CO₂, and 93% N₂ (Trager and Jensen, 1976). To evaluate the antimalarial activity of the hybrids, the 5% D-sorbitol synchronized ring-stage parasites (Lambros and Vanderberg, 1979) were adjusted to a final parasitemia of 2% and 2% hematocrit, to which serial dilutions of the hybrids and positive control (atovaquone) were added followed by incubation for 72 hrs. Uninfected erythrocytes and drug-free parasitised erythrocytes were used as negative controls. Following 72 hours of incubation period, the plates were frozen at -70°C for 1 hour and thawed after 1 hour. The lysate (25 µl) was transferred to a second plate, to which 100 µl Malstat™ and 20 µl nitroblue tetrazolium-phenazine thiosulphate (1:1) mixture was added to each well and incubated for 40 min at 37°C to determine the parasite lactate dehydrogenase (pLDH) activity (Makler et al., 1993). Thereafter, 5% acetic acid (50 µl) was added to each well, and the absorbance of the formazan products recorded at 620 nm, as an indicator of parasite viability. The percentage of parasite growth, taking the appropriate controls into account, was calculated and used to determine the concentration required to inhibit parasite growth by 50% (IC₅₀ value) from log sigmoid dose–response curves using the GraphPad Prism® 5.0.0 software (GraphPad Software, CA, USA). A four-parameter logistic regression model was applied to characterize the dose-response relationship, accommodating asymptotic behaviour at both low and high concentration extremes where parasite growth inhibition did not converge to absolute 0% or 100%. This modelling approach permits estimation of non-fixed lower and upper asymptotes, thereby capturing intrinsic biological variability and enhancing the robustness of curve fitting in biological assay conditions (Straetemans, 2012). Each experiment was repeated at least in triplicate, with the average and standard deviation (S.D) reported (van Zyl et al., 2002).

5.2.3 *In silico* studies

Due to the unknown allelic status of the *Pf*FCR3 strain used in the *in vitro* study, the *in silico* model was constructed based on the most prevalent mutation (Y268S) reported in the resistant isolates (Akhoon et al., 2014; Fisher et al., 2012; Plucinski et al., 2014). While this approach provides a reasonable approximation, we acknowledge this as a limitation of the study, as the actual genotype may differ.

5.2.3.1 Molecular modelling

3D structures of ligands were prepared using LigPrep program of Schrödinger Maestro software (Schrödinger Release Notes - Release 2023-2) and OPLS4 force field, with protonation states being predicted using Epik with a target pH of 7.0 ± 2.0 (Schrödinger Release 2023-2: Epik) (Shelley et al., 2007). The protein structure was prepared using the protein preparation wizard from Schrödinger (Schrödinger Release 2023-2: Protein Preparation Workflow) (Solmaz and Hunte, 2008). All extra chains and ligands were deleted, keeping only the chains C and P involved in the binding pocket. To build the mutant model, tyrosine at position 279 (equivalent to position 268 of *Plasmodium* parasite) in the wild type (WT) model was replaced by serine to account for the Y279S mutation (equivalent to Y268S in *P. falciparum*) (Chebon-Bore et al., 2021; Jacobsen et al., 2021; Song et al., 2015). The protein was later pre-processed and minimised to relieve unfavourable constraints.

Molecular docking was performed using Glide extra precision (XP) scoring function, and ligand structures were treated as flexible, which allowed for sampling of ring conformations (including input ring conformation) and nitrogen inversion, to obtain five poses per ligand (Friesner et al., 2004, 2006). Prime MM-GBSA (Molecular Mechanics/Generalized Born Model and Solvent Accessibility) v3.000 program was used to estimate the binding free energy (or ΔG) for each docked pose complex with the VSGB solvation model and OPLS4 force field (Genheden and Ryde, 2015; Li et al., 2011; Mulakala and Viswanadhan, 2013).

The complexes with the best binding pose score (most negative ΔG value) were submitted for 100ns molecular dynamic (MD) simulations using the Desmond program found within the Schrödinger software suite and the OPLS4 force field (Abdullah et al., 2023). Water molecules present in the binding pocket from the docking procedure were retained, and the system was solvated using the TIP4P water model. An orthorhombic simulation box was set up with boundaries 10 Å away from the protein, containing more than 130,000 atoms. The rest of the settings were kept on default and MD simulation was done for 100ns with NPT conditions (300 K, 1.103 bar and 2000 bar.Å). The MD

simulations were analysed using the simulation interaction diagram tool within the Schrödinger Suite (Schrödinger Release 2023-2: Desmond Molecular Dynamics System).

5.2.3.2 *In silico* ADMET and Drug-likeness

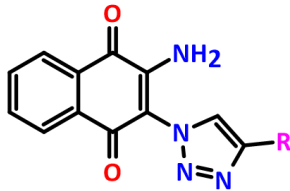
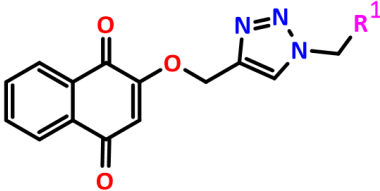
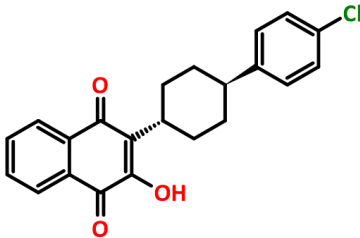
ADMET properties, including the molecular properties that are incorporated in Lipinski's rule of five (Ro5), gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, along with number of rotatable bonds (RB), topological polar surface area (TPSA), and bioavailability were calculated using SwissADME program (Daina et al., 2017; SwissADME). ProTox-3.0 tool was used to predict the potential toxicity of chemical hybrids (Drwal et al., 2014; ProTox-3.0) with acute toxicity (rodent) predicted as LD₅₀ in mg/kg body weight, organ toxicity and toxicological endpoints.

5.3 Results and Discussion

5.3.1 Antimalarial activity and SAR

All the test hybrids, along with atovaquone, evaluated for *in vitro* antimalarial activity against the FCR3 strain of *P. falciparum* displayed a dose dependent effect (**Table 5.1**).

Table 5.1: *In vitro* assay results and summary of docking and MM-GBSA scores for hybrids docked in 3CX5 (Y279S mutant model) and atovaquone.

Hybrid ID	Structures of hybrids	Antimalarial activity <i>Pf</i> FCR3 (IC ₅₀ ±S.D.; μM)	3CX5 mutant (Y279S) model		Key interactions
			Docking score	MM-GBSA (kcal/mol)	
 D3, D7, D8  D12 – D18  Atovaquone					
D3	R = Phe	0.471 ± 0.128	-8.94	-38.76	Glu ²⁷² (water bridge, aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D7	R = 3,5-diCF ₃ Phe	0.596 ± 0.003	-8.27 (pose 1); -8.07 (pose 2)	-44.56 (pose 1); -45.13 (pose 2)	No interactions with His ¹⁸¹ , Glu ²⁷² and Ser ²⁷⁹ . Only intramolecular aromatic H-bond was observed.
D8	R = CH ₂ SPhe	0.651 ± 0.153	-9.62	-50.87	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge)
D12	R ¹ = Phe	0.028 ± 0.006	-10.37 (pose 1); -10.31 (pose 2)	-40.02 (pose 1); -40.00 (pose 2)	Glu ²⁷² (water bridge and aromatic H-bond), Met ¹³⁹ (aromatic H-bond) / His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge and aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D13	R ¹ = 4-Me Phe	0.033 ± 0.004	-10.77	-56.54	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge)

D14	R ¹ = 4-tBu Phe	0.401 ± 0.077	-12.09	-47.86	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge and aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D15	R ¹ = 4-Br Phe	0.191 ± 0.049	-10.34 (pose 1); -10.12 (pose 2)	-44.49 (pose 1), -49.43 (pose 2)	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge, aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D16	R ¹ = 3-Br, 5-F Phe	0.074 ± 0.021	-10.65	-42.47	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge and aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D17	R ¹ = 4-NO ₂ Phe	0.259 ± 0.026	-9.96	-45.65	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge, aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D18	R ¹ = 4-CF ₃ Phe	0.110 ± 0.023	-10.91 (pose 1); -10.62 (pose 2)	-41.80 (pose 1); -49.32 (pose 2)	Glu ²⁷² (water bridge and aromatic H-bond), Met ¹³⁹ (aromatic H-bond) / His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge, aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
Atova-quone	-	1.713 ± 0.014	-8.230	-21.66	No interactions with His ¹⁸¹ , Glu ²⁷² and Ser ²⁷⁹

All the hybrids showed promising antiparasmodial activities with IC_{50} values $<1 \mu M$ against the FCR3 strain. Hybrids **D12** and **D13** exhibited the best activities with IC_{50} values respectively, 61-fold and 52-fold lower than atovaquone (**Table 5.1**). Examination of the bioactivity data for the FCR3 plasmodial strain disclosed interesting SAR among this set of tested hybrids. It was observed that there were two series of hybrids – 2-amino-3-(1H-1,2,3-triazol-1-yl)-1,4-naphthoquinones (**D3**, **D7** and **D8**) and 2-[(1H-1,2,3-triazol-4-yl)methoxy]-1,4-naphthoquinones (**D12** to **D18**) (**Table 5.1**). According to the findings of the *in vitro* assay, hybrids **D12** to **D18** belonging to 2-[(1H-1,2,3-triazol-4-yl)methoxy]-1,4-naphthoquinones exhibited better antiparasmodial activities compared to hybrids **D3**, **D7** and **D8** (2-amino-3-(1H-1,2,3-triazol-1-yl)-1,4-naphthoquinones), suggesting that the presence of methoxy oxygen played a key role in the binding of hybrids at the binding pocket such that the hybrids could produce their inhibitory effects. Further analysis revealed that the antiparasmodial activity depended on the electron-donating and electron-withdrawing nature of the substituent attached to the phenyl ring. Hybrid **D12**, with the best antiparasmodial activity, had no substituent on the phenyl ring. Hybrid **D12** has also been reported to inhibit F32 Tanzania (chloroquine-sensitive) strain of *P. falciparum* with IC_{50} value of $17.9 \pm 2.5 \mu M$ (Oramas-Royo et al., 2019). Hybrid **D13** with a 4-methyl phenyl group showed a slight increase in the IC_{50} value (around 1.2 fold) compared to **D12** with unsubstituted phenyl. While hybrid **D14** with the *tert*-butyl group at the para position displayed a further increase in the IC_{50} value, which could be accounted for by the bulky and electron-donating nature of the *tert*-butyl group. The presence of halogen groups (F or Br) improved the inhibitory activities of hybrids **D15**, **D16**, and **D18**. The presence of a halogen atom was also observed to enhance the antimalarial activities of quinolone-based compounds, as reported against the atovaquone-resistant *P. falciparum* TM90-C2B strain (Cross et al., 2010). Di-substitution improved the inhibitory efficiency of hybrid **D16** compared to mono-substituted **D15**. The presence of the nitro group at the para position in **D17** did not further improve the activity of the hybrid. Whilst, hybrid **D18**, with a para-trifluoromethyl substituent, displayed improved activity compared to **D14**, likely attributed to the presence of halogen atoms.

From hybrids **D3**, **D7** and **D8**, hybrid **D3** with an unsubstituted phenyl ring displayed improved antimalarial activity. On the other hand, hybrid **D7** with two trifluoromethyl groups might face binding hindrance at the active site due to the bulky nature of the compound (Báez-Grez and Pino-Rios, 2023; Huber et al., 2023; Iversen et al., 2001).

5.3.2 *In silico* studies

5.3.2.1 Molecular modelling

To verify that the hybrids targeted the cyt *bc*₁ complex, molecular docking studies were performed to investigate their binding modes within the yeast cyt *bc*₁ binding pocket (Vallières et al., 2013) (**Table 5.1**). The docking scores for all test hybrids demonstrated higher ranked values (ranging from -8.07 to -12.09 kcal/mol) compared to atovaquone (-8.23 kcal/mol), suggesting stronger binding affinities towards the active site of cyt *bc*₁ complex (**Table 5.1**). The best binding affinity was displayed by hybrid **D14** with a docking score of -12.09 kcal/mol. However, MM-GBSA binding free energy calculations showed that hybrid **D13** (docking score -10.77 kcal/mol) displayed a more favourable ΔG_{bind} value of -56.54 kcal/mol compared to hybrid **D14** (ΔG_{bind} = -47.86 kcal/mol). The higher negative docking score of hybrid **D14** suggests strong initial binding poses, likely due to favourable hydrogen bonds and hydrophobic interactions from the *tert*-butyl group. However, MM-GBSA binding energy, which incorporates solvation effects and molecular mechanics, revealed that the strong docking poses may not translate into energetically favourable binding complexes. Conversely, hybrids with poorer docking scores, like hybrid **D13**, might have achieved lower desolvation penalties and better overall stabilization, as reflected by its more favourable MM-GBSA ΔG_{bind} value.

On analysing the interactions with the key residues His¹⁸¹ (chain P) and Glu²⁷² (chain C), it was observed that hybrid **D7** displayed no interactions with either His¹⁸¹ or Glu²⁷² and was observed to be binding at a site of the pocket away from these key residues. This seemed to be energetically more favourable for hybrid **D7**, as suggested by its MM-GBSA score (ΔG_{bind} = -45.13 kcal/mol).

All the other test hybrids interacted with His¹⁸¹ and Glu²⁷² via hydrogen bond and water bridge formation, respectively, supporting that these two key interactions are important for keeping the ligand stable within the binding pocket of cyt *bc*₁ and impart their inhibitory activities (**Figure 5.1**). Compared to hybrid **D12**, hybrids **D15**, **D16** and **D18** displayed slightly improved docking and MM-GBSA scores with similar initial interactions with the binding pocket residues His¹⁸¹, Glu²⁷², and Met¹³⁹ (chain C). However, as suggested by the *in vitro* results, hybrid **D12** outperformed the rest in the antiplasmodial assay. This discrepancy highlights the inherent limitations of computational scoring functions, which primarily account for static binding interactions within the binding pocket and often overlook critical biological factors that may account for favourable pharmacokinetic properties. Further analysis of the molecular properties presented in **Table 5.2** revealed that hybrids **D15** (LogP 3.00), **D16** (LogP 3.29) and **D18** (LogP 3.45) possess higher lipophilicity compared to **D12** (LogP 2.45) which might have negatively impacted the bioavailability of these compounds. Notably,

the LogP values of these hybrids are better compared to atovaquone (LogP 4.64), suggesting a potentially improved bioavailability and pharmacokinetic profile. This observation highlights the importance of integrating computational predictions with experimental validation, as molecular docking scores alone may not fully predict biological potency of compounds.

To support the study findings, atovaquone was analysed in two separate models, one for wild type (WT) and another accounting for Y279S mutations, to assess and observe the differences in its binding modes in the two models using docking and MD simulations; and further using those results to compare with the results of the test hybrids. It was observed that atovaquone interacted with His¹⁸¹ (via H-bond) and Glu²⁷² (via water bridge) when docked in the WT model (docking score -9.98), whereas in the resistant model, it lost these key interactions and moved away from the binding pocket to an energetically more favourable site as supported by its MM-GBSA score in resistant model ($\Delta G_{\text{bind}} = -21.66$ kcal/mol) vs MM-GBSA score in the WT model of ($\Delta G_{\text{bind}} = -12.63$ kcal/mol) (**Figure 5.1**). These observations were in agreement with the literature (Akhoon et al., 2014; Birth et al., 2014; Fisher et al., 2012; Kessl et al., 2005), and were also supported by the *in vitro* antiparasmodial assay performed to assess atovaquone activity against the sensitive *PfNF54* strain (IC₅₀ value: 0.021 ± 0.011 μM), further supporting that the *PfFCR3* strain is resistant to atovaquone.

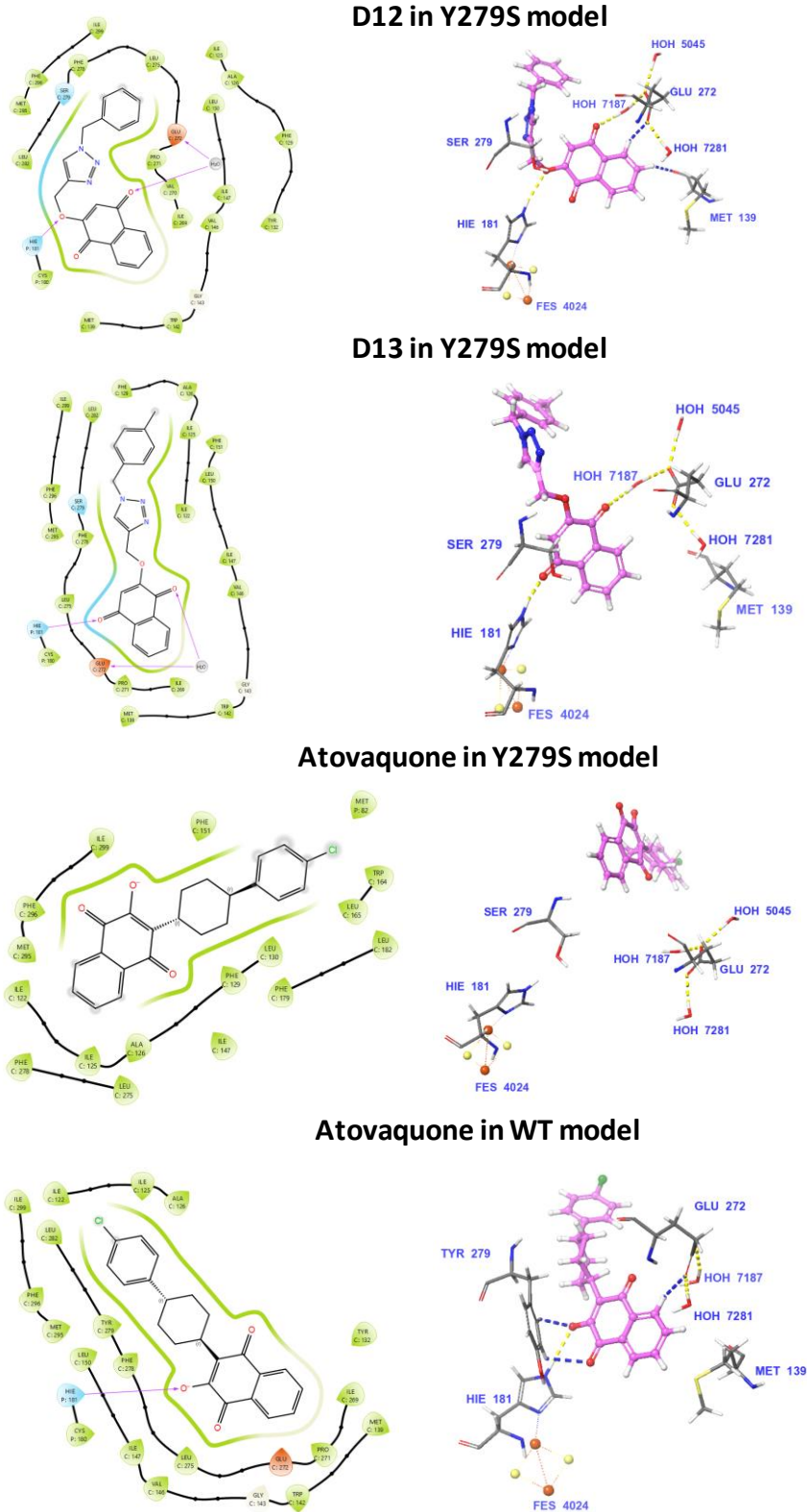


Figure 5.1: Ligand interaction diagram (LID) for hybrids **D12**, **D13** and **atovaquone** in **3CX5 (Y279S) mutant model** and their comparison with **atovaquone** in **3CX5 (WT) model** with 2-dimensional (2D) LIDs on the left-hand side and 3-dimensional (3D) LIDs on the right-hand side.

For further assessment, test compounds were subjected to 100 ns MD simulations. The results for the two most active hybrids, **D12** and **D13**, are presented to understand their trajectory and key ligand-protein interactions in the Y279S mutant model where Tyr²⁷⁹ (chain C) was replaced by Ser²⁷⁹ (chain C).

Protein-Ligand Root Mean Square Deviation (RMSD)

RMSD determination helped examine the target protein's conformational changes when combined with ligand molecules for a 100 ns MD simulation. At the beginning of the simulation, there was a jump in the RMSD as the system equilibrated and reached a stationary state. This jump was higher for hybrid **D13** compared to hybrid **D12**, but subsequently the RMSD remained relatively stable until the end of the simulation (**Figure 5.2**). The core structure of the protein remained stable, as indicated by localised analysis of key domains in the protein root mean square fluctuation (RMSF) values (supplementary information, **Figure S.3**). The RMSD value for both ligands changed within a narrow range and remained lower than the RMSD of the protein. This indicated that both ligands remained securely within the binding pocket and did not diffuse away, aligning with effective binding interactions typically observed in stable protein-ligand complexes (Gohlke and Klebe, 2002).

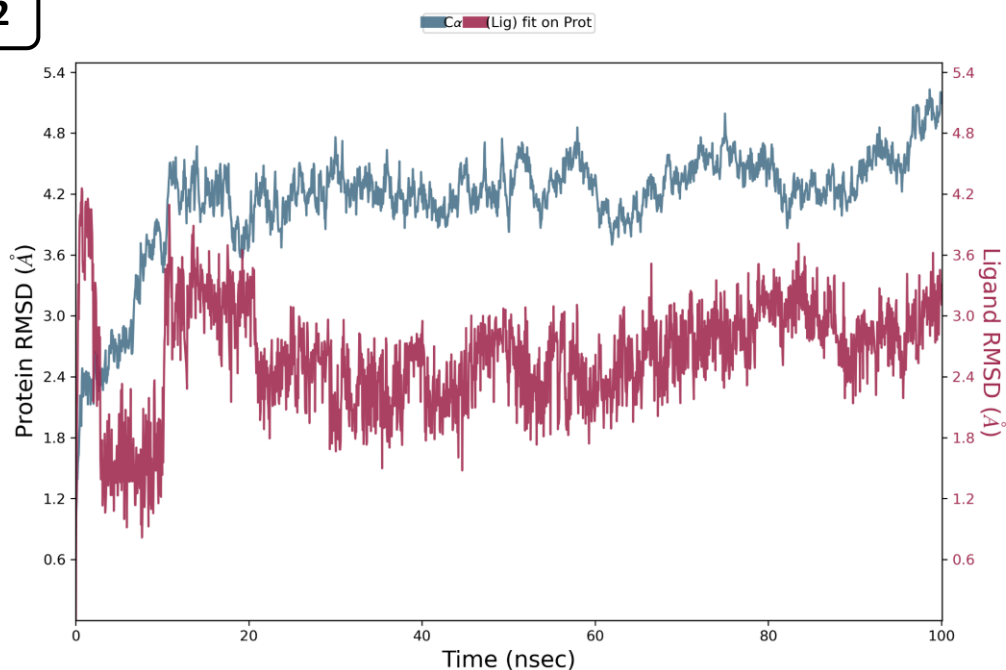
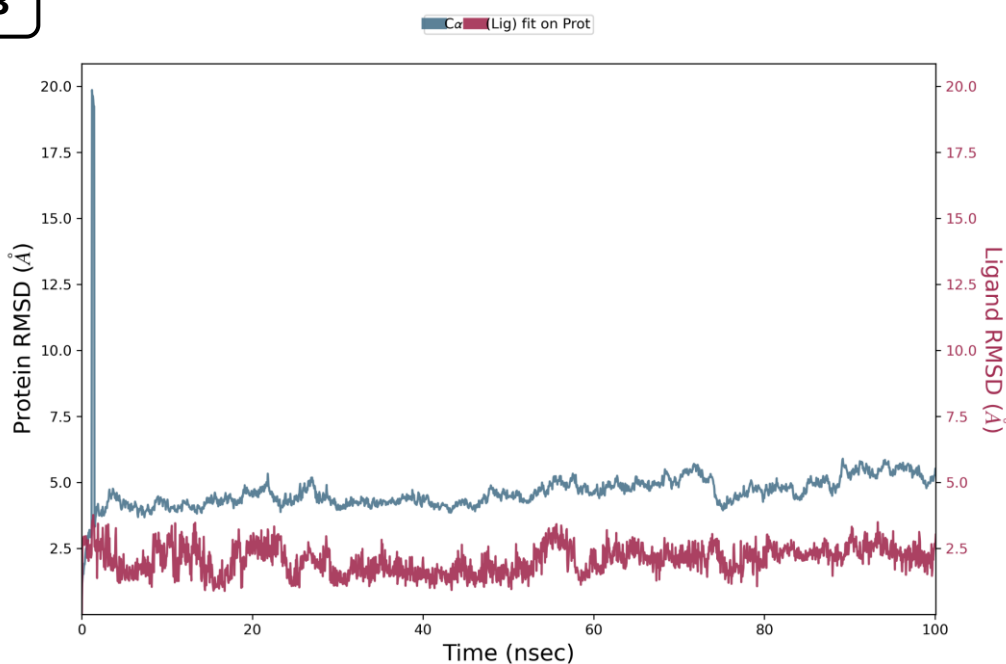
D12**D13**

Figure 5.2: Protein-Ligand (PL) Root Mean Square Deviation (RMSD) plots for hybrids **D12** and **D13** in complex with 3CX5 (Y279S) resistant-model over 100 ns of MD simulation obtained using Schrödinger Desmond. The blue curve represents the RMSD of the protein C α atoms, providing insights into the backbone stability and overall conformational changes during the simulation. The red curve corresponds to the ligand RMSD, showing its movement relative to the protein binding site. A steady RMSD after 20 ns suggests system equilibration, indicating that both the protein and ligands had stabilized in their respective conformations.

Ligand protein contacts

The ligand-protein interactions between cyt *bc*₁ and **D12** and **D13** (**Figure 5.3**), indicated that the residues with the highest interaction fractions was His¹⁸¹ (chain P) for both hybrids **D12** and **D13**. In addition to His¹⁸¹, hybrid **D12** also displayed major interactions with Ser²⁷⁹ (chain C) and Phe¹²⁹ (chain C), whereas hybrid **D13** exhibited additional interactions with Phe¹²⁹ (chain C) and Glu²⁷² (chain C).

The ligand-protein contacts analysis indicated that hybrids **D12** and **D13** interacted with His¹⁸¹ (chain P, via H-bonds) for 78% and 70% of the simulation time, respectively (**Figure 5.3**). In addition, hybrid **D12** also interacted with Ser²⁷⁹ for 49% of the simulation time via a H-bond and with Phe¹²⁹ for 34% of the simulation time via π - π stacking. On the other hand, hybrid **D13** had additional interactions with Glu²⁷² via water bridge for 40% of the simulation time and with Phe¹²⁹ for 10% to 28% of the time via π - π stacking. Both hybrids showed comparable interactions with the binding pocket residues, with hybrid **D12** showing slightly better interactions in terms of time duration and with Ser²⁷⁹ which was present in the mutant model. These *in silico* results support the marginally better IC₅₀ value of hybrid **D12** compared to hybrid **D13** in the antiplasmodial assay.

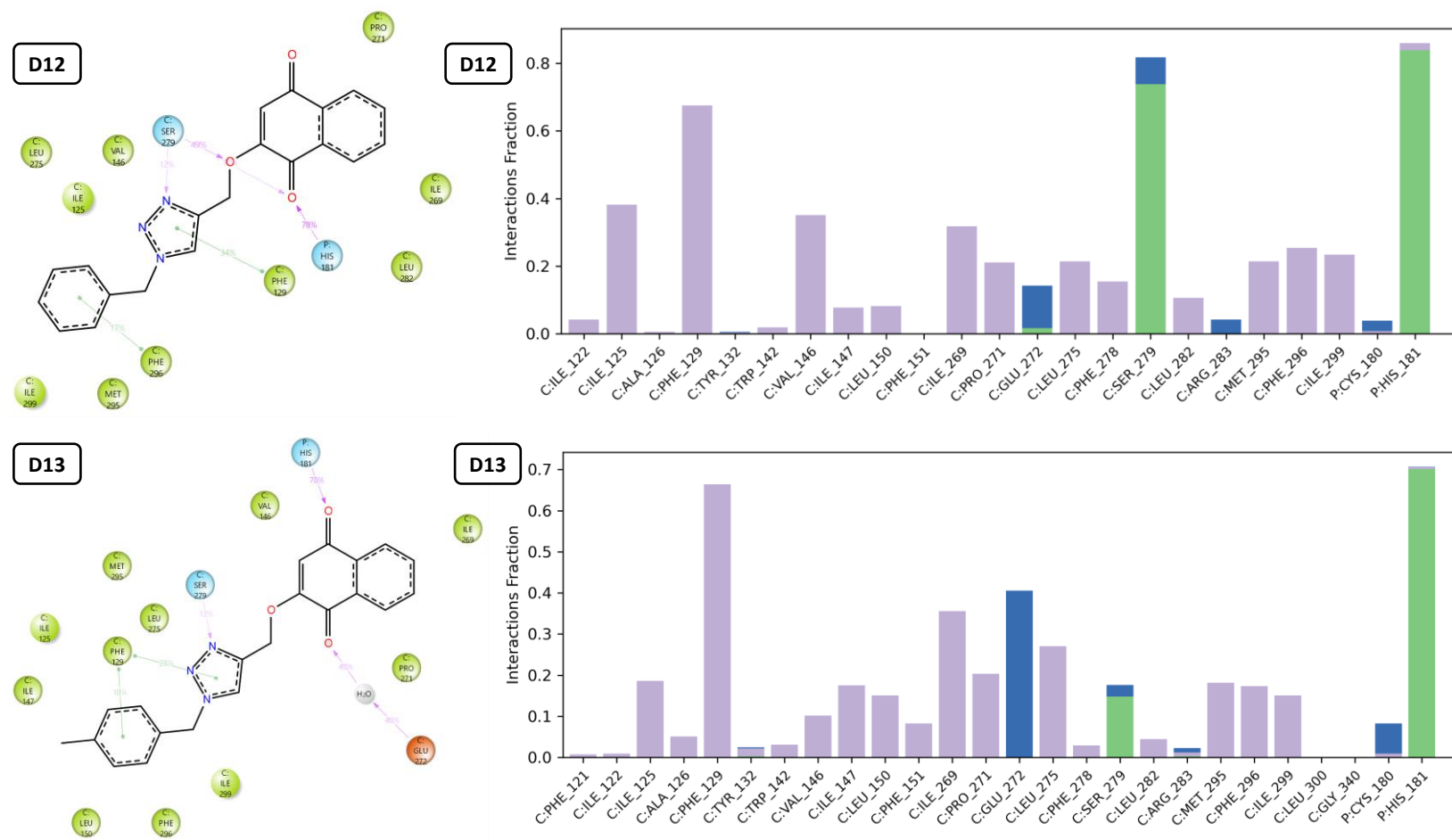


Figure 5.3: Ligand-protein (LP) contact analysis from MD simulation for hybrids **D12** and **D13**, represented by LP contacts 2D summary diagrams (left) and LP contacts histograms (right) in 3CX5 (Y279S) resistant model. The 2D schematic interaction diagram visualising key interactions between the ligand and protein residues happening more than 10% of the simulation time are shown. The bar chart represents the frequency of the protein-ligand interactions categorised into four types: hydrogen bonds (green), hydrophobic bonds (purple), ionic bonds (red) and water bridges (blue). Residues with higher interaction frequencies indicate key binding site contacts contributing to ligand stability.

5.3.3 Drug- likeness:

All test hybrids, including atovaquone, complied with Lipinski's rule of five (Ro5) with no violations, indicating favourable drug-likeness (**Table 5.2**) (Lipinski, 2002). A bioavailability score of ≥ 0.55 further supported their potential as drug candidates. Though all these hybrids displayed satisfactory molecular properties and Lipinski's parameters within acceptable limits, hybrid **D7** exhibited #HBAs touching the highest permitted value of 10, along with the highest MW (452.31 g/mol) and the highest LogP value of 4, suggesting that it might face challenges with absorption. Hybrids **D8** and **D17** displayed highest topological polar surface area (TPSA) of 116.17 Å² and 119.90 Å², respectively, of all the test hybrids, suggesting that these two hybrids were more polar compared to other test hybrids and hence would be less extensively absorbed with low bioavailability. This observation was supported by their low predicted bioavailability score of 0.55 and 0.56, respectively.

Table 5.2: Summary of physicochemical, drug-likeness and pharmacokinetic properties of hybrids compared to atovaquone.

Ligand ID	PHYSICOCHEMICAL PROPERTIES						DRUG-LIKENESS		PHARMACOKINETIC PROPERTIES	
	MW	LogP	#HBA	#HBD	#RB	TPSA	Lipinski #violations	Bioavailability Score	GI absorption	BBB permeant
Criteria	≤ 500 g/mol (Lipinski)	≤ 5 (Lipinski)	≤ 10 (Lipinski)	≤ 5 (Lipinski)	≤ 10 (Veber)	≤ 140 Å ² (Veber)	≤ 1 violation	≥ 0.55	High is favourable	No is favourable
D3	316.31	1.93	4	1	2	90.87	0	0.55	High	No
D7	452.31	4	10	1	4	90.87	0	0.55	Low	No
D8	362.41	2.34	4	1	4	116.17	0	0.55	High	No
D12	345.35	2.42	5	0	5	74.08	0	0.85	High	Yes
D13	359.38	2.74	5	0	5	74.08	0	0.85	High	Yes
D14	401.46	3.62	5	0	6	74.08	0	0.85	High	Yes
D15	424.25	3	5	0	5	74.08	0	0.85	High	Yes
D16	442.24	3.29	6	0	5	74.08	0	0.85	High	Yes
D17	390.35	1.82	7	0	6	119.90	0	0.56	High	No
D18	413.35	3.45	8	0	6	74.08	0	0.85	High	No
Atova-quone	366.84	4.64	3	1	2	54.37	0	0.85	High	Yes

MW = molecular weight; LogP = log of octanol/water partition coefficient; #HBA = number of hydrogen bond acceptor(s); #HBD = number of hydrogen bond donor(s); #RB = number of rotatable bond(s); TPSA = topological polar surface area; Lipinski #violations = number of violations of Lipinski's rule; GI = gastrointestinal; BBB = blood-brain barrier

In comparing the number of rotatable bonds (#RB), hybrid **D3** had the least #RB = 2, which was the same as that of the reference drug, atovaquone. Since the #RB helps to determine the conformational flexibility of the molecule, hybrid **D3** was the most rigid whereas hybrids **D14**, **D17** and **D18** were the most flexible with #RB = 6. Hybrids **D12**, **D13**, **D15** and **D16** had a balanced #RB = 5. A molecule with excessive rotatable bonds may be too flexible to achieve a stable binding with the target protein, while one with too few rotatable bonds may be overly rigid, limiting its ability to fit optimally into the binding site (Veber et al., 2002).

All hybrids were predicted to have high GI absorption except hybrid **D7** as supported by its high MW and high LogP values. Hybrids **D12**, **D13**, **D14**, **D15**, and **D16** were predicted to cross the BBB, making them suitable for the central nervous system (CNS) targeted therapies like cerebral malaria (Bensalel and Gallego-Delgado, 2024; Postels and Birbeck, 2013). Hybrids **D3**, **D7**, **D8**, **D17** and **D18** were predicted to not cross the BBB, limiting their CNS-related applications.

BOILED Egg (or Brain Or Intestinal Estimated) Plot

Like atovaquone, hybrids **D12**, **D13**, **D14**, **D15**, and **D16** were plotted in the yellow core suggesting their lipophilic nature and low polarity (TPSA <90 Å²), facilitating their ability to penetrate the BBB, and making them suitable as CNS drug candidates (**Figure 5.4**). Hybrids **D3**, **D8**, **D17** and **D18** were plotted in the white region suggesting their good oral bioavailability and exhibiting a balance between lipophilicity and polarity required for absorption across the intestinal barrier. Hybrid **D7** was plotted outside the egg, in the grey region, exhibiting poor pharmacokinetics for oral administration or BBB permeability. It seemed to be very lipophilic hybrids (high WLogP) and might face issues like low solubility. None of the test hybrids falling in the egg regions were marked as a P-gp substrate, suggesting that their absorption or BBB permeability was unaffected by P-glycoprotein's active efflux.

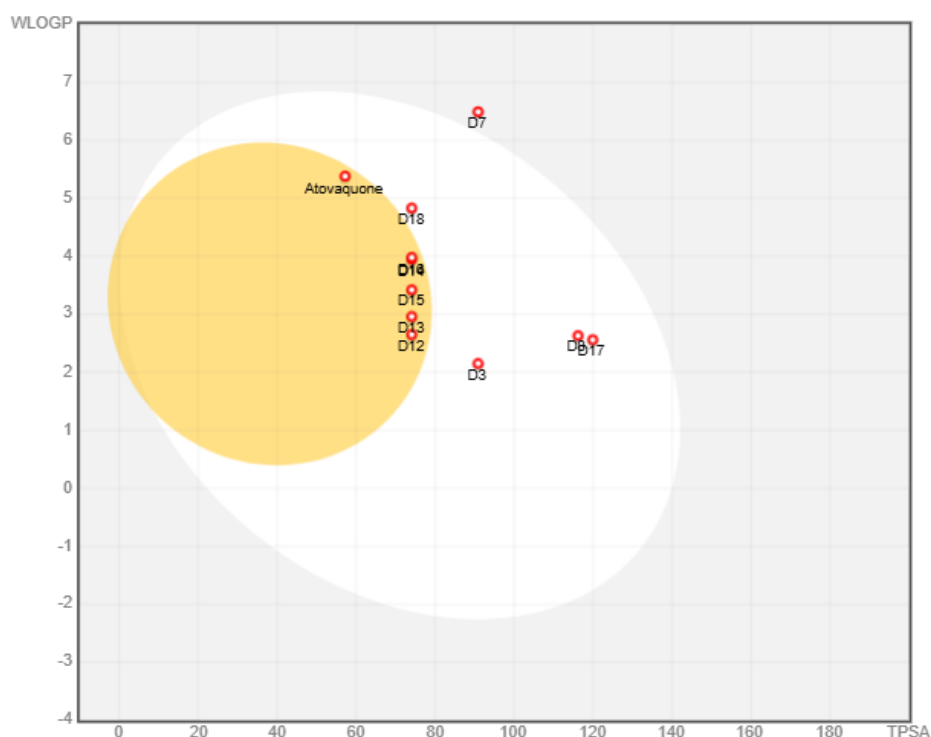


Figure 5.4: BOILED Egg (or Brain Or Intestinal Estimated) Plot of WLogP vs TPSA for atovaquone and the test hybrids. Yellow indicates BBB-permeant, white indicates human intestinal absorption (HIA), i.e. gastrointestinal permeant; blue indicates P-glycoprotein positive, and red indicates P-glycoprotein negative.

Toxicity:

Hybrids were evaluated for their safety profiles, LD₅₀ values, toxicity class, organ toxicity, and toxicity endpoints. Hybrids **D12**, **D13** and **D14** were the safest of all with LD₅₀ = 2000 mg/kg and toxicity class 4 which posed them to be harmful if swallowed (**Table 5.3**). Like atovaquone, hybrids **D12** and **D13** were predicted to be non-carcinogenic, non-immunotoxic and non-cytotoxic, with potential of respiratory and nephrotoxicity. Unlike atovaquone, these hybrids were flagged as mutagenic and neurotoxic raising concerns for their prolonged use.

The cytotoxicity of these hybrids was previously evaluated against HEK293 human embryonic kidney cell line, where hybrids **D13** and **D14**, along with atovaquone exhibited low toxicity with IC₅₀ values of 32.4 µM, 36.4 µM and 55.8 µM, respectively (**Table 5.3**) (Erasmus et al., 2021). All other tested hybrids displayed IC₅₀ values exceeding 100 µM, indicating minimal cytotoxic effects against the human HEK293 cells.

Table 5.3: Summary of the toxicity profiles for hybrids in comparison to atovaquone, based on ProTox 3.0 predictions.

Hybrid ID	HEK-293 (IC ₅₀ ; µM) (Erasmus et al., 2021)	LD ₅₀ (mg/kg)	Toxicity Class	Organ Toxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
Atovaquone	55.85	2000	Class 4	Nephrotoxic Respiratory toxic	Non-carcinogenic	Non- immunotoxic	Non- mutagenic	Non- cytotoxic
D3	>100	400	Class 4	Hepatotoxic, Neurotoxic, Respiratory toxic	Carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
D7	>100	1260	Class 4	Hepatotoxic, Neurotoxic	Carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
D8	>100	1260	Class 4	Neurotoxic, Respiratory toxic	Carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
D12	>100	2000	Class 4	Neurotoxic, Nephrotoxic, Respiratory toxic	Non-carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
D13	32.4	2000	Class 4	Neurotoxic, Nephrotoxic, Respiratory toxic	Non-carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic

D14	36.42	2000	Class 4	Neurotoxic, Respiratory toxic	Non-carcinogenic	Immunotoxic	Non- mutagenic	Non- cytotoxic
D15	>100	2000	Class 4	Neurotoxic, Nephrotoxic, Respiratory toxic	Non-carcinogenic	Immunotoxic	Mutagenic	Non- cytotoxic
D16	>100	2000	Class 4	Hepatotoxic, Neurotoxic, Nephrotoxic, Respiratory toxic	Non-carcinogenic	Immunotoxic	Mutagenic	Non- cytotoxic
D17	>100	2000	Class 4	Respiratory toxic	Carcinogenic	Immunotoxic	Mutagenic	Non- cytotoxic
D18	>100	2000	Class 4	Hepatotoxic, Neurotoxic, Respiratory toxic	Non-carcinogenic	Immunotoxic	Mutagenic	Non- cytotoxic

Additionally, observation of the supernatant while performing the antimalarial assay did not present any signs of blood lysis, indicating that the hybrids displayed inhibitory activity by targeting the parasite and not by causing haemolysis.

5.4 Conclusions

This study investigated a series of 1,4-naphthoquinone-1,2,3-triazole hybrids using a combination of *in vitro* and *in silico* approaches to identify potential antimalarial candidates. All hybrids demonstrated potent antiplasmodial activity against the *P. falciparum* FCR3 strain, with IC₅₀ values ranging from 0.028 μ M to 0.651 μ M, markedly superior to the reference drug atovaquone (IC₅₀ 1.713 μ M). Among these, hybrids **D12** and **D13** emerged as the most promising hybrids with respective activities around 61- and 52-fold more than atovaquone, showing strong potential for further development against atovaquone-resistant strains. Molecular docking further supported these findings, showing that hybrids **D12** and **D13** established stable interactions with key residues His¹⁸¹ (chain P) and Glu²⁷² (chain C) within the binding pocket of the mutant model. In contrast, these interactions were lost when atovaquone was docked with the Y279S resistant model, indicating that stable binding to His¹⁸¹ (chain P) and Glu²⁷² (chain C) may be critical for a ligand's effectiveness against the resistant strain.

Cytotoxicity data against human HEK-293 cells revealed that all hybrids, except hybrids **D13** and **D14**, exhibited improved safety profile compared to atovaquone, particularly highlighting hybrid **D12** as a favourable candidate based on the *in vitro* and *in silico* results. Additionally, predicted LogP values of the hybrids suggested improved bioavailability compared to atovaquone. Furthermore, *in silico* assessments of drug-likeness and toxicity profiles were consistent with the experimental results, underscoring the utility of computational tools in early-stage drug discovery.

Overall, these findings offer a strong foundation for future structural optimisation and preclinical development. Future studies should aim to enhance the pharmacokinetic profiles of these hybrid compounds and validate their efficacy *in vivo* to accelerate the development of effective antimalarial therapies against emerging drug resistance.

Author contributions: Conceptualization: PA and RvZ; Data curation: PA; Formal analysis: PA; Funding acquisition: RvZ; Investigation: PA; Methodology: PA; Project administration: RvZ; Resources: RvZ; Supervision: RvZ; Validation: PA, RvZ; Visualisation: PA, RvZ; Writing - original draft: PA; Writing - review & editing: PA, DDN, CJvdW, RvZ.

Ethics approval: This study used *Plasmodium falciparum* and human blood for culturing and performing *in vitro* assay with ethical clearance granted by the WITS Human Research Ethics Committee (Clearance Certificate No. M210121) and WITS Institutional Biosafety Committee (Clearance Certificate No. 20190404). Funding was obtained from the Faculty of Health Sciences Research Office.

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Supplementary information:

- **Supplementary Information S1:** MD simulation report for D12-3CX5 (Y279S) complex
- **Supplementary Information S2:** MD simulation report for D13-3CX5 (Y279S) complex
- **Figure S.3:** Protein RMSF plots for hybrids D12 and D13 with RMSF values (Å) for key residues His¹⁸¹, Glu²⁷² and Ser²⁷⁹.

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Supplementary information:

Supplementary Information S1: MD simulation report for D12-3CX5 (Y279S) complex (**Appendix H.2**)

Supplementary Information S2: MD simulation report for D13-3CX5 (Y279S) complex (**Appendix H.3**)

Supplementary Figure S.3:

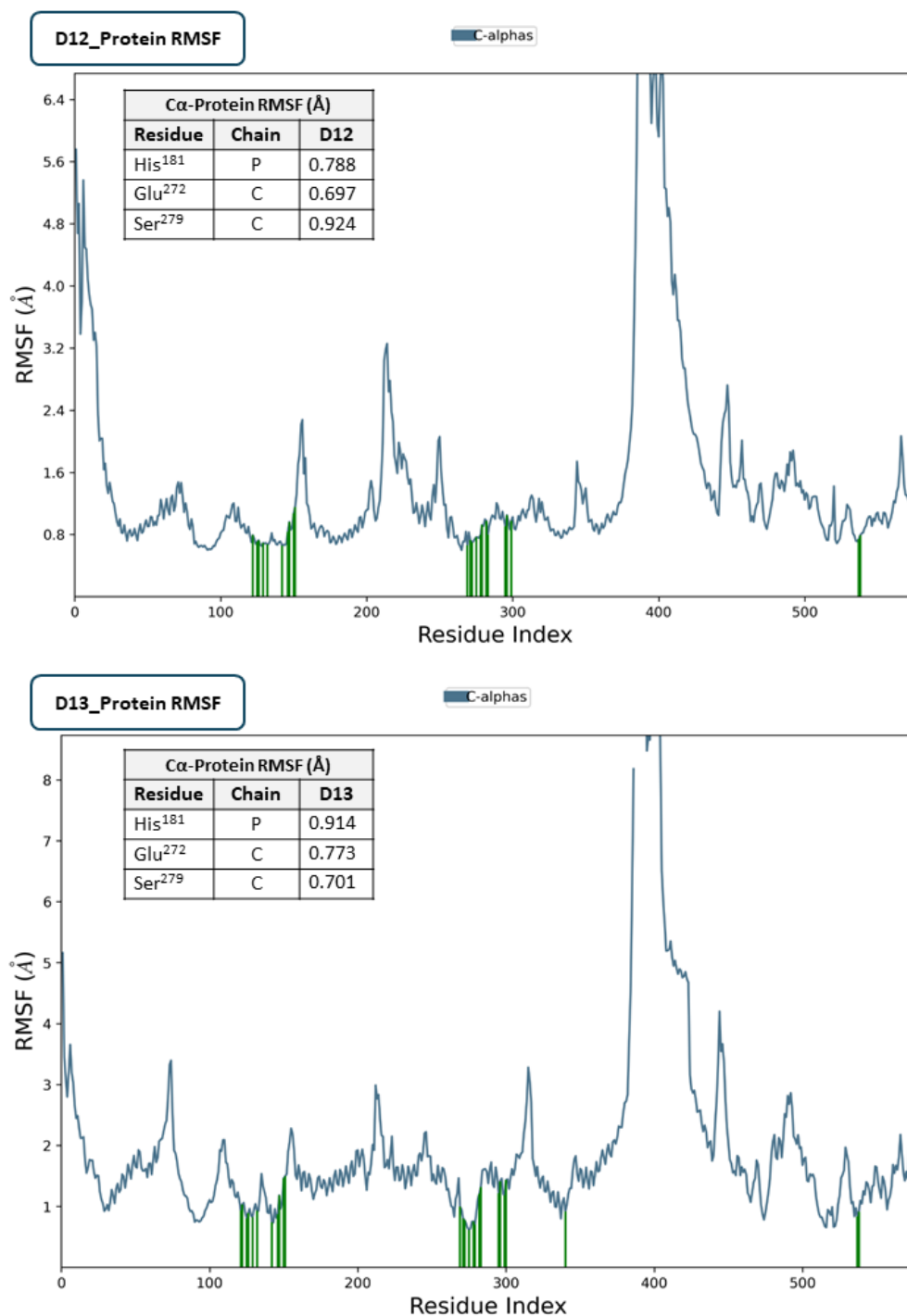


Figure 5.5: Protein RMSF plots for hybrids D12 and D13 with RMSF values (Å) for key residues His¹⁸¹, Glu²⁷² and Ser²⁷⁹.

Chapter 6

Evaluation of naphthyl bearing 1,3,4-thiadiazoleacetamide hybrids as *Plasmodium* cytochrome *bc*₁ complex inhibitors

This chapter focuses on the computational evaluation of a set of naphthyl bearing 1,3,4-thiadiazoleacetamide compounds previously studied in collaboration to assess the *in vitro* antimalarial and preliminary cytotoxicity activity of the compounds. In the published paper (1), the target site was *P. falciparum* dihydrofolate reductase (PfDHFR), which was investigated by the PhD student, Kavita Pal (Drug Design and Synthesis Laboratory, Department of Chemistry, Jamia Millia Islamia, New Delhi 110025). The collective findings were published in the paper entitled “Naphthyl bearing 1,3,4-thiadiazoleacetamides targeting the parasitic folate pathway as anti-infectious agents: *in silico*, synthesis, and biological approach” in the Royal Society of Chemistry, Medicinal Chemistry 2023. DOI: 10.1039/d3md00423f (**Appendix F**).

Building on this foundational work, I undertook a new computational investigation to determine whether these hybrids exhibited inhibitory potential against the cytochrome *bc*₁ (cyt *bc*₁) complex and correlated the results with already determined antimalarial activity against ATQ-S NF54 strain. In addition, the *in vitro* toxicity profile was extended and the *in silico* drug-likeness and pharmacokinetic properties were determined too.

By repurposing compounds with known biological activity and acceptable cytotoxicity profiles, this study aimed to determine if new lead compounds could also target the *Plasmodium bc*₁ complex. The findings offered valuable insights into the structural and pharmacological features that could be further optimised for the development of next-generation antimalarial therapies.

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Student's contributions:

Contributions	Priyanka Agarwal	C. Johan van der Westhuizen	Robyn L. van Zyl	Published	Data reported in this chapter
Conceptualisation	✓				
Cytotoxicity assay (HEK293)	✓			✓	✓
Cytotoxicity assay (K562)	✓				✓
Computational studies against cytochrome <i>bc</i> ₁ complex	✓				✓
Computational studies to determine drug-likeness and toxicity profile	✓				✓
Data analysis of cytotoxicity assay and computational results	✓		✓		✓
Supervision		✓	✓		

Naphthyl bearing 1,3,4-thiadiazoleacetamides targeting the *Plasmodium falciparum* cytochrome *bc*₁ complex

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Abstract:

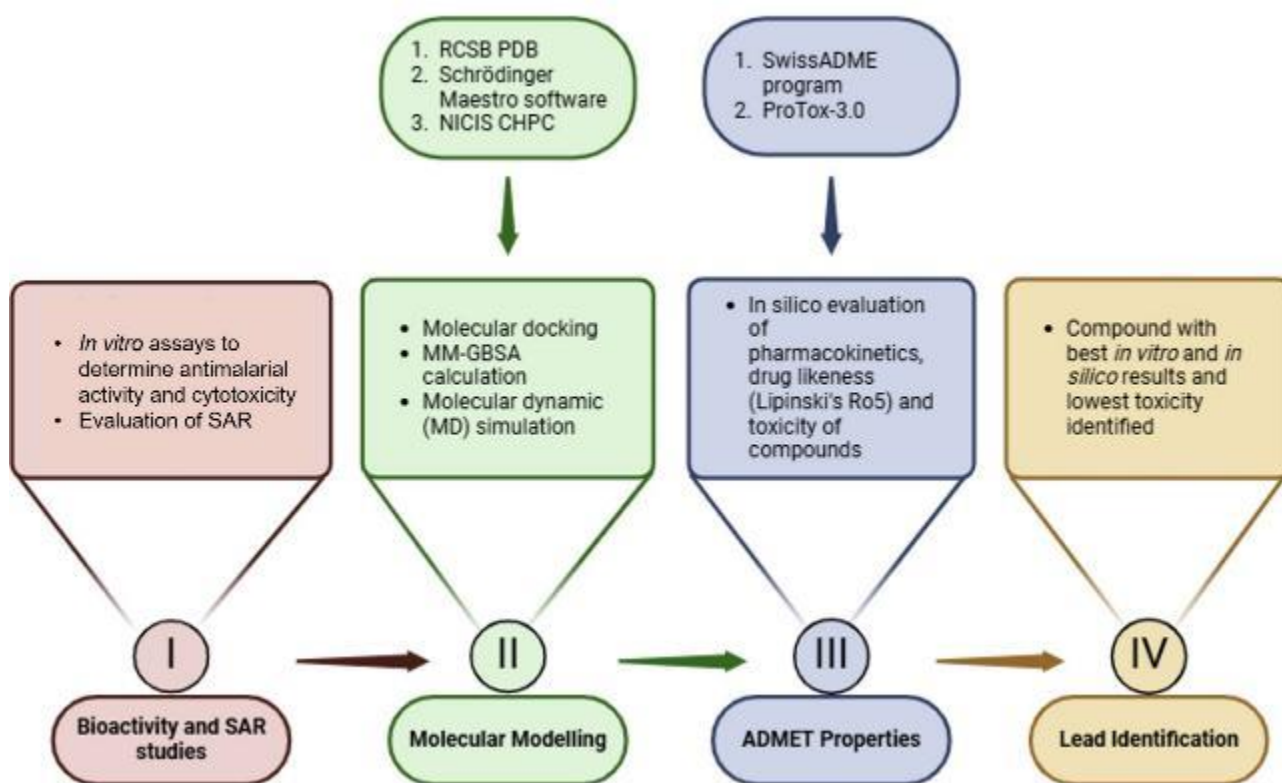
Malaria remains a global health concern, necessitating the development of novel therapeutic strategies to overcome drug resistance. Resistance of malaria parasites to current treatments necessitates new antimalarials targeting *Plasmodium falciparum*. One promising approach is the design of dual-target inhibitors, which can simultaneously act on multiple essential pathways within the parasite, thereby reducing the likelihood of resistance development. Dihydrofolate reductase (DHFR) inhibitors have been widely studied as antimalarial agents; however, targeting multiple essential pathways including folate biosynthesis and mitochondrial electron transport chain (mETC) within *P. falciparum* could enhance treatment efficacy and reduce the likelihood of resistance. In this study, a series of 2-(4-substituted piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide hybrids previously reported as *P. falciparum* DHFR inhibitors were computationally evaluated to determine their potential to also target the cytochrome *bc*₁ (cyt *bc*₁) complex, a crucial component of the parasite's mETC. Molecular docking, molecular dynamics (MD) simulations, and MM-GBSA binding free energy calculations were employed to assess their binding affinity, stability, and key interactions with *bc*₁ complex. Additionally, drug-likeness and ADMET predictions were performed to evaluate their pharmacokinetic properties. The *in silico* results identified hybrid **T6** for its inhibitory potential against the *Plasmodium* cyt *bc*₁ complex enzyme suggesting a dual-targeting mechanism. The *in silico* findings presented in this study provide predictive insights into the binding affinity, drug-likeness, and pharmacokinetic properties of the investigated ligands, and require further validation through *in vitro* experiments to confirm their potential as viable drug candidates.

Keywords: malaria, *Plasmodium falciparum*, atovaquone, cytochrome *bc*₁, naphthalene hybrids

Highlights of the article:

- Naphthyl bearing 1,3,4-thiadiazoleacetamide hybrids inhibit *Plasmodium* cytochrome *bc*₁ complex
- *In vitro* antimalarial activity compared to *in silico* docking scores
- Two of the active hybrids had favourable interactions with key residues His¹⁸¹ and Glu²⁷² in target
- ADMET properties of hybrids with favourable drug-likeness and *in silico* toxicity profiles

GRAPHIC ABSTRACT:



6.1 Introduction

Malaria is a severe protozoal disease prevalent in 83 malaria-endemic countries resulting in 263 million malaria cases and 597,000 deaths in the year 2023, with WHO African countries, namely Nigeria (26%), the Democratic Republic of Congo (13%), Uganda (5%), Ethiopia (4%) and Mozambique (4%), collectively contributing to more than 50% of this disease burden (2). One of the major reasons for the increase in malaria cases is the development of resistance to the currently available chemotherapy and insecticides. Hence, there is an urgent need to develop novel hybrids that are effective to fight against resistance and also have a clear understanding of the pathway that are approved drug targets. One promising approach is to design dual-target inhibitors, which can simultaneously act on multiple essential pathways within the parasite, thereby reducing the likelihood of resistance development.

P. falciparum dihydrofolate reductase (*PfDHFR*) is a critical enzyme in the parasite's folate biosynthesis pathway, catalysing the conversion of dihydrofolate to tetrahydrofolate, which is essential for deoxyribonucleic acid (DNA) synthesis. Inhibition of *PfDHFR* disrupts these vital processes, leading to impaired parasite growth and replication. Consequently, *PfDHFR* has been a prominent target for antimalarial drugs such as cycloguanil (prodrug proguanil) (3).

Another critical pathway is the *Plasmodium* mitochondrial electron transport chain (*PmtETC*). The erythrocytic stage of the parasite depends mainly on glycolysis for energy supply, and the *PmtETC* regenerate ubiquinone that acts as an electron acceptor for dihydroorotate dehydrogenase (DHODH), which is essential for biosynthesis of pyrimidine (4,5). The mitochondrial enzyme cyt *bc*₁ complex (EC 1.10.2.2), also known as complex III forms an important component of the respiratory chain and is responsible for the oxidation of ubiquinol to ubiquinone and catalytic transfer of electrons that maintains mitochondrial membrane potential (6,7). Atovaquone is a hydroxynaphthoquinone analogue of ubiquinone that is approved for the treatment and prevention of *P. falciparum* malaria (in combination with proguanil) (8,9). It is a competitive inhibitor of ubiquinol, inhibiting the mitochondrial electron transport chain at the *bc*₁ complex level, resulting in loss of mitochondrial function (10). However, the parasite has developed resistance to atovaquone (11,12). Hence, there is a need to develop novel hybrids that can help to fight the emerging resistance.

Targeting both the mETC and folate biosynthesis, could enhance efficacy by disrupting crucial metabolic processes required for parasite survival. Such dual-acting compounds may offer the

advantage of increasing treatment potency while mitigating resistance by decreasing the parasite's ability to adapt through single-target mutations. With this aim, the current study evaluated a series of inhibitors originally designed for DHFR inhibition, for potential repurposing against the cyt *bc*₁ complex. The hybrids were evaluated for their binding affinity against *Plasmodium bc*₁ complex. Computational approaches provided a valuable means to identify and optimize such multi-target inhibitors by evaluating their binding affinity, stability, and pharmacokinetic properties, paving the way for the development of more effective antimalarial therapies.

6.2 Methodology

6.2.1 *In vitro* antimalarial activity

The chloroquine and atovaquone-sensitive strain of *P. falciparum* (NF54) was maintained continuously *in vitro* in supplemented RPMI-1640 culture media at 37 °C and gassed with a mixture of 5% CO₂, 3% O₂ and 92% N₂ (13). The culture was synchronised in the ring stage with 5% D-sorbitol (14). To determine the antimalarial activity of test hybrids, the synchronised ring stage parasites were adjusted to a final parasitaemia of 2% and 2% haematocrit, to which serial dilutions of the hybrids and positive control, were added after a 24 h incubation. Negative controls included uninfected erythrocytes and drug-free parasitised erythrocytes. Following a further 48 h incubation period, the plates were frozen at -70°C for 1 h and thawed for another hour. 25 µl of lysate was transferred to a non-sterile plate, to which 100 µl Malstat™ and 20 µl nitroblue tetrazolium and phenazine ethosulphate (1:1) mixture was added to each well and incubated for 40 min at 37 °C to quantify the parasite lactate dehydrogenase (pLDH) activity (15). Thereafter, 50 µl of 5% acetic acid was added to each well and the absorbance of the formazan products were read at 620 nm, as an indicator of parasite viability. The percentage parasite growth, taking the appropriate controls into account were calculated and used to determine the concentration required to inhibit parasite growth by 50% (IC₅₀ value) from log sigmoid dose response curves using the GraphPad Prism 5.0 software. Each experiment was repeated in triplicate (16).

6.2.2 *In silico* studies

6.2.2.1 Molecular modelling

3D structures of ligands were prepared using LigPrep program of Schrödinger Maestro software (17) and OPLS4 force field, with protonation states being predicted using Epik with a target pH of 7.0 ±

2.0 (Schrödinger Release 2023-2: Epik) (18). The protein structure (PDB 3CX5) was prepared using protein preparation wizard from Schrödinger (Schrödinger Release 2023-2: Protein Preparation Workflow) (19). All extra chains and ligands were deleted, keeping only the chains C and P involved in the binding pocket. The protein was pre-processed and minimised to relieve unfavourable constraints. The model was treated as the wild type (WT) model.

Molecular docking was performed using Glide extra precision (XP) scoring function, and ligand structures were treated as flexible, which allowed for sampling of ring conformations (including input ring conformation) and nitrogen inversion, to obtain five poses per ligand (20,21). Prime MM-GBSA (Molecular Mechanics/Generalized Born Model and Solvent Accessibility) v3.0.000 program was used to estimate the binding free energy (or ΔG) for each docked pose complex with the VSGB solvation model and OPLS4 force field (22–24).

The complexes with the best binding pose score (lowest ΔG value) were submitted for 50 ns MD simulations using the Desmond program found within the Schrödinger software suite and the OPLS4 force field (25). Water molecules present in the binding pocket from the docking procedure were retained, and the system was solvated using the TIP4P water model. An orthorhombic simulation box was set up with boundaries 10 Å away from the protein, containing more than 130,000 atoms. The rest of the settings were kept on default and MD simulation was done for 50 ns with NPT conditions (300 K, 1.013 bar and 2000 bar.Å). The MD simulations were analysed using the simulation interaction diagram tool within the Schrödinger Suite (Schrödinger Release 2023-2: Desmond Molecular Dynamics System).

6.2.3 *In silico* ADMET and Drug-likeness

ADMET properties, including the molecular properties that are incorporated in Lipinski's rule of five (Ro5), gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, along with number of rotatable bonds (RB), topological polar surface area (TPSA), and bioavailability were calculated using SwissADME program (26,27). ProTox-3.0 tool was used to predict the potential toxicity of chemical hybrids (28,29). It predicted acute toxicity (rodent) as LD₅₀ in mg/kg body weight, organ toxicity and toxicological endpoints.

6.2.4 Cytotoxicity assay

The cytotoxicity of the test hybrids was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by (30). The normalized human embryonic kidney epithelial (HEK-293) and human erythroleukemia (K562) cell lines were preserved at 37°C in a humidified environment with 5% CO₂ as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. A cell suspension (10,000 cells per well) was incubated at 37°C for 48 h with serial dilutions of hybrids/positive control. A final concentration of less than 1% DMSO did not affect the viability of the cells. Thereafter, 40 µl of MTT (5mg/ml in phosphate buffer saline (pH 7.3)) was added to each well and incubated for a further 4 hours. DMSO was used to dissolve the formazan crystals and then quantified spectrophotometrically at an absorbance of 540 nm with a reference wavelength of 690 nm. Cellular viability (%) was determined using the appropriate controls and used to calculate the IC₅₀ values which were compared to the positive control, atovaquone. The experiment was repeated in triplicate.

6.3 Results and Discussion

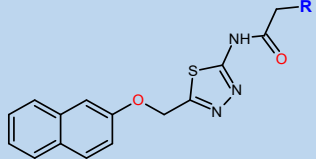
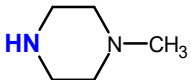
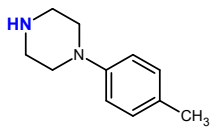
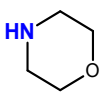
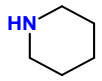
6.3.1 *In vitro* antimalarial activity

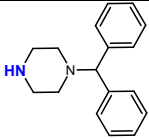
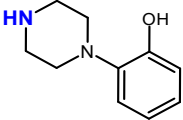
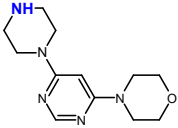
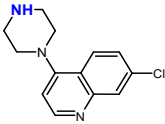
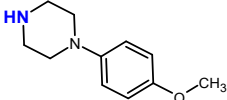
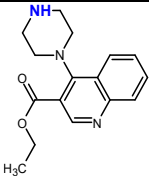
The antimalarial activity for the test hybrids and atovaquone against the ATQ-S NF54 strain indicated that hybrid **T5** was the most active (**Table 6.1**) (1).

6.3.2 Molecular modelling comparison to *in vitro* antimalarial activity

Naphthalene-based 1,3,4-thiadiazoleacetamides were analysed using molecular docking against 3CX5 (WT) model to determine if *Plasmodium* cyt *bc*₁ complex could be a potential target for these hybrids and to understand their binding modes within the cyt *bc*₁ binding pocket (**Table 6.1**). To achieve this, docking scores were analysed and binding poses were visually inspected to assess interactions with key residues His¹⁸¹ and Glu²⁷². The docking score values reflect the predicted binding affinity, with more negative values indicating stronger ligand-target interaction.

Table 6.1: Summary table for naphthyl bearing 1,3,4-thiadiazoleacetamide with *in silico* results docked in the 3CX5 (WT) model and *in vitro* antimalarial activity against the ATQ-S *P. falciparum* NF54 strain.

Hybrid Id	Substituent R = 	3CX5 (WT) model		Key interactions	Antimalarial activity (IC ₅₀ μM) (1)
		Docking score (kcal/mol)	MM-GBSA ΔG _{Bind} (kcal/mol)		
Atovaquone		-9.98	-12.63	His ¹⁸¹ (via H-bond) and Glu ²⁷² (via water bridge)	0.02 ± 0.01
T1		-9.78 (pose 1)/ -9.64 (pose 2)	-33.76 (pose 1)/ -38.40 (pose 2)	His ¹⁸¹ (H-bond), Phe ¹²⁹ (π-π stacking) (pose 1) / His ¹⁸¹ (H-bond), Phe ¹²⁹ (π-π stacking), Tyr ²⁷⁹ (aromatic H-bond) (pose 2)	7.83 ± 1.78
T2		-7.32 (pose 1)/ -7.18 (pose 2)	-42.11 (pose 1)/ -52.79 (pose 2)	Glu ²⁷² (aromatic H-bond), Tyr ²⁷⁹ (π-π stacking) (pose 1)/ no interaction (Pose 2)	63.67 ± 1.69
T3		-8.65 (pose 1) / -7.85 (pose 2)	-59.79 (pose 1)/ -8.50 (pose 2)	His ¹⁸¹ (H-bond), Phe ¹⁵¹ (π-π stacking), Tyr ²⁷⁹ (aromatic H-bond) (pose 1) / Phe ¹²⁹ (π-π stacking) (pose 2)	10.69 ± 1.12
T4		-8.11 (pose 1)/ -7.98 (pose 2)	-43.84 (pose 1)/ -44.26 (pose 2)	His ¹⁸¹ (H-bond), Phe ²⁹⁶ (π-π stacking) (pose 1) / His ¹⁸¹ (H-bond) (pose 2)	21.48 ± 1.33

T5		-8.36 (pose 1) / -8.10 (pose 2)	-61.52 (pose 1)/ -51.56 (pose 2)	Tyr ²⁷⁹ (π-π stacking) (pose 1) / No interaction (pose 2)	0.94 ± 2.03
T6		-8.55 (pose 1) / -7.84 (pose 2)	-50.02 (pose 1)/ -51.45 (pose 2)	Phe ¹⁷⁹ (π-π stacking), Phe ¹²⁹ (π-π stacking) (pose 1) / His ¹⁸¹ (H-bond), Tyr ²⁷⁹ (aromatic H-bond and π-π stacking), Glu ²⁷² (aromatic H-bond), Phe ¹⁵¹ (π-π stacking) intramolecular bonding (pose 2)	3.47 ± 1.25
T7		-7.13 (pose 1)/ -6.56 (pose 2)	-35.40 (pose 1)/ -55.91 (pose 2)	No interaction (pose 1) / Glu ²⁷² (aromatic H-bond), Phe ¹⁵¹ (π-π stacking), Tyr ²⁷⁹ (π-π stacking) (pose 2)	61.33 ± 3.66
T8		-7.33 (pose 1)/ -7.32 (pose 2)	-53.41 (pose 1)/ -44.63 (pose 2)	Glu ²⁷² (salt bridge) (pose 1) / His ¹⁸¹ (H-bond), Tyr ²⁷⁹ (aromatic H-bond), Glu ²⁷² (aromatic H-bond) (pose 2)	3.93 ± 1.25
T9		-12.10 (pose 1)/ -10.13 (pose 2)	-50.95 (pose 1)/ -50.15 (pose 2)	His ¹⁸¹ (H-bond), Phe ¹²⁹ (π-π stacking) with Phe ¹²⁹ (pose 1) / H-bonded interaction with His ¹⁸¹ , no other interaction (pose 2)	>200
T10		-8.26 (pose 1) / -8.12 (pose 2)	-38.73 (pose 1)/ -50.05 (pose 2)	Phe ¹⁵¹ (π-π stacking) (pose 1) / Tyr ²⁷⁹ (π-π stacking) (pose 2)	28.86 ± 1.61

Atovaquone, known to target the *Plasmodium* cyt *bc*₁ complex, displayed a docking score of -9.98 kcal/mol and $\Delta G_{\text{Bind}} = -12.63$ kcal/mol with a potent IC₅₀ value of 0.02 ± 0.01 μM on the ATQ-S *P. falciparum* strain (**Table 6.1**). On visual inspection of the docked pose, it was observed that atovaquone interacted with His¹⁸¹ (via H-bond) and Glu²⁷² (via water bridge) when docked in the WT model. This supported the fact that these two key interactions play a crucial role in stabilizing the ligand within the binding pocket and contributing to its inhibitory activity (**Figure 6.1**) (31–34).

The docking scores for all tested naphthalene-based 1,3,4-thiadiazoleacetamides, except hybrid **T9**, demonstrated values of lower ranking order (ranging from -9.78 to -6.56 kcal/mol) compared to the reference drug atovaquone (-9.98 kcal/mol), suggesting lower binding affinities towards the active site of cyt *bc*₁ complex (**Table 6.1**). The best binding affinity was displayed by hybrid **T9** with a docking score of -12.10 kcal/mol. The higher negative docking score of hybrid **T9** indicated strong initial binding poses, likely attributed to its interaction with His¹⁸¹. However, its poor antimalarial activity suggested that despite a strong binding affinity towards cyt *bc*₁ complex, there was lack of effective target engagement. This highlights the limitation of docking scores, as it only estimate binding affinity and do not confirm whether the interaction successfully disrupts the target's function. This further suggests that the hybrid may not have efficiently reached or interacted with the target. The MM-GBSA binding free energy calculations showed that all hybrids displayed a more favourable ΔG_{Bind} value (range: -61.52 to -8.50 kcal/mol) compared to atovaquone ($\Delta G_{\text{Bind}} = -12.63$ kcal/mol), but the antimalarial activities of hybrids did not support this observation. MM-GBSA binding energy, which incorporates solvation effects and molecular mechanics, revealed that weak docking poses may translate into energetically favourable binding complexes. Hybrids with poorer docking scores, might have achieved lower desolvation penalties and better overall stabilization, as reflected by more favourable MM-GBSA ΔG_{Bind} value, but it did not translate into the antimalarial potential of hybrids.

Visual inspection of the binding poses of the naphthalene hybrids revealed that hybrid **T5** displayed no interactions with His¹⁸¹ or Glu²⁷² indicating that cyt *bc*₁ was possibly not the target responsible for its antimalarial activity (**Figure 6.1**). However, visual inspection of the docked poses for two other hybrids, **T6** and **T8**, did display some interactions with key residues His¹⁸¹ (H-bond), Tyr²⁷⁹ (aromatic H-bond), and Glu²⁷² (aromatic H-bond or salt bridge) (**Figure 6.1**). Overall, the interactions did not appear to be sufficiently favourable to suggest cyt *bc*₁ as the primary target for the inhibitory activity as hybrids predominantly engaged in π - π stacking with the binding pocket residues.

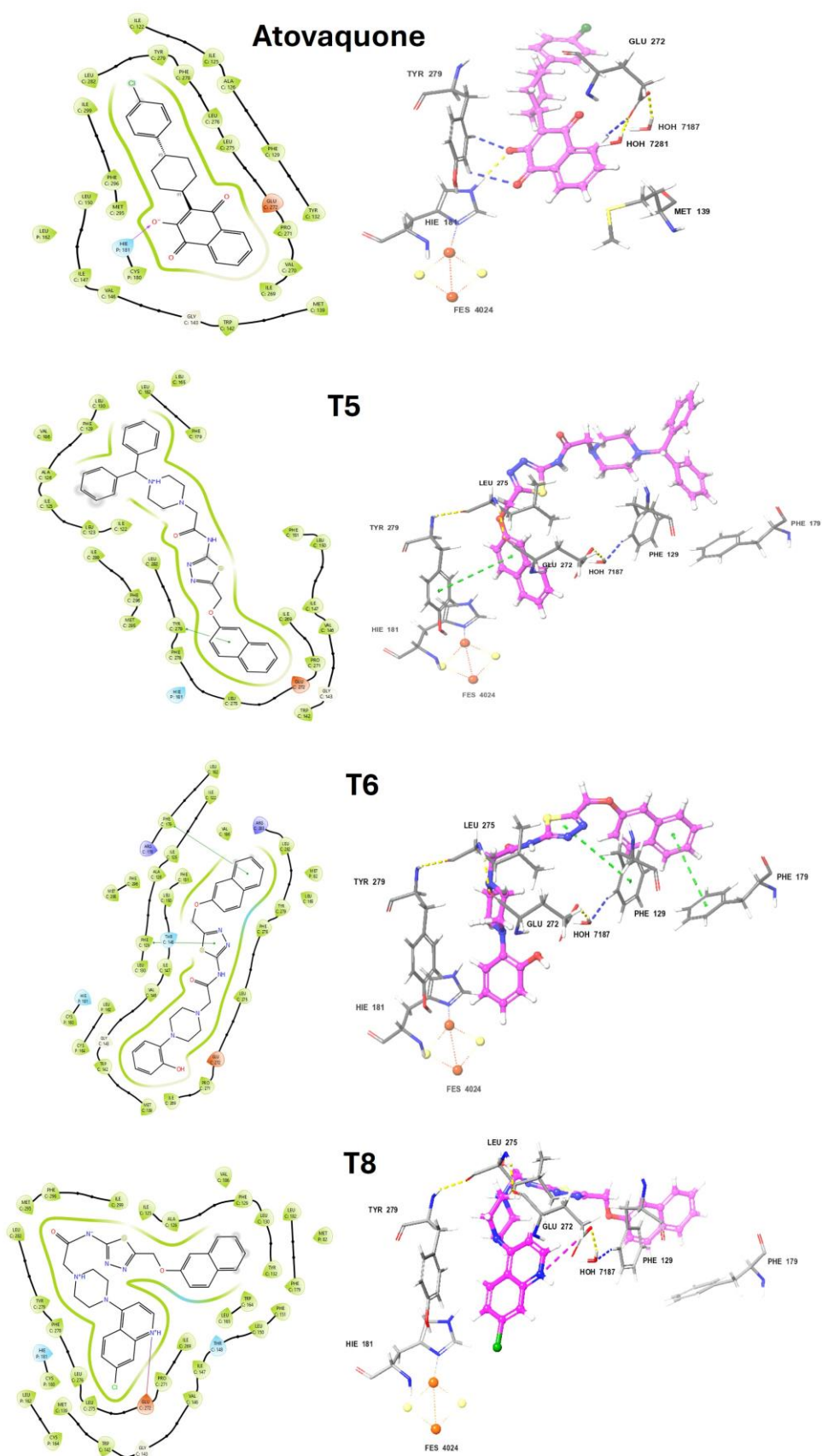


Figure 6.1: Ligand interaction diagrams (LID) for **atovaquone**, **T5**, **T6**, and **T8** in **3CX5 (WT) model**; 2-dimensional (2D) LIDs on the left-hand side and 3-dimensional (3D) LIDs on the right-hand side.

MD Simulation: A 50 ns MD simulations were performed on three most active hybrids **T5**, **T6** and **T8** which displayed the best anti-plasmodial activity against the ATQ-S NF54 strain. These results were compared with atovaquone and are discussed as below.

Protein-Ligand Root Mean Square Deviation (RMSD)

RMSD determination helped examine the target protein's conformational changes when combined with ligand molecules for a 50 ns MD simulation. At the beginning of the simulation, there was a jump in the RMSD as the system equilibrated and reached a stationary state. This jump was higher for hybrid **T8** compared to hybrids **T5** and **T6**, but subsequently the RMSD remained relatively stable until the end of the simulation (**Figure 6.2**). Once the system was stabilised, the core structure of the protein in complex with hybrid **T8** appeared to remain stable. However, greater protein fluctuations were observed for the 3CX5 (WT) and hybrid **T6** complex, as suggested by the continuous increase in the protein and the ligand RMSD values.

The ligand RMSD values for hybrids **T5** and **T8** changed within a narrow range, indicating greater stability. In contrast, the RMSD for ligand **T6** exhibited significant variations. For all three protein-ligand complexes, the ligand RMSDs remained lower than the protein RMSD values, indicating that the three ligands and more specifically hybrids **T5** and **T8** remained securely within the binding pocket and did not diffuse away, aligning with effective binding interactions typically observed in stable protein-ligand complexes (35).

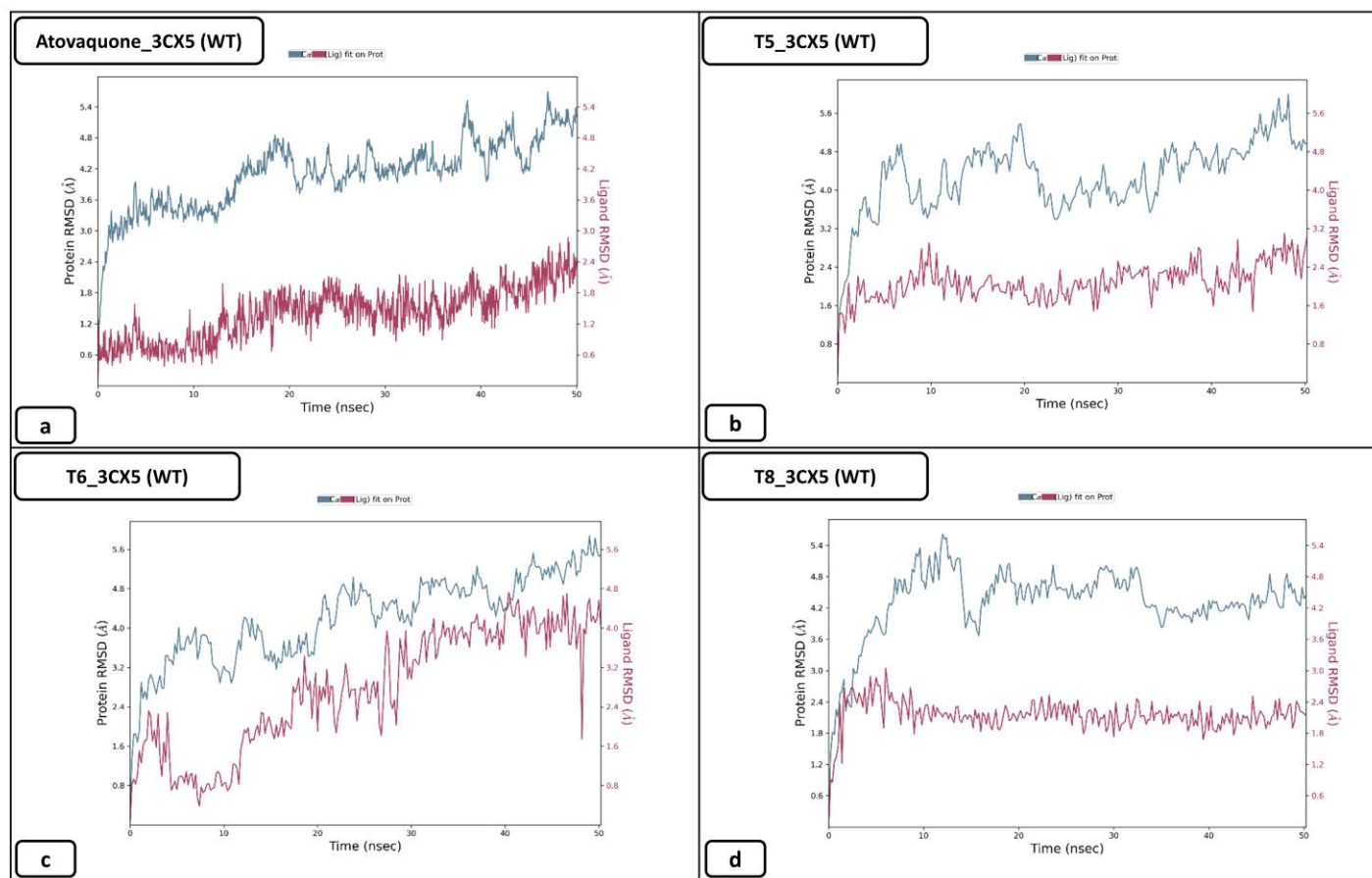


Figure 6.2: Protein-Ligand (PL)-Root Mean Square Deviation (RMSD) plots for **atovaquone** (a), hybrids **T5** (b), **T6** (c) and **T8** (d) in complex with 3CX5 (WT) model over 50 ns of MD simulation obtained using Schrödinger Desmond. The blue curve represents the RMSD of the protein C α atoms, providing insights into the backbone stability and overall conformational changes during the simulation. The red curve corresponds to the ligand RMSD, showing its movement relative to the protein binding site.

Ligand protein contacts

The ligand-protein interactions between cyt *bc*₁ and atovaquone (a), hybrids **T5** (b), **T6** (c) and **T8** (d) (**Figure 6.3**), indicated that hybrids **T6** and **T8** interacted with His¹⁸¹ via H-bonds. No water bridge interaction with Glu²⁷² was observed in case of hybrids **T6** and **T8**, however there was some hydrophobic interaction with Tyr²⁷⁹ (**Figure 6.3 (c, d)**). In contrast, hybrid **T5** did not form any H-bonds and very small contribution was observed via a water bridge interaction with Glu²⁷² (**Figure 6.3 (b)**).

The ligand-protein contacts analysis indicated that hybrid **T5** interacted via π - π interaction with His¹⁸¹ for 45% of the simulation time (**Figure 6.4 (b)**). In addition, hybrid **T5** interacted with Ile¹²⁵ for 75% of the simulation time via water bridge and with Phe¹²⁹ and Phe¹⁵¹ via π -cation and π - π stacking for up to 30% and 50% of the simulation time, respectively. Since hybrid **T5** was not seen forming any interaction with His¹⁸¹ and Glu²⁷², the observations indicated that the cyt *bc*₁ complex was not the target for the antimalarial activity of hybrid **T5** (**Figure 6.4 (b)**).

On the other hand, hybrid **T6** interacted with His¹⁸¹ via H-bond for 94% of the simulation time and with Tyr²⁷⁹ for 54% via π - π stacking. Additionally, it interacted with Met²⁹⁵ via water bridge for 48% of the simulation time (**Figure 6.4 (c)**). Similarly, hybrid **T8** interacted with His¹⁸¹ via H-bond for 71% of the simulation time. Additionally, it also interacted with Tyr²⁷⁹ via π - π stacking for 58% of the simulation time (**Figure 6.4 (d)**). Though both hybrids **T6** and **T8** displayed higher IC₅₀ values compared to hybrid **T5** against ATQ-S *P. falciparum* NF54 strain (**Table 6.1**), the MDS studies revealed that cyt *bc*₁ could be an additional potential target for these two hybrids. This requires further exploration to confirm the cyt *bc*₁ enzyme inhibitory activity of these hybrids and their further optimisation. Although hybrid **T6** exhibited stronger interactions than hybrid **T8**, the latter demonstrated greater stability in the binding pocket, as indicated by the RMSD plot of ligand T8-3CX5 (WT) complex (**Figure 6.2 (c, d)**).

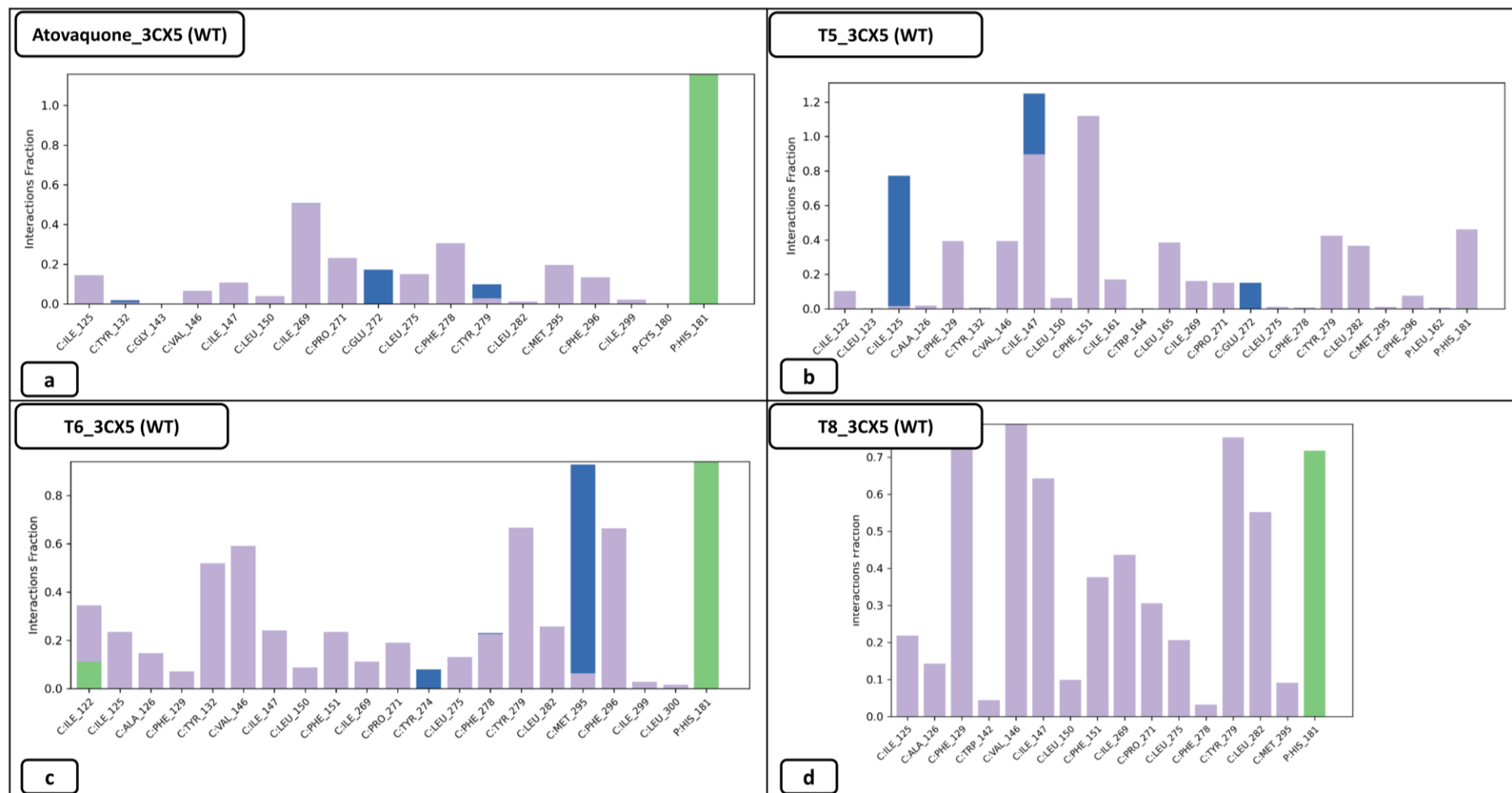


Figure 6.3: Ligand-protein (L-P) contact analysis from MD simulation for **atovaquone** (a) and hybrids **T5** (b), **T6** (c) and **T8** (d), represented by LP contacts 2D summary diagrams in 3CX5 (WT) model. The bar chart represents the frequency of the protein-ligand interactions categorised into four types: hydrogen bonds, hydrophobic bonds, ionic bonds and water bridges. Residues with higher interaction frequencies indicate key binding site contacts contributing to ligand stability.

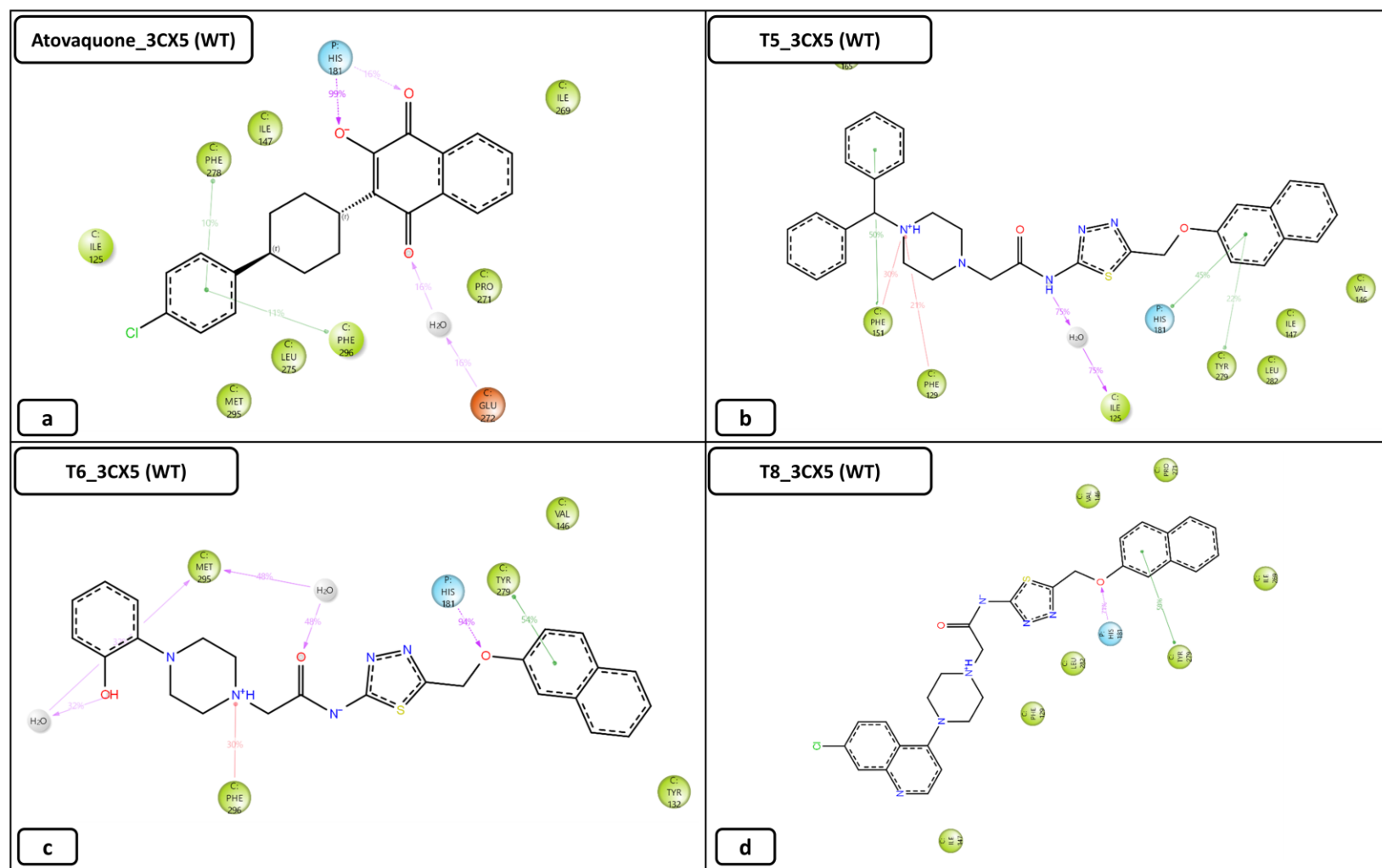


Figure 6.4: Ligand-protein (L-P) contact analysis from MD simulation for **atovaquone** (a), hybrids **T5** (b), **T6** (c) and **T8** (d), represented by LP contacts 2D summary diagrams in 3CX5 (WT) model. The 2D schematic interaction diagram visualising key interactions between the ligand and protein residues happening more than 10% of the simulation time are shown.

6.3.3 *In silico* ADMET and Drug-likeness

All hybrids, except hybrids **T5**, **T7**, **T8** and **T10**, complied with Lipinski's rule of five (Ro5) with no violations, indicating favourable drug-likeness (**Table 6.2**) (36). Hybrids **T5**, **T7**, **T8** and **T10** violated one rule with higher MW (>500 g/mol). Additionally, hybrids **T7** and **T10** exhibited the highest values for TPSA, indicating their poor pharmacokinetics and non-permeability of the BBB. All hybrids displayed a higher number of rotatable bonds (#RB), more specifically hybrids **T5**, **T7**, **T9** and **T10**. Since the #RB helps to determine the conformational flexibility of the molecule, a molecule with excessive rotatable bonds may be too flexible to achieve a stable binding with the target protein, while one with too few rotatable bonds may be overly rigid, limiting its ability to fit optimally into the binding site (37). These predictions suggest that these hybrids were significantly flexible to bind efficiently to the binding pocket site, thus negatively impacting their binding affinity towards the cyt *bc*₁ by reducing stable interactions with key residues.

All hybrids were predicted to have high GI absorption except hybrid **T10**, supported by its high MW. None of the hybrids were predicted to cross the BBB, making them non-suitable candidates for the central nervous system (CNS) targeted therapies like cerebral malaria (38,39).

Table 6.2: Summary of molecular properties and drug-likeness parameters of test hybrids.

Ligand id	PHYSICOCHEMICAL PROPERTIES						DRUG-LIKENESS PROPERTIES		PHARMACOKINETICS	
	Lipinski Rule of 5				Veber					
	MW ≤ 500 g/mol	LogP ≤ 5	#HBA ≤ 10	#HBD ≤ 5	#RB ≤ 10	TPSA ≤ 140 Å²	Lipinski #violations ≤ 1 violation	Bioavailability Score ≥ 0.55	GI absorption High is favourable	BBB permeant No is favourable
T1	397.49	2.16	6	1	7	98.83	0	0.55	High	No
T2	473.59	3.79	5	1	8	98.83	0	0.55	High	No
T3	384.45	2.35	6	1	7	104.82	0	0.55	High	No
T4	382.48	3.21	5	1	7	95.59	0	0.55	High	No
T5	549.69	4.69	6	1	10	98.83	1 (MW>500)	0.55	High	No
T6	475.56	3.14	6	2	8	119.06	0	0.55	High	No
T7	546.64	2.33	8	1	9	137.08	2 (MW>500)	0.17	High	No
T8	545.06	4.19	6	1	8	111.72	1 (MW>500)	0.55	High	No
T9	489.59	3.37	6	1	9	108.06	0	0.55	High	No
T10	582.67	3.93	8	1	11	138.02	1 (MW>500)	0.55	Low	No
Atova- quone	366.84	4.64	3	1	2	54.37	0	0.85	High	Yes

MW = molecular weight; **LogP** = log of octanol/water partition coefficient; **#HBA** = number of hydrogen bond acceptor(s); **#HBD** = number of hydrogen bond donor(s); **#RB** = number of rotatable bond(s); **TPSA** = topological polar surface area; Lipinski **#violations** = number of violations of Lipinski's rule; **GI** = gastrointestinal; **BBB** = blood-brain barrier.

BOILED Egg (or Brain Or Intestinal Estimated) Plot

Unlike atovaquone, none of the hybrids were plotted in the yellow core, suggesting that these hybrids were not lipophilic enough or had higher TPSA values (ranging from 95.59 Å² to 138.02 Å²) to penetrate the BBB, making them non-suitable as CNS drug candidates to target cerebral malaria; where TPSA <90 Å² is considered as an optimal cut off value for CNS drug candidate (**Figure 6.5**) (40,41). Unlike atovaquone, all the test hybrids were plotted in the white region suggesting good oral bioavailability (bioavailability score: 0.55), which complies with the MMV requirement that all new test compounds be orally absorbed. Of the set, hybrid **T7** was seen towards the outer edge of the white region, with the lowest bioavailability score of 0.17 (**Figure 6.5**). Hybrid **T10** was plotted outside the egg, in the grey region, exhibiting poor pharmacokinetics for oral administration or BBB permeability. This could be due to its high MW (582.67 g/mol) and TPSA (138.02 Å²) which resulted in low GI absorption and inability to cross the BBB. All test hybrids positioned in the egg white region, except for hybrid **T4**, were marked as a P-gp substrate, suggesting that their absorption or BBB permeability was affected by P-glycoprotein's active efflux. These predictions indicate towards poor pharmacokinetic profile of these hybrids.

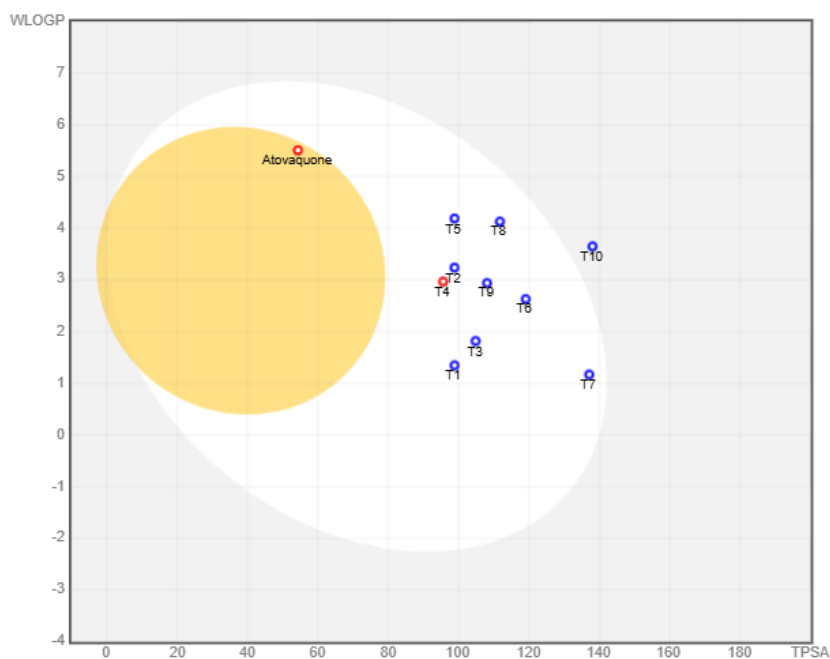


Figure 6.5: BOILED Egg (or Brain Or Intestinal Estimated) Plot of WlogP vs TPSA for atovaquone and the test hybrids. Yellow indicates BBB-permeant, white indicates human intestinal absorption (HIA), i.e. gastrointestinal permeant; blue indicates P-glycoprotein positive, and red indicates P-glycoprotein negative.

6.3.4 Toxicity

All the naphthalene-based test hybrids and atovaquone were evaluated against the normalized human embryonic kidney epithelial (HEK293) and human erythroleukemia (K562) cell lines to determine their cytotoxicity (**Table 6.3**). Hybrid **T5**, which exhibited the most potent antimalarial activity against the ATQ-S *P. falciparum* NF54 strain ($IC_{50} = 0.94 \pm 2.03 \mu M$), also demonstrated the most favourable toxicity profile against the HEK cell line and a moderate profile against the K562 cell line. This translated into a high safety index and strong selectivity for the malaria parasite over the human cells. Similarly, hybrids **T6** and **T8** displayed moderate antimalarial activity (IC_{50} values of $3.47 \mu M$ and $3.93 \mu M$, respectively), and toxicity profile with HEK293 cell viability at $50 \mu M$ recorded at 92.90% and 43.3%, respectively. Although the latter two hybrids displayed similar cytotoxicity to atovaquone against the K562 cell line.

The naphthalene hybrids were evaluated for their *in silico* safety profiles, namely, LD_{50} values (rodent model), toxicity class, organ toxicity, and toxicity endpoints. It was noted that all test hybrids (**T1** to **T9**) were predicted to be safe with $LD_{50} = 2580$ mg/kg and toxicity class 5, indicating these hybrids might be harmful if swallowed (**Table 6.3**). Hybrid T10 was predicted to be more toxic with $LD_{50} = 1100$ mg/kg and classified under toxicity class 4, indicating that it may be harmful if swallowed. Though the two hybrids: **T6** and **T10**, similar to atovaquone, were predicted to be non-carcinogenic, non-immunotoxic, non-mutagenic and non-cytotoxic, with potential nephrotoxicity, and respiratory toxicity, along with neurotoxicity; hybrid T6 appeared considerably safer, with LD_{50} of 2580 mg/kg and belonging to toxicity class 5 (**Table 6.3**). All hybrids were predicted to be non-cytotoxic and non-immunotoxic, except hybrid **T8**, which was flagged to be immunotoxic. The remaining hybrids T1 to T5, were flagged to be potentially carcinogenic, mutagenic, neurotoxic along with respiratory toxicity, raising concerns over their prolonged use. This may be circumvented by the fact that the duration of malaria treatment is over a short period (2). Hybrid T7 and T9 were predicted to be mutagenic along with neurotoxic and respiratory toxic.

Table 6.3: Summary of the preliminary *in vitro* and *in silico* toxicity profile of atovaquone and naphthalene hybrids predicted by ProTox3.0.

Hybrid ID	Cytotoxicity HEK293 %cell growth ±S.D. at 50µM	Cytotoxicity K562 %cell growth ±S.D. at 50µM	LD ₅₀ (mg/kg)	Toxicity Class	Organ Toxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
Atovaquone	52.34 ± 7.92	11.19 ± 1.75	2000	Class 4	Nephrotoxic Respiratory toxic	Non- carcinogenic	Non- immunotoxic	Non- mutagenic	Non- cytotoxic
T1	42.75 ± 4.82	28.47 ± 11.11	2580	Class 5	Neurotoxic, Respiratory toxic	Carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
T2	66.40 ± 2.71	43.43 ± 6.33	2580	Class 5	Neurotoxic, Respiratory toxic	Carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
T3	69.99 ± 3.29	73.10 ± 1.92	2580	Class 5	Neurotoxic, Respiratory toxic	Carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
T4	86.90 ± 4.02	68.21 ± 1.14	2580	Class 5	Neurotoxic, Respiratory toxic	Carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
T5	94.38 ± 8.44	67.01 ± 1.58	2580	Class 5	Neurotoxic, Respiratory toxic	Carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
T6	92.90 ± 5.34	14.46 ± 4.15	2580	Class 5	Neurotoxic, Nephrotoxic, Respiratory toxic	Non- carcinogenic	Non- immunotoxic	Non- mutagenic	Non- cytotoxic

T7	87.41 ± 9.46	53.50 ± 8.54	2580	Class 5	Neurotoxic, Respiratory toxic	Non- carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
T8	43.26 ± 1.75	9.51 ± 3.12	2580	Class 5	Neurotoxic, Respiratory toxic	Non- carcinogenic	Immunotoxic	Mutagenic	Non- cytotoxic
T9	57.99 ± 5.30	44.56 ± 12.34	2580	Class 5	Neurotoxic, Respiratory toxic	Non- carcinogenic	Non- immunotoxic	Mutagenic	Non- cytotoxic
T10	59.76 ± 9.80	37.83 ± 8.73	1100	Class 4	Neurotoxic, Nephrotoxic, Respiratory toxic	Non- carcinogenic	Non- immunotoxic	Non- mutagenic	Non- cytotoxic

6.4 Conclusions

Following the evaluation of a series of naphthyl bearing 1,3,4-thiadiazoleacetamides hybrids through a combination of *in vitro* and *in silico* approaches, the potential of these hybrids as antimalarial drugs targeting the *Plasmodium* cyt *bc*₁ complex was determined. The *in vitro* antimalarial activity indicated that hybrid **T5** exhibited the best antimalarial activity (IC₅₀ value: 0.94 µM) against the ATQ-S NF54 strain, followed by hybrids **T6** and **T8** (IC₅₀ value of 3.47 µM and 3.93 µM, respectively, **Table 6.1**). *In silico* assessment suggested that due to the lack of interaction between hybrid **T5** and key residues His¹⁸¹ and Glu²⁷², the cyt *bc*₁ complex was not the potential target responsible for its antimalarial activity. Additionally, hybrid T5 can be explored further for its activity against *Pf*DHFR, where it exhibited highest binding affinity against both the WT *Pf*-DHFR-TS (PDB ID: 3QGT) and quadruple mutant *Pf*-DHFR-TS (PDB ID: 3QG2) models (Pal, et al., 2023). On the other hand, hybrids **T6** and **T8** were seen interacting with His¹⁸¹ via H-bonds suggesting that *Plasmodium* cyt *bc*₁ could be a potential or secondary target for the observed inhibitory activity of these two hybrids. However, the IC₅₀ values for their antimalarial activities against the NF54 strain were very high compared to atovaquone and hybrid **T5**, which could possibly be due to their poor pharmacokinetic profile. Further, the hybrid **T6** was predicted to be non-carcinogenic, non-immunotoxic, non-mutagenic and non-cytotoxic, whereas hybrid **T8** was predicted to be immunotoxic.

The insights gained from this study provide a rational basis for further evaluation of hybrid **T6** for its inhibitory potential against the *Plasmodium* cyt *bc*₁ complex enzyme to ascertain the target for the inhibitory activity of this hybrids and to improve on the toxicological and pharmacokinetic profiles.

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Chapter 7

Designing novel *Plasmodium falciparum* cytochrome *bc*₁ inhibitors using computer-aided drug discovery (CADD)

This chapter explores the design and evaluation of novel chalcone ligands as potential inhibitors of the *Plasmodium* cytochrome *bc*₁ (cyt *bc*₁) complex using computer-aided drug discovery (CADD) techniques. A set of chalcones, identified as promising yet underexplored compounds, was initially screened through molecular docking. Based on binding affinity, one ligand was selected as a template for designing new derivatives. Molecular modelling studies were then performed to examine the binding mode of atovaquone, a known cyt *bc*₁ inhibitor, alongside the newly designed compounds, comparing their interactions with key binding pocket residues. Additionally, *in silico* tools were used to predict pharmacokinetic properties and drug-likeness. This study highlights the significance of *in silico* approaches in early-stage drug discovery, facilitating the design, binding analysis, and pharmacokinetic evaluation of potential inhibitors.

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Student's contributions:

Contributions	Priyanka Agarwal	C. Johan van der Westhuizen	Robyn L. van Zyl
Conceptualisation	✓		
Methodology	✓		
Building cyt <i>bc</i> ₁ WT and mutant model, <i>in silico</i>	✓		
Computational studies against cytochrome <i>bc</i> ₁ complex	✓		
Computational studies to determine drug-likeness and toxicity profile	✓		
Visualisation and Data analysis	✓		✓
Supervision		✓	✓

Computational design, molecular modelling, and pharmacokinetics profiling of chalcones as novel inhibitors of *Plasmodium falciparum* cytochrome *bc*₁

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Abstract:

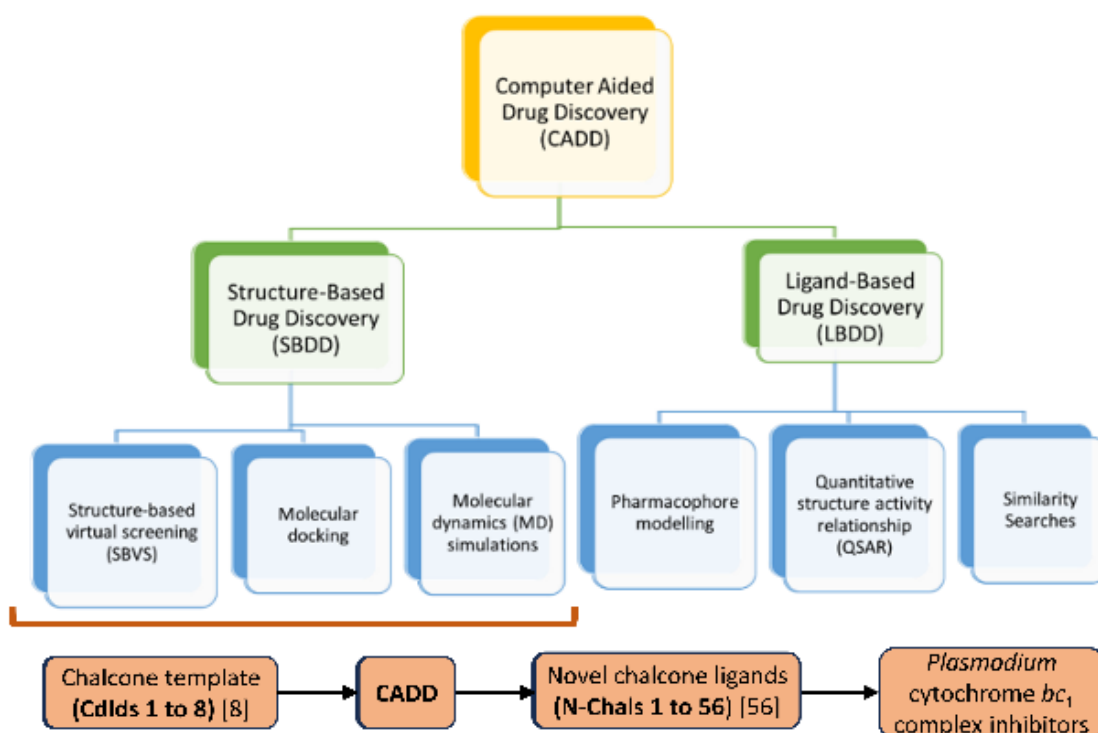
With the rise of artemisinin resistance, the search for alternative lead compounds has intensified. Chalcones have been identified as potential inhibitors of the *Plasmodium* cytochrome *bc*₁ (cyt *bc*₁) complex but remain largely unexplored. This study aimed to design and evaluate novel chalcones for their inhibitory potential against atovaquone-resistant (ATQ-R) *Plasmodium bc*₁. Using rational drug design principles, new chalcone derivatives were developed and assessed for their antimalarial activity against the ATQ-R (Y279S) model. The Schrödinger software suite was employed for molecular modelling, alongside *in silico* analysis of drug-likeness and ADMET properties. The ATQ-R (Y279S) mutant model was built by substituting the residue Tyr²⁷⁹ (equivalent to Tyr²⁶⁸ of *P. falciparum*) in the wild-type model, with Ser²⁷⁹ to account for Y279S mutation (equivalent to Y268S in *P. falciparum*). Fifty-six ligands were designed using interactive pose prediction and the R-Group enumeration tool, followed by molecular docking and molecular dynamic (MD) simulations on selective compounds against Y279S mutant model built using the *Saccharomyces cerevisiae* X-ray structure (PDB: 3CX5) representing the cyt *bc*₁ complex. Based on the MD simulations and key binding pocket interactions, this study highlights the antimalarial potential of three novel chalcones (**N-Chal-19**, **N-Chal-45** and **N-Chal-46**) as effective candidates against ATQ-R *Plasmodium* strains. The *in silico* findings of this study offer predictive insights into the binding affinity, drug-likeness, and pharmacokinetic properties of the investigated ligands. However, further *in vitro* validation is necessary to confirm their biological activity and potential as viable drug candidates.

Keywords: malaria, *Plasmodium falciparum*, atovaquone, chalcones, cytochrome *bc*₁, CADD

Highlights:

- Chalcones identified as promising *Plasmodium* cytochrome *bc*₁ complex inhibitors
- Computer-aided drug discovery was used to design novel compounds targeting atovaquone-resistant strains
- Chalcones interacted favourably with key residues His¹⁸¹, Glu²⁷² in ATQ-R Y279S mutant model
- Novel chalcones possessed favourable drug-likeness properties

Graphical abstract



7.1 Introduction

Malaria is a protozoal disease prevalent in 83 endemic countries and is caused by five *Plasmodium* species, with *P. falciparum* responsible for the most severe cases and fatalities [1]. A key factor in the rising malaria burden is resistance to current insecticides and therapeutic drugs, including artemisinin derivatives, the first-line treatments [2]. This underscores the urgent need for novel antimalarial drugs with well-defined mechanisms of action and efficacy against resistant strains.

The *Plasmodium* mitochondrial electron transport chain (PmtETC) is a validated and approved target for the development of antimalarial drugs [3]). It contains five key enzymes, namely, the NADH:ubiquinone oxidoreductase (Complex I or *Pf*NDH2), succinate dehydrogenase (Complex II or SDH), glycerol-3-phosphate dehydrogenase (G3PDH), malate:quinone oxidoreductase (MQO), and dihydroorotate dehydrogenase (DHODH). Additionally, the ETC also includes *bc*₁ (cytochrome *bc*₁ complex), cytochrome *c*, aa3 cytochrome *c* oxidase, and F1Fo-ATPase (complex V) [4].

The mitochondrial cyt *bc*₁ complex (EC 1.10.2.2), or complex III, is a key component of the respiratory chain, facilitating oxidation of ubiquinol to ubiquinone and electron transfer to maintain mitochondrial membrane potential [5]. This dimeric, multi-subunit enzyme comprises up to 11 subunits in eukaryotes, with its catalytic core consisting of cytochrome *b*, cytochrome *c*₁ and Reiske iron-sulfur protein ([2Fe2S] ISP), which drive electron transfer [6]. The *bc*₁ complex transfer electrons from ubiquinol to cytochrome *c*, coupled with proton translocation across the mitochondrial membrane, generating transmembrane electrochemical gradient ($\Delta\Psi_m$). The series of redox reactions is known as Q-cycle [7].

Atovaquone is a hydroxynaphthoquinone analogue of ubiquinone that was approved for the prevention and treatment of malaria [8], [9]. It is a competitive inhibitor of ubiquinol, inhibiting the mtETC at the *bc*₁ complex level, resulting in the loss of mitochondrial function [10]. However, the parasite has developed resistance to this drug [11], [12], [13], highlighting the need for novel compounds targeting the cyt *bc*₁ complex in the resistant *Plasmodium* strains.

As a part of this study to identify and develop compounds effective against ATQ-R strains, chalcones were identified as promising yet under explored scaffold with potential efficacy against these strains. Licochalcone A (**Figure 7.1**) has been shown to inhibit the *Plasmodium bc*₁ complex III more effectively than complex II, targeting the cyt *bc*₁ complex at very low concentrations. This positions licochalcone A as a promising scaffold for the development of novel antimalarial drugs [14], [15].

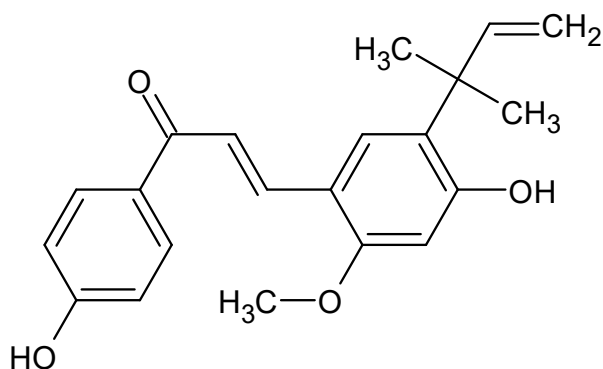


Figure 7.1: Licochalcone A [14]

As such, chalcone based compounds were investigated using Computer-Aided Drug Discovery (CADD) that aimed to improve both the speed and cost-effectiveness of the drug development process [16]. Among the CADD methodologies, Structure-Based Drug Design (SBDD) was employed to design and evaluate novel chalcones with the potential to target cyt *bc*₁ complex, thus highlighting its practical applications. Building upon the *in vitro* activities of the chalcone compounds reported in the literature [17] and their *in silico* analyses of their binding affinities, additional derivatives were designed and evaluated using the molecular modelling tools to investigate their binding modes within the ATQ-R (Y279S) mutant model developed for this study. The pharmacokinetic properties and drug-likeness of all compounds were assessed and compared with those of atovaquone and previously reported compounds [18]. This comparison aimed to determine whether the newly designed compounds demonstrated improved performance in *in silico* studies, supporting their potential advancement as antimalarial drug candidates targeting the *Plasmodium* cyt *bc*₁ complex.

7.2 Methods

7.2.1 Data collation and SAR of chalcones

While compiling a database of *Plasmodium* cyt *bc*₁ complex inhibitors [19] (unpublished), the most active set of eight chalcones from Gutteridge et al. (2006) [18] were collated and drawn using Instant JChem software, version: 22.19.0 from Chemaxon. Based on the structure-activity relationship (SAR) analysis, 56 compounds were designed and selected for further molecular modelling studies.

7.2.2 Molecular modeling

An initial screening of eight chalcone compounds was conducted on a wild type (WT) model using molecular docking in Schrödinger Release 2021-1 (Maestro version 12.7). Following which all advanced molecular modelling studies, including the development of truncated models representing both the WT and the mutant (Y279S) cyt *bc*₁ complex, docking, MM-GBSA and MD simulations were performed on Schrödinger Release 2023-2 (Maestro version 13.3).

Ligand preparation: 3D structures of ligands were prepared using LigPrep program of Schrödinger Maestro software and OPLS4 force field, with protonation states being predicted using Epik with a target pH of 7.0 ± 2.0 (Schrödinger Release: Epik) [20].

Protein preparation: The protein structure (PDB 3CX5) was prepared using protein preparation wizard from Schrödinger Release 2021-1 (for initial docking model) and Schrödinger Release 2023-2 (for truncated models used for advanced studies) [21]. For the truncated models, all extra chains and ligands were deleted, keeping only chains C and P that were involved in the binding pocket. The protein was pre-processed and minimised to relieve unfavourable constraints, resulting in the wild-type (WT) model. To build the Y279S mutant model, residue Tyr²⁷⁹ (equivalent to Tyr²⁶⁸ of *P. falciparum*) in the WT model was replaced by Ser²⁷⁹ to account for Y279S mutation (equivalent to Y268S in *P. falciparum*) [22], [23].

Grid generation: Separate grids were generated for WT and Y279S mutant models using Glide Receptor Grid Generation tool available within Schrödinger.

Docking: Molecular docking was performed using Glide extra precision (XP) scoring function, and ligands structures were treated as flexible, which allowed for sampling of ring conformations (including input ring conformation) and nitrogen inversion, to obtain 5 poses per ligand [24], [25].

MM-GBSA: Prime MM-GBSA (Molecular Mechanics/Generalized Born Model and Solvent Accessibility) v3.000 program was used to estimate the binding free energy (or ΔG) for each docked pose complex with the VSGB solvation model and OPLS4 force field [26], [27], [28].

MD simulation: The ligand-protein complexes with the best binding pose score (lowest ΔG value) were submitted for a 100 ns MD simulations using Desmond program found within the Schrödinger software suite and the OPLS4 force field [29]. Water molecules present in the binding pocket from the docking procedure were retained, and the system was solvated using the TIP4P water model. An orthorhombic simulation box was set up with boundaries 10 Å away from the protein, containing more than 130,000 atoms. Rest of the settings were kept on default and MD simulation was done for 100 ns with NPT conditions (300 K, 1.103 bar and 2000 bar.Å). The MD simulations were analysed using the simulation interaction diagram (SID) tool within the Schrödinger Suite (Schrödinger Release 2023-2: Desmond Molecular Dynamics System).

Ligand designing: Interactive pose prediction and R-Group Enumeration tools available within the Schrödinger Maestro were used to design novel compounds. For enumeration, 'R-groups to Create a Hydrogen Bond' was selected so that the new compounds could be developed with the potential to form H-bonds with key binding site residues, His¹⁸¹ and Glu²⁷². The latter residues are crucial for stabilizing atovaquone in the binding pocket, allowing it to exhibit its inhibitory activity against the cyt *bc*₁ complex [6], [30], [31]

7.2.3 *In silico* ADMET and drug-likeness

ADMET properties, including the molecular properties that are incorporated in Lipinski's rule of five (Ro5), gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, along with number of rotatable bonds (#RB), topological polar surface area (TPSA), and bioavailability were calculated using SwissADME program [32], [33]. ProTox-3.0 tool was used for predicting the potential toxicity of the ligands where it predicted acute toxicity in rodents as LD₅₀ values in mg/kg body weight, organ toxicity and toxicological endpoints [34], [35], [36].

7.3. Results and Discussion

7.3.1 Chalcone compounds SAR study

The dataset collated from the literature [17], [18] yielded a total of eight chalcone compounds (**Table 7.1**) with antiplasmodial activities against *P. falciparum* W2 strain (IC₅₀ range: 0.23 nM to 3.2 nM) and D6 strain (IC₅₀ range: 0.19 nM to 3.8 nM). Atovaquone has been reported to inhibit *P. falciparum* D6 and W2 strains with IC₅₀ values ranging from 0.1 to 1.2 nM [17], [37].

Table 7.1: *In silico* molecular modelling analysis of chalcone compounds active against two ATQ-S *P. falciparum* strains (chloroquine-resistant (W2), chloroquine-sensitive (D6)) strains [18].

Cdid	Ring B	Ring A	IC ₅₀ nM (Pf W2)	IC ₅₀ nM (Pf D6)	Docking score (3CX5; WT) (kcal/mol)	HBond score (3CX5; WT)
1	3,4-di-OMe phenyl	quinolin-4-yl	0.50	0.88	-10.52	-0.45
2	2,5-di-Cl phenyl	quinolin-3-yl	1.7	0.62	-11.13	-0.70
3	2,5-di-Cl phenyl	quinolin-4-yl	0.23	0.19	-10.76	-0.43
4	3,4-di-Cl phenyl	quinolin-4-yl	0.80	1.7	-10.57	-0.44
5	2-OMe, 5-F phenyl	quinolin-2-yl	0.67	3.8	-11.23	-0.70
6	2-OMe, 5-F phenyl	quinolin-3-yl	1.1	0.46	-11.37	-0.70
7	3-F, 4-OMe phenyl	quinolin-4-yl	3.2	0.93	-10.40	-0.70
8	2-OMe, 5-Cl phenyl	quinolin-3-yl	0.59	0.95	-10.74	-0.47
Atovaquone			0.3 to 1.2	0.1 to 0.3	-8.33	0.00

The dataset was too small to draw any conclusion about the SAR. In general, it was observed that compounds where Ring A was either quinolin-3-yl or quinolin-4-yl exhibited slightly better antimalarial activity compared to compounds with quinolin-2-yl (**Table 7.1**). Amongst the quinolin-3-yl compounds, the best docking score was determined for **Cddid-6**, where Ring B was 2-OMe-5-F-phenyl. While for the quinolin-4-yl compounds, **Cddid-3** with a 2,5-di-Cl phenyl (as Ring B) displayed the best activity, but had a low ranked docking score and hydrogen-bond (H-bond) score compared to **Cddid-6** (**Table 7.1**).

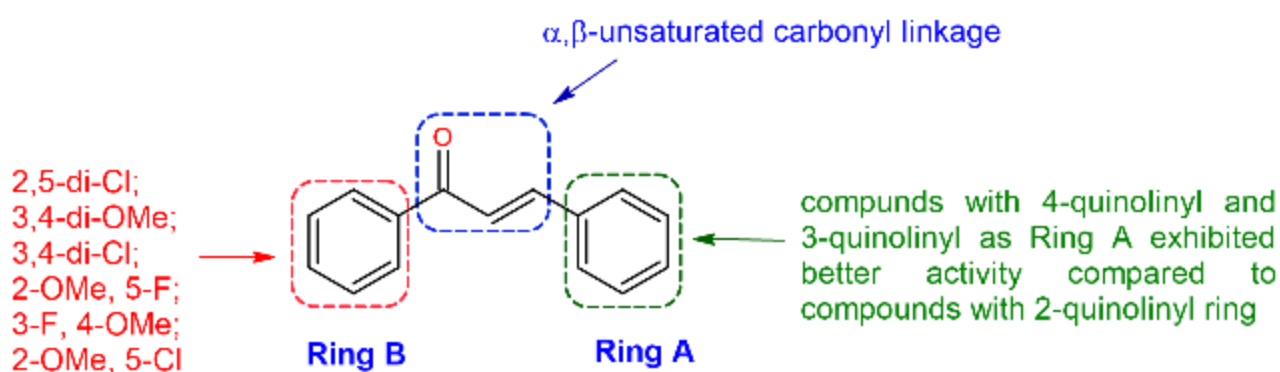


Figure 7.2: Structural framework of chalcone compounds (Adapted from Rodrigues et al., 2010).

7.3.2 Molecular modeling and designing novel compounds

7.3.2.1 Docking collated compounds

According to the initial docking results in the 3CX5 (WT) model, **Cdld-6** displayed the best docking score of -11.37 kcal/mol along with the H-bond score of -0.7 (**Table 7.1**). On visual inspection of the docked pose, the ligand was seen interacting with His¹⁸¹ via H-bond (yellow dotted line), and with Tyr²⁷⁹ and Leu²⁷⁵ via aromatic H-bonds (blue dotted line) (**Figure 7.3**). Additionally, there was π - π stacking between Phe²⁹⁶ and the quinolone ring of the ligand.

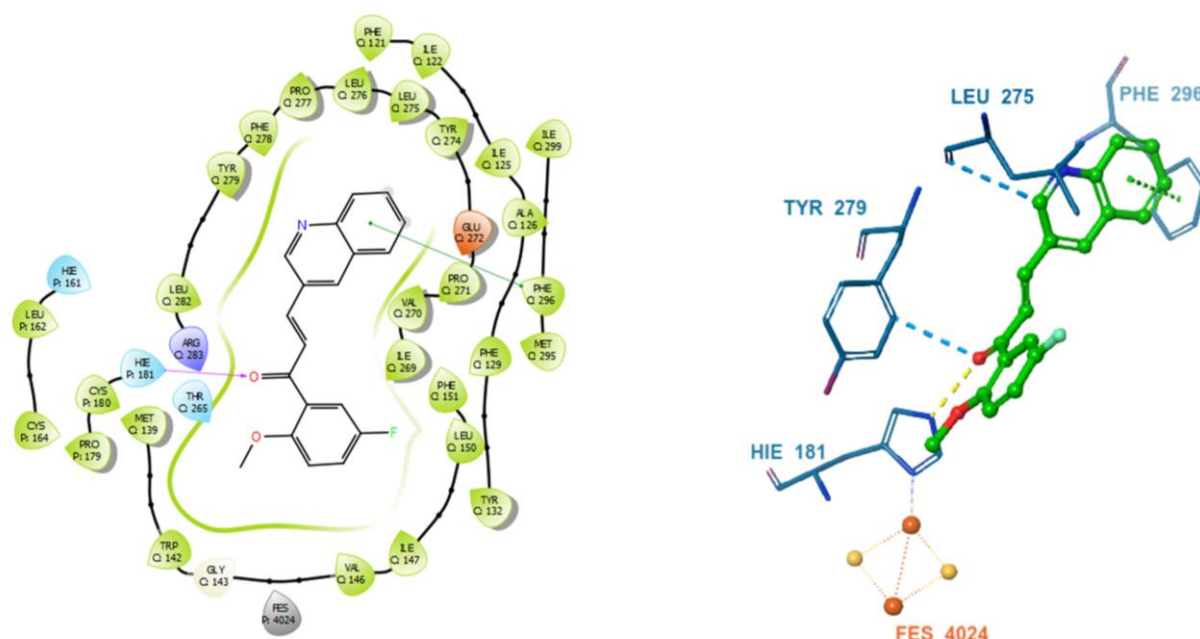


Figure 7.3: Ligand interaction diagram for **Cdld-6** docked in 3CX5 (WT) model.

7.3.2.2 Designing novel compounds

As seen in **Figure 7.3**, the ligand **Cdld-6** interacted with His¹⁸¹ via H-bond and with Phe²⁹⁶ via π - π interaction. Additionally, it was surrounded by the hydrophobic residues which included Ile²⁹⁹, Ile¹²⁵, Phe²⁷⁸, Leu²⁷⁵, Phe¹²⁹, Tyr¹³², Ile¹⁴⁷, Pro²⁷¹, Ile¹⁶⁹, Trp¹⁴², Val¹⁴⁶, Leu¹⁶², Tyr²⁷⁹, Leu²⁸², and Leu¹⁵⁰. No interaction between the ligand and Glu²⁷² via a water-bridge was observed. Any structural modification to **Cdld-6** that enhances the interactions with the binding pocket residues thereby increasing the H-bond score, could improve the docking score and the overall binding of the ligand to the binding site. With this rational approach, modifications of **Cdld-6** were designed with the potential to form H-bonds with the key residues His¹⁸¹ and Glu²⁷² that could also aid to increase the compounds ranking based on the H-bond scores. Building on this, 56 compounds were designed and their docking, MM-GBSA and H-Bond scores were determined (**Table 7.S.1**). This was followed by a

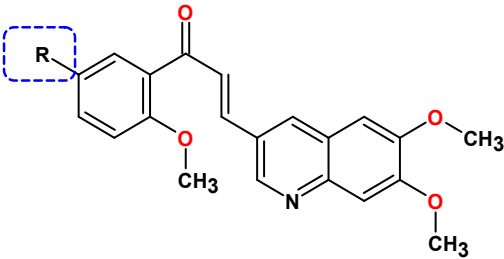
visual inspection of all docked poses to identify those interacting with the key residues His¹⁸¹ and Glu²⁷². Based on all these observations, three ligands were selected, namely **N-Chal-19**, **N-Chal-45** and **N-Chal-46** to be further investigated using *in silico* methods. Other higher ranked ligands with superior docking scores compared to these three ligands were not considered for further analysis due to their unfavourable MM-GBSA, H-bond score, and lack of interactions with key residues.

7.3.2.3 Docking of novel compounds

Table 7.2 presents the docking results for ligands in the atovaquone-resistant (Y279S) model, indicating that the designed compounds demonstrated superior docking, MM-GBSA and H-Bond scores compared to atovaquone and **CdId-6**. Among them, ligand **N-Chal-45** exhibited the most favourable results, followed by **N-Chal-46** and **N-Chal-19**. Based on these results, the three ligands were further analysed using MD simulations to assess their effectiveness against atovaquone-resistant Y279S model and their drug-likeness properties.

The 2D and 3D LIDs for atovaquone in 3CX5 (WT) and Y279S mutant models, along with **CdId-6** and novel chalcones in the mutant model are shown for comparison in **Figure 7.4**. For the third selected ligand, **N-Chal-19**, the analysis is included in supplementary information (**Figure 7.S.1**).

Table 7.2: Summary of three selected novel designed chalcones in comparison to **CdId-6** and atovaquone with their structural differences and their docking and MM-GBSA results against 3CX5 (Y279S) model.

Ligand Id	R group	Docking score 3CX5 (Y279S) (kcal/mol)	MM-GBSA ΔG_{Bind} 3CX5 (Y279S) (kcal/mol)	H-bond score
				
N-Chal-19	-OCH ₃	-10.53	-47.48	-0.89
N-Chal-45	-OH	-11.91	-53.37	-1.39
N-Chal-46	-CH ₂ OH	-11.50	-51.43	-1.28
CdId-6	5-F	-10.68	-44.36	-0.70
Atovaquone		-8.230	-21.66	0

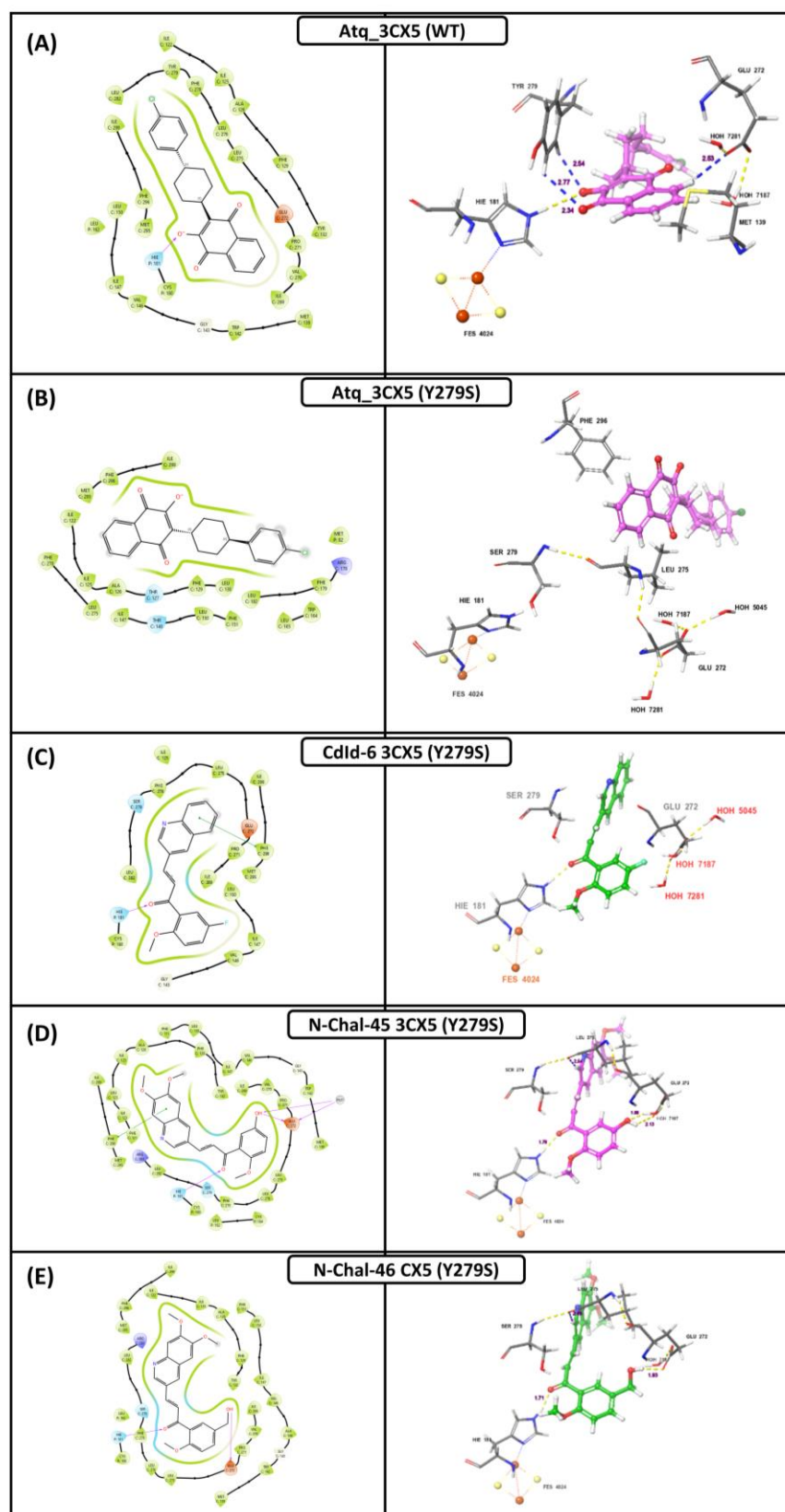


Figure 7.4: Comparison of 2D and 3D Ligand interaction diagrams (LIDs) for **atovaquone** in the **3CX5 (WT)** (A) and **3CX5 (Y279S) mutant** (B) models, along with **Cdlid-6** and novel compounds in the **3CX5 (Y279S) mutant** (C to E) model. The table presents key interactions observed in each model, highlighting H-bonds and water bridge, and other relevant interactions with active site residues.

In the wild type (WT) model (**Figure 7.4.A**), His¹⁸¹ interacted with the hydroxyl oxygen of atovaquone via a H-bond (yellow dashed line). Additionally, Tyr²⁷⁹ interacted with the hydroxyl and carbonyl oxygen of the naphthoquinone ring via aromatic H-bonds (blue dashed line), and Glu²⁷² interacted via aromatic H-bond with the naphthoquinone hydrogen. In contrast, in the Y279S mutant model, atovaquone moved away from the key residues to an energetically more favoured site of the binding pocket (**Figure 7.4.B**), as supported by the higher negative MM-GBSA value ($\Delta G = -21.66$ kcal/mol; **Table 7.2**) compared to its docking score (-9.98 kcal/mol) and MM-GBSA ($\Delta G = -12.63$ kcal/mol) in the 3CX5 (WT) model.

As a result of this movement, atovaquone lost its interaction with the key residue His¹⁸¹, which aligned with previously reported findings explaining why atovaquone loses its effectiveness in mutant strains [6], [30]. Given the difference in binding modes of atovaquone in the WT and Y279S mutant model, the study then focussed on determining whether the designed ligands could establish interactions with the key residues His¹⁸¹ and Glu²⁷² in the mutant model, enabling them to work as effectively as atovaquone does in the WT strains.

Analysis of the 2D and 3D LIDs (**Figure 7.4.C**) for the ligand **Cdid-6** revealed that it interacted with His¹⁸¹ via H-bond. Further, no interactions were observed between Glu²⁷² and the ligand. However, there were interactions between the His¹⁸¹ and carbonyl oxygen of α,β -unsaturated chains for ligands **N-Chal-45** (**Figure 7.4.D**) and **N-Chal-46** (**Figure 7.4.E**). Additionally, both ligands interacted with Glu²⁷² through either a water bridge or H-bond, and with Leu²⁷⁵ via an aromatic H-bond, indicating stronger interactions between the ligands and the residues.

7.3.2.4 MD simulations

MD simulations were performed for 100 ns on atovaquone in the 3CX5 WT and Y279S mutant models, along with **Cdid-6**, **N-Chal-45**, **N-Chal-46** and **N-Chal-19** in the mutant model, and the results compared with atovaquone in the 3CX5 (WT) model. This analysis aimed to observe the binding behaviour of the novel ligands and assess their potential effectiveness against the Y279S resistance. The goal was to determine whether these compounds could interact in the similar manner as atovaquone in the non-mutated strain by interacting with key residues His¹⁸¹ and Glu²⁷² to be effective. The MD simulation results were evaluated on the following three parameters.

Protein-Ligand Root Mean Square Deviation (RMSD): To examine the conformational changes of the target protein when combined with the ligand molecule, the corresponding RMSD value was determined in the 100 ns MD simulation. According to the protein-ligand RMSD plot for **atovaquone** in the 3CX5 (WT) model (**Figure 7.5.a**), along with **CdId-6** (**Figure 7.5.b**), **N-Chal-46** (**Figure 7.5.c**) and **N-Chal-46** (**Figure 7.5.d**) in the 3CX5 (Y279S) mutant model, there was a jump in the protein RMSD at the beginning of the simulation, as the system equilibrated. Thereafter, the RMSD stabilised, with a few fluctuations in between reflecting the inherent flexibility of certain loop regions. These fluctuations were associated more with **CdId-6**, indicating more conformational changes with the **CdId-6**-3CX5 mutant model. The core structure of the protein remained stable, as indicated by localized analysis of key domains in the RMSF plot. The ligand RMSD value of atovaquone, along with test ligands changed within a very narrow range, and remained stable until the end of the simulation, indicating that the ligands remained securely within the binding pocket in their respective complexes and did not diffuse away, aligning with effective binding interactions typically observed in stable protein-ligand complexes [38].

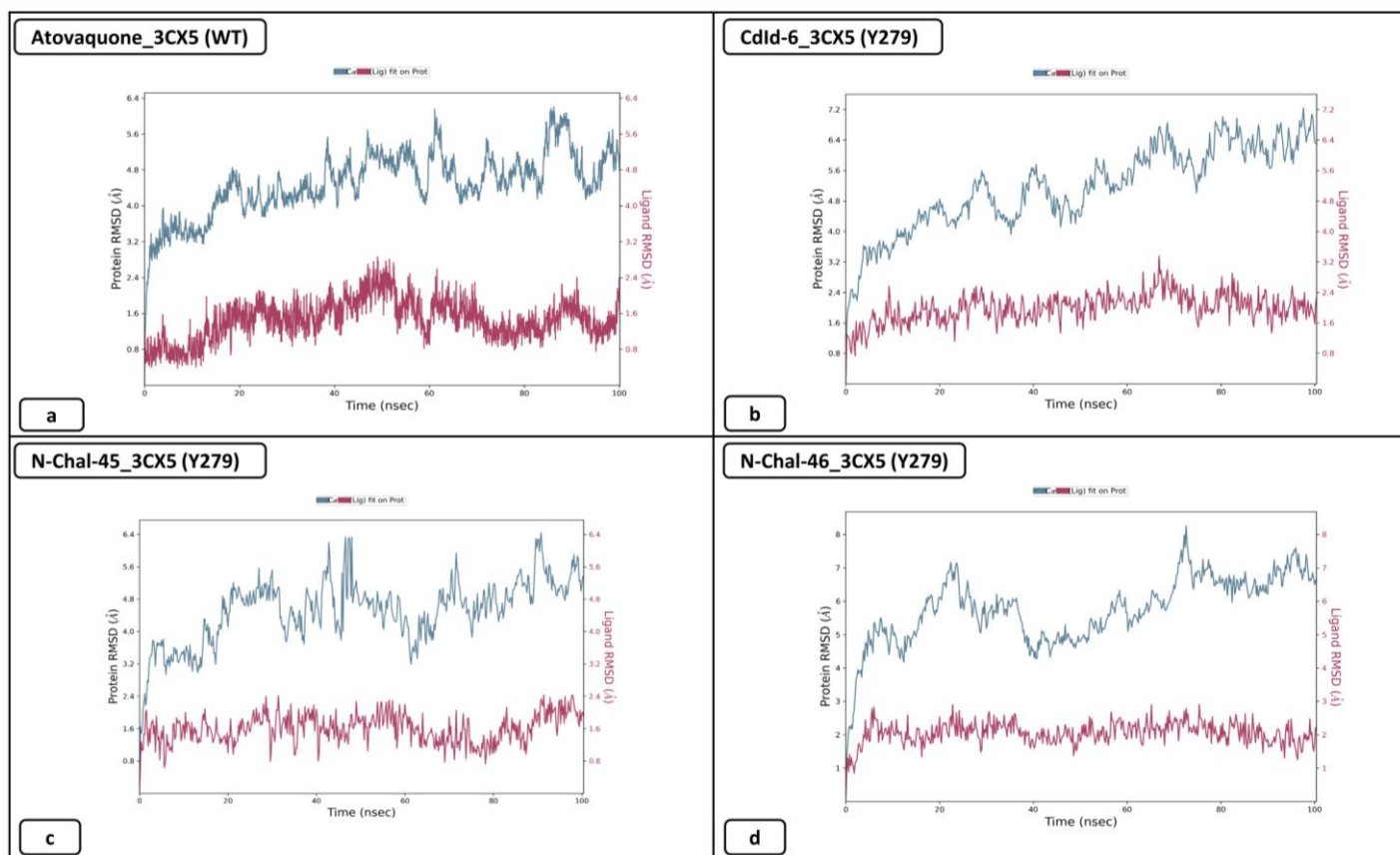


Figure 7.5: RMSD plot of the protein-ligand complex over 100 ns of MD simulation for atovaquone (a) in complex with 3CX5 (WT), along with **Cdlid-6** (b), **N-Chal-45** (c), and **N-Chal-46** (d) in complex with 3CX5 (Y279S) models. The blue curve represents the RMSD of the protein C α atoms, providing insights into the backbone stability and overall conformational changes during the simulation; while the red curve corresponds to the ligand RMSD, showing its movement relative to the protein binding site. Initial deviations suggested the ligand accommodation and binding site adaptation. A steady state or plateau in the RMSD values indicated that the system had reached equilibrium, with minimal changes in the protein-ligand complex.

Protein Root Mean Square Fluctuation (RMSF): The protein RMSF helped to identify and characterize the local changes that occurred within the protein's configuration when the ligand interacted with binding pocket residues, more specifically noting the RMSF values for His¹⁸¹, Glu²⁷², and Tyr²⁷⁹ or Ser²⁷⁹. The RMSF was determined to analyse the structural changes in the protein resulting from the binding of ligands to particular residue sites, with the highest fluctuation observed at around residue index 400 (**Figure 7.6**). The higher fluctuations observed in the loop regions aligned with their inherent dynamic behaviour, which did not impact the stability of the binding pocket as these regions did not contribute to ligand binding, and their flexibility might facilitate conformational adjustments during the simulation.

Table 7.3 presents the RMSF data for the interaction between key residues for atovaquone in the 3CX5 (WT) model (**Figure 7.6.a**), along with **CdId-6** (**Figure 7.6.b**), **N-Chal-45** (**Figure 7.6.c**), **N-Chal-46** (**Figure 7.6.d**) and **N-Chal-19** (**Figure 7.S.2**) in the 3CX5 (Y279S) mutant model. Comparing the RMSF data with atovaquone, all ligands established contacts with key residue His¹⁸¹ with favourably low RMSF values indicating stable interactions. Similarly, **N-Chal-45**, **N-Chal-46** and **N-Chal-19** also interacted with Glu²⁷², however **CdId-6** did not make any contact with it, indicating favourable interactions of the designed ligands in comparison to **CdId-6**. Further, all ligands interacted with Ser²⁷⁹ that replaced Tyr²⁷⁹ in the mutant model, with favourably low RMSF data.

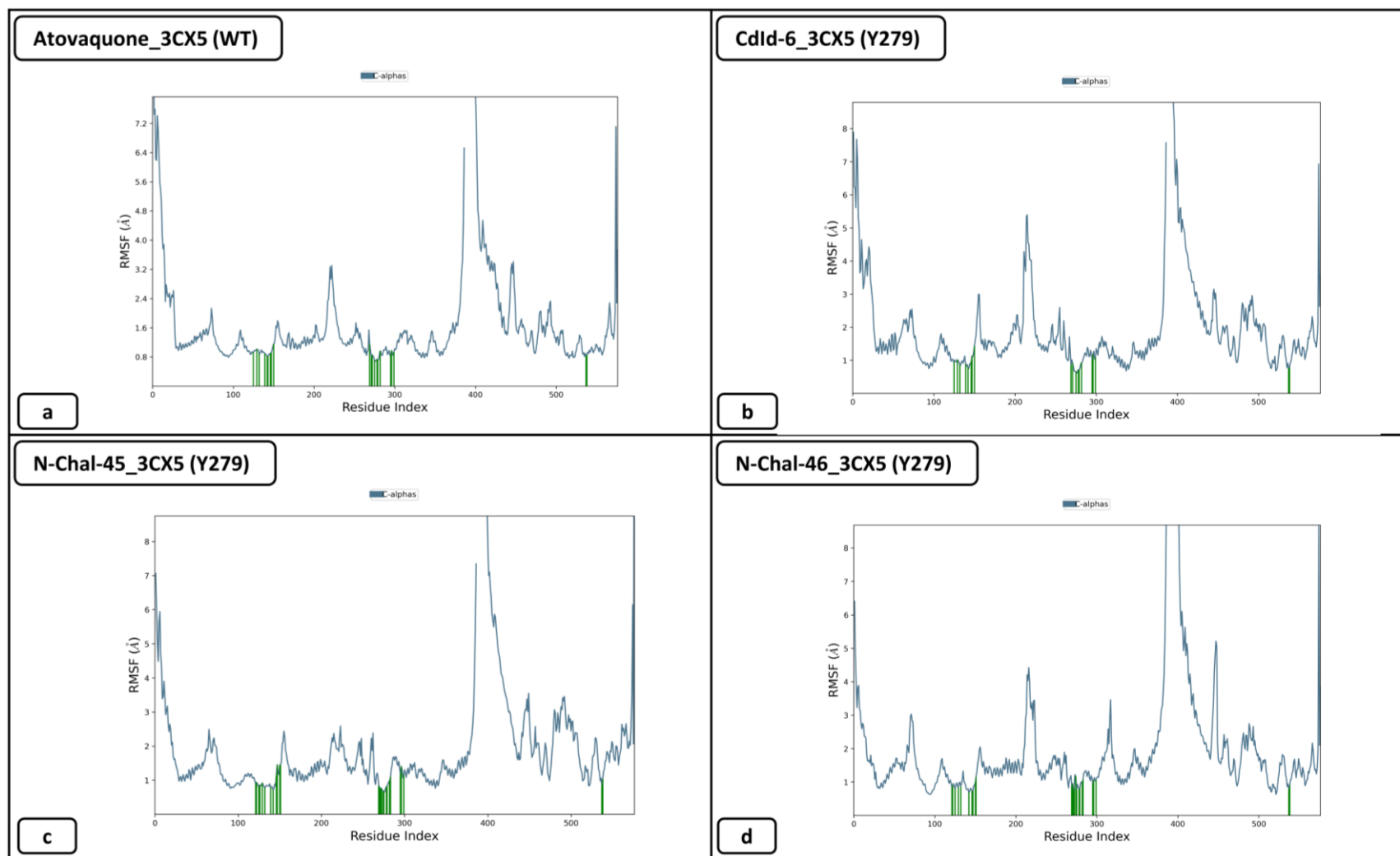


Figure 7.6: Protein RMSF plot for characterizing local fluctuations along the protein chain for **atovaquone** (a) in 3CX5 (WT) model, along with **Cdid-6** (b), **N-Chal-45** (c), and **N-Chal-46** (d) in the 3CX5 (Y279S) mutant model. Green vertical bars show the protein residues interacting with the ligand, and peaks are the loop regions that fluctuate the most during simulation.

Table 7.3: RMSF values (Å) of key residues interacting with atovaquone, **Cdid-6**, **N-Chal-46**, **N-Chal-45**, and **N-Chal-19**.

Residue	Chain	C α -Protein RMSF (Å)				
		Atovaquone	Cdid-6	N-Chal-46	N-Chal-45	N-Chal-19
His ¹⁸¹	P	0.85	0.79	0.89	1.04	0.65
Glu ²⁷²	C	0.86	No ligand contact	0.81	0.77	0.81
Tyr ²⁷⁹	C	0.72		-	-	-
Ser ²⁷⁹	C	-	0.70	0.81	0.83	0.67

Protein-Ligand (PL) contacts: Throughout the 100 ns simulation, ligand formed numerous connections with the target protein via hydrogen, hydrophobic, and water bridge bonds which were analysed with the help of P-L histogram and 2D contact summary.

For atovaquone in complex with the 3CX5 (WT) model (**Figure 7.7.a**), the maximum interactions were seen with His¹⁸¹ via the H-bonds and with Glu²⁷² via water bridges. These observations were in agreement with the interactions of atovaquone reported in the literature, as these residues play a significant role and have been vital for the stabilisation of atovaquone inside the cavity [39], [40], [41], [31], [23]. The rest of the interactions were mainly hydrophobic. From the simulation interaction diagram (SID) (**Figure 7.8.a**), it was observed that His¹⁸¹ acted as a H-bond donor and interacted with hydroxyl oxygen for 99% of the simulation time and with carbonyl oxygen for 10% of the simulation time. Glu²⁷² acted as a H-bond donor and interacted with the carbonyl oxygen of the naphthoquinone ring via a water bridge for 43% of the simulation time. These MD simulation results were very important for this study and hence were used to compare and predict the binding stability of the designed ligands.

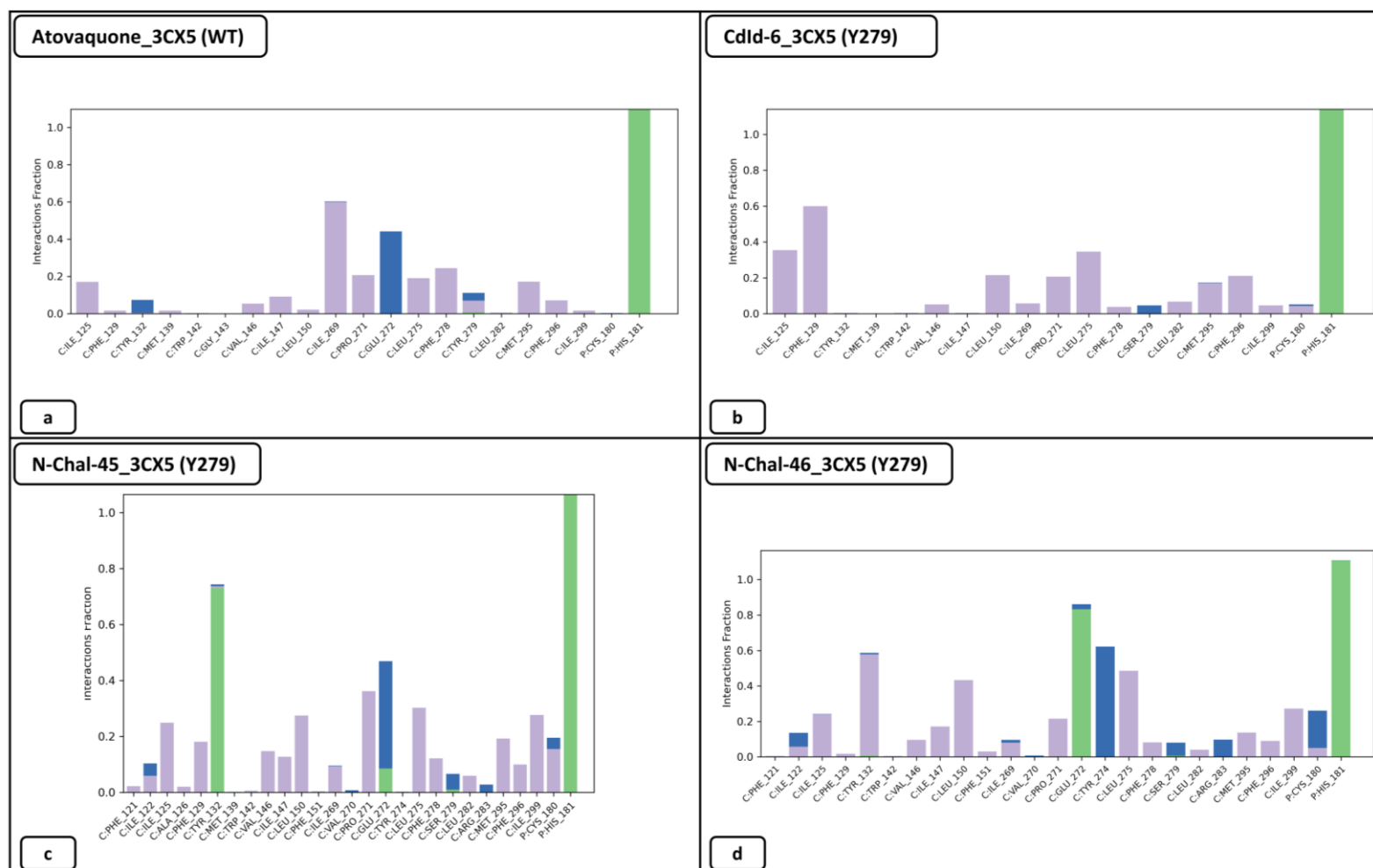


Figure 7.7: Protein-ligand contact histogram for **atovaquone (a)** in complex with **3CX5 (WT)** and for **CdId-6 (b)**, **N-Chal-45 (c)**, and **N-Chal-46 (d)** in complex with **3CX5 (Y279S)** models, representing the frequency of the interactions categorised into four types: hydrogen bonds, hydrophobic bonds, ionic bonds and water bridges. Residues with higher interaction frequencies indicate key binding site contacts contributing to ligand stability.

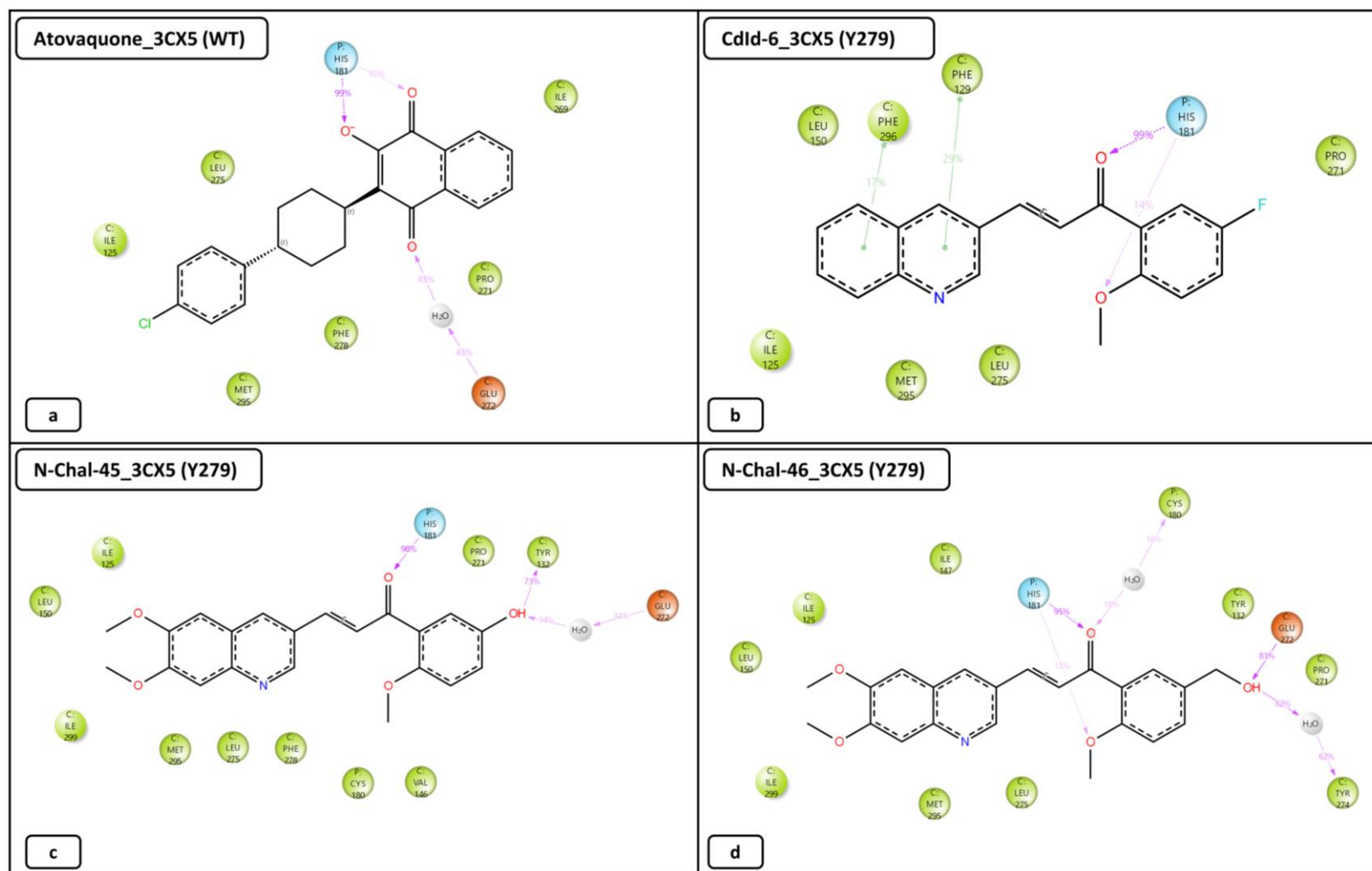


Figure 7.8: Schematic diagram showing ligand atoms interacting with the protein residues for more than 10% of the simulation for **atovaquone (a)** in complex with **3CX5 (WT)** and for **Cdid-6 (b)**, **N-Chal-45 (c)**, and **N-Chal-46 (d)** in complex with **3CX5 (Y279S)** models.

Analysing the P-L contacts for other ligands, it was observed that all ligands including **CdId-6** (Figure 7.7.b, 7.8.b), **N-Chal-45** (Figure 7.7.c, 7.8.c), and **N-Chal-46** (Figure 7.7.d, 7.8.d), interacted with His¹⁸¹ the most, supported by the simulation interaction diagram (SID) which displayed that His¹⁸¹ interacted with carbonyl oxygen of α,β -unsaturated chain via H-bonds for more than 95% of the simulation time, suggesting strong interaction between the ligands and the key residue His¹⁸¹. Additionally, **N-Chal-45** interacted with Glu²⁷² via water bridge for 34% of the simulation time and with Tyr¹³² for 73% of the simulation time. Further, **N-Chal-46** interacted with Glu²⁷² via H-bond for 81% of the simulation time and with Tyr²⁷⁴ for 62% of the simulation time via a water bridge. No interaction between **CdId-6** and Glu²⁷² was observed. The overall L-P interaction for **N-Chal-45** and **N-Chal-46** was observed to be very strong and favourable, indicating towards their strong potential to bind to the cyt *bc*₁ complex active site and highlighting their capability to target atovaquone-resistant malaria.

7.3.3 ADMET and drug-likeness

All compounds presented in Table 7.4 complied with Lipinski's rule of five (Ro5) and Veber's rules with no violations, indicating favourable drug-likeness [42], [43]. All the compounds displayed satisfactory molecular properties and Lipinski's parameters like lipophilicity (LogP), molecular weight (MW), hydrogen bond properties, and polar surface area (TPSA) within acceptable limits. Though, the bioavailability score was low compared to atovaquone, it still supported their potential as drug candidates. Medicinal chemists strive to design compounds that can balance flexibility and rigidity for optimal target binding [43], [44]. Comparing the number of rotatable bonds (#RB) with atovaquone, all compounds exhibited a high number of rotatable bonds suggesting that these ligands could be too flexible to bind tightly to the targeted binding site, and in turn might affect their efficacy.

Table 7.4: Summary of molecular properties, drug-likeness parameters and ADME properties of atovaquone, along with **N-Chal-19**, **N-Chal-45**, **N-Chal-46** and **CdId-6**.

Ligand id	Criteria	N-Chal-19	N-Chal-45	N-Chal-46	CdId-6	Atovaquone
MW	≤ 500 g/mol (Lipinski)	379.41	365.38	379.41	307.32	366.84
LogP	≤ 5 (Lipinski)	3.55	3.16	3.10	3.91	4.64
#HBA	≤ 10 (Lipinski)	6	6	6	4	3
#HBD	≤ 5 (Lipinski)	0	1	1	0	1
#RB	≤ 10 (Veber)	7	6	7	4	2
TPSA	≤ 140 Å ² (Veber)	66.88	77.88	77.88	39.19	54.37
Lipinski #violations	≤ 1 violation	0	0	0	0	0
Bioavailability Score	≥ 0.55	0.55	0.55	0.55	0.55	0.85
GI absorption	High is favourable	High	High	High	High	High
BBB permeant	Yes is CNS-targeted	Yes	No	Yes	Yes	Yes
CYP Inhibitor 1A2	No is favourable	No	No	Yes	Yes	Yes
CYP Inhibitor 2C19	No is favourable	Yes	Yes	Yes	Yes	Yes
CYP Inhibitor 2C9	No is favourable	Yes	Yes	Yes	Yes	Yes
CYP Inhibitor 2D6	No is favourable	No	No	No	No	No
CYP Inhibitor 3A4	No is favourable	Yes	Yes	Yes	No	Yes

Where, MW = molecular weight; LogP = log of octanol/water partition coefficient; #HBA = number of hydrogen bond acceptor(s); #HBD = number of hydrogen bond donor(s); #RB = number of rotatable bond(s); TPSA = topological polar surface area; Lipinski #violations = number of violations of Lipinski's rule; GI = gastrointestinal; BBB = blood-brain barrier.

All ligands were predicted to have high gastrointestinal (GI) absorption suggesting favourable oral bioavailability (**Table 7.4**). Like atovaquone, ligands CdId-6, **N-Chal-19** and **N-Chal-46** were predicted to cross the blood-brain barrier (BBB), making them suitable for central nervous system (CNS) targeted therapies like cerebral malaria. However, **N-Chal-45** was non-permeant, indicating potential limitations for CNS-related applications. All ligands were predicted to be non-inhibitors for

CYP2D6. Additionally, **N-Chal-19** and **N-Chal-45** were non-inhibitor of CYP1A2. All ligands were predicted to be inhibitors of CYP2C19 and CYP2C9, suggesting potential drug-drug interaction risks with drugs metabolised by these two CYP enzymes.

All ligands (CdId-6, **N-Chal-19**, **N-Chal-45** and **N-Chal-46**) belonged to class 4 with LD₅₀ = 1000 mg/kg, indicating them unsafe compared to atovaquone, which also belong to class 4 with a higher LD₅₀ = 2000 mg/kg (**Table 7.5**,). The three N-Chal ligands were predicted to have an increased risk of causing respiratory or kidney toxicity, along with being immunogenic and mutagenic.

Table 7.5: Summary of toxicity profiles of three N-Chal ligands, CdId-6 and atovaquone based on ProTox 3.0 predictions.

Compound ID	N-Chal-19	N-Chal-45	N-Chal-46	CdId-6	Atovaquone
LD ₅₀ (mg/kg)	1000	1000	1000	1000	2000
Toxicity Class	Class 4	Class 4	Class 4	Class 4	Class 4
Organ Toxicity	Respiratory toxic	Respiratory toxic	Nephrotoxic; Respiratory toxic	Hepatotoxic; Neurotoxic	Nephrotoxic; Respiratory toxic
Carcinogenicity	Non-carcinogenic	Non-carcinogenic	Non-carcinogenic	Non-carcinogenic	Non-carcinogenic
Immunotoxicity	Immunotoxic	Immunotoxic	Immunotoxic	Immunotoxic	Non-immunotoxic
Mutagenicity	Mutagenic	Mutagenic	Mutagenic	Mutagenic	Non-mutagenic
Cytotoxicity	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic	Non-cytotoxic

Class 1: fatal if swallowed (LD₅₀ ≤ 5 mg/kg); **Class 2:** fatal if swallowed (5 < LD₅₀ ≤ 50 mg/kg); **Class 3:** toxic if swallowed (50 < LD₅₀ ≤ 300 mg/kg); **Class 4:** harmful if swallowed (300 < LD₅₀ ≤ 2000 mg/kg); **Class 5:** may be harmful if swallowed (2000 < LD₅₀ ≤ 5000 mg/kg); **Class 6:** non-toxic (LD₅₀ > 5000 mg/kg)

These results are based on *in silico* predictions and may include false positives or negatives. Therefore, experimental validation is recommended, along with further optimization of the compounds to address the toxicological profile.

7.4 Conclusion

In the present study, novel chalcone compounds were designed by the molecular modification approach of the rational drug design. The designed compounds were investigated using molecular modelling studies that included molecular docking, MD simulations, drug-likeness, and ADMET profiling. Among the designed chalcones, three ligands, namely **N-Chal-19**, **N-Chal-45** and **N-Chal-46** were identified to exhibit the highest binding affinity against the 3CX5 (Y279S) mutant model. Along with this, the three ligands were predicted to exhibit satisfactory *in silico* drug-likeness and ADMET properties with no violations of the Lipinski's Ro5. MD simulation studies further supported the antimalarial potential of these compounds with the formation of well-defined and stable protein-ligand interactions against the Y279S mutant model. All three ligands were identified as non-carcinogenic and non-cytotoxic; however, they were predicted to exhibit immunotoxic and mutagenic potential. Based on the *in silico* findings and the predicted immunotoxic and mutagenic liabilities, the newly designed ligands **N-Chal-19**, **N-Chal-45** and **N-Chal-46** warrant careful consideration. Further *in vitro* validation is necessary to assess their efficacy and safety profile before establishing their potential as antimalarial agents effective against resistant strains of *P. falciparum*.

Author contributions: Conceptualization: PA and RvZ; Data curation: PA; Formal analysis: PA; Funding acquisition: RvZ; Investigation: PA; Methodology: PA; Project administration: RvZ; Resources: RvZ; Supervision: RvZ; Validation: PA, RvZ; Visualization: PA, RvZ; Writing - original draft: PA; Writing - review & editing: PA, CJvdW, RvZ.

Ethics approval: No ethics was required since it was all computational studies.

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Supplementary information:

Table 7.S.1.1: Summary of select novel designed chalcones with the highest docking and MM-GBSA results against truncated 3CX5 (Y279S) model (Schrödinger 2023-2).

Table 7.S.1.2: Summary of chalcones (CdId 1 to CdId 8) with the docking scores and MM-GBSA results against truncated 3CX5 (Y279S) model (Schrödinger 2023-2).

Figure 7.9: 2D and 3D docked poses of N-Chal-19.

Figure 7.10: MD simulation of ligand **N-Chal-19** in complex with **3CX5 (Y279S)** mutant model..

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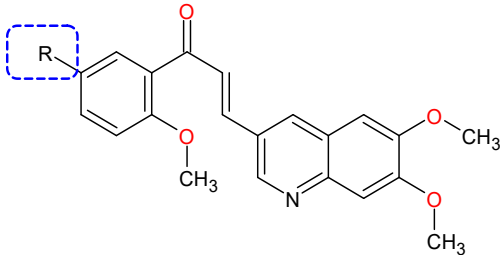
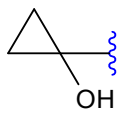
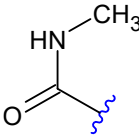
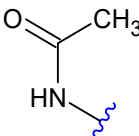
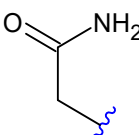
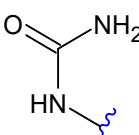
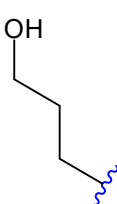
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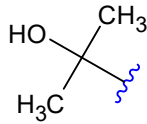
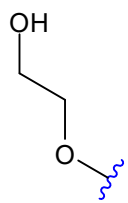
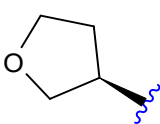
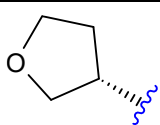
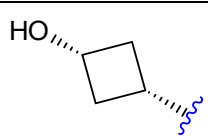
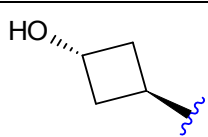
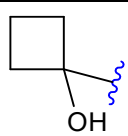
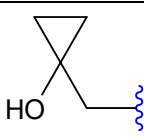
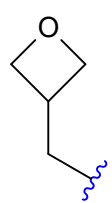
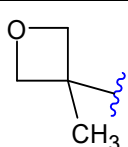
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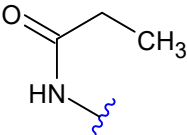
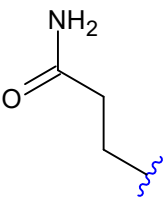
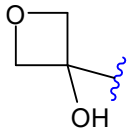
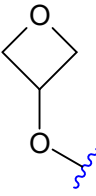
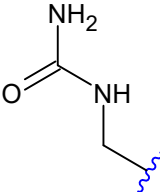
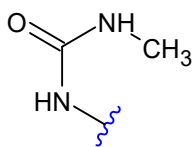
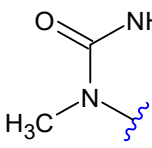
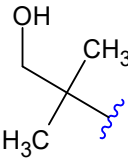
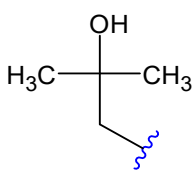
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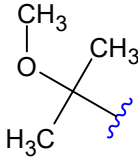
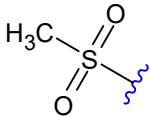
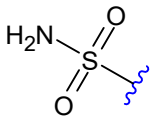
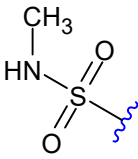
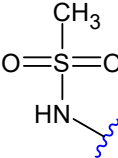
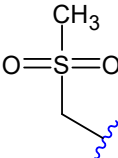
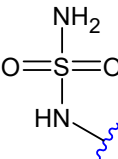
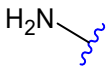
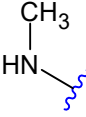
Table 7.6: Summary of select novel designed chalcones with the highest docking and MM-GBSA results against truncated 3CX5 (Y279S) model (Schrödinger 2023-2).

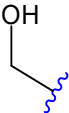
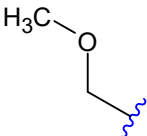
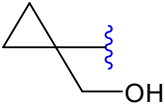
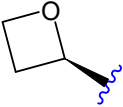
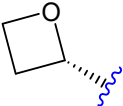
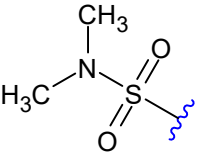
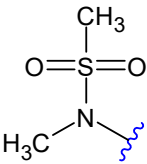
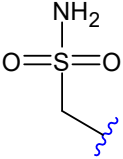
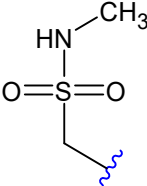
Ligand Id	R group	Docking score 3CX5 (Y279S) (kcal/mol)	MM-GBSA ΔG_{Bind} 3CX5 (Y279S) (kcal/mol)	H-bond score
				
N-Chal-1		-11.49	-38.72	-1.54
N-Chal-2		-9.22	-48.10	-0.57
N-Chal-3		-8.36	-47.53	0.00
N-Chal-4		-11.02	-51.48	-0.46
N-Chal-5		-10.51	-45.67	-0.81
N-Chal-6		-10.86	-58.80	-0.70

N-Chal-7		-10.40	-36.40	-0.72
N-Chal-8		-10.72	-41.63	-1.16
N-Chal-9		-11.12	-30.61	-0.60
N-Chal-10		-11.58	-29.03	-0.70
N-Chal-11		-10.83	-36.98	0.00
N-Chal-12		-11.02	-45.82	-0.25
N-Chal-13		-12.26	-38.39	-1.57
N-Chal-14		-10.76	-42.80	0.00
N-Chal-15		-10.84	-51.28	0.00
N-Chal-16		-10.23	-34.63	-0.42

N-Chal-17		-11.33	-41.92	-0.15
N-Chal-18		-11.17	-52.43	0.00
N-Chal-19		-10.53	-47.48	-0.89
N-Chal-20		-11.69	-39.65	-1.11
N-Chal-21		-11.42	-50.21	0.00
N-Chal-22		-10.38	-42.51	-0.41
N-Chal-23		-9.09	-46.55	-0.31
N-Chal-24		-8.53	-51.23	0.00
N-Chal-25		-10.52	-47.70	0.00
N-Chal-26		-9.33	-47.53	-0.70

N-Chal-27		-10.07	-48.29	0.00
N-Chal-28		-11.71	-45.41	-0.93
N-Chal-29		-12.25	-33.75	-1.95
N-Chal-30		-8.66	-45.44	0.00
N-Chal-31		-10.56	-51.76	-0.47
N-Chal-32		-9.67	-44.65	-0.27
N-Chal-33		-10.79	-38.12	-0.99
N-Chal-34		-10.67	-26.31	-0.07
N-Chal-35		-10.44	-33.15	0.00

N-Chal-36		-9.85	-40.06	-0.34
N-Chal-37		-9.83	-31.69	-0.66
N-Chal-38		-9.32	-35.65	-0.54
N-Chal-39		-9.36	-38.72	0.00
N-Chal-40		-7.98	-38.09	0.00
N-Chal-41		-10.56	-39.91	-0.10
N-Chal-42		-10.26	-49.19	-0.65
N-Chal-43		-9.87	-39.14	-0.54
N-Chal-44		-10.421	-49.37	-0.23
N-Chal-45		-11.91	-53.37	-1.39

N-Chal-46		-11.50	-51.43	-1.28
N-Chal-47		-10.17	-52.52	0.00
N-Chal-48		-11.37	-16.66	-0.63
N-Chal-49		-11.04	-33.43	-0.66
N-Chal-50		-10.71	-35.95	-0.60
N-Chal-51		-6.29	-32.53	0.00
N-Chal-52		-8.74	-48.29	0.00
N-Chal-53		-11.14	-49.31	-1.08
N-Chal-54		-9.13	-37.13	0.00

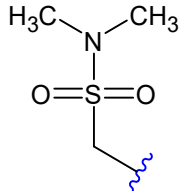
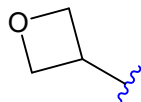
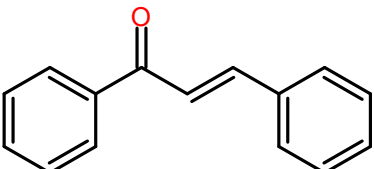
N-Chal-55		-10.58	-43.51	-0.10
N-Chal-56		-11.04	-51.60	0.00
Atovaquone		-8.23	-21.66	0.00

Table 7.S.1.2: Summary of chalcones (Cdlid 1 to Cdlid 8) with the docking scores and MM-GBSA results against truncated 3CX5 (Y279S) model (Schrödinger 2023-2)

 Ring B Ring A					
Cdlid	Ring B	Ring A	Docking score 3CX5 (Y279S) (kcal/mol)	MM-GBSA ΔG_{Bind} 3CX5 (Y279S) (kcal/mol)	H-Bond score
1	3,4-di-OMe phenyl	quinolin-4-yl	-10.42	-41.89	-0.61
2	2,5-di-Cl phenyl	quinolin-3-yl	-10.38	-45.06	-0.46
3	2,5-di-Cl phenyl	quinolin-4-yl	-11.29	-47.89	-0.65
4	3,4-di-Cl phenyl	quinolin-4-yl	-10.72	-47.57	-0.70
5	2-OMe, 5-F phenyl	quinolin-2-yl	-10.72	-44.25	-0.70
6	2-OMe, 5-F phenyl	quinolin-3-yl	-10.68	-44.36	-0.70
7	3-F, 4-OMe phenyl	quinolin-4-yl	-10.09	-39.62	-0.67
8	2-OMe, 5-Cl phenyl	quinolin-3-yl	-10.48	-38.49	-0.83

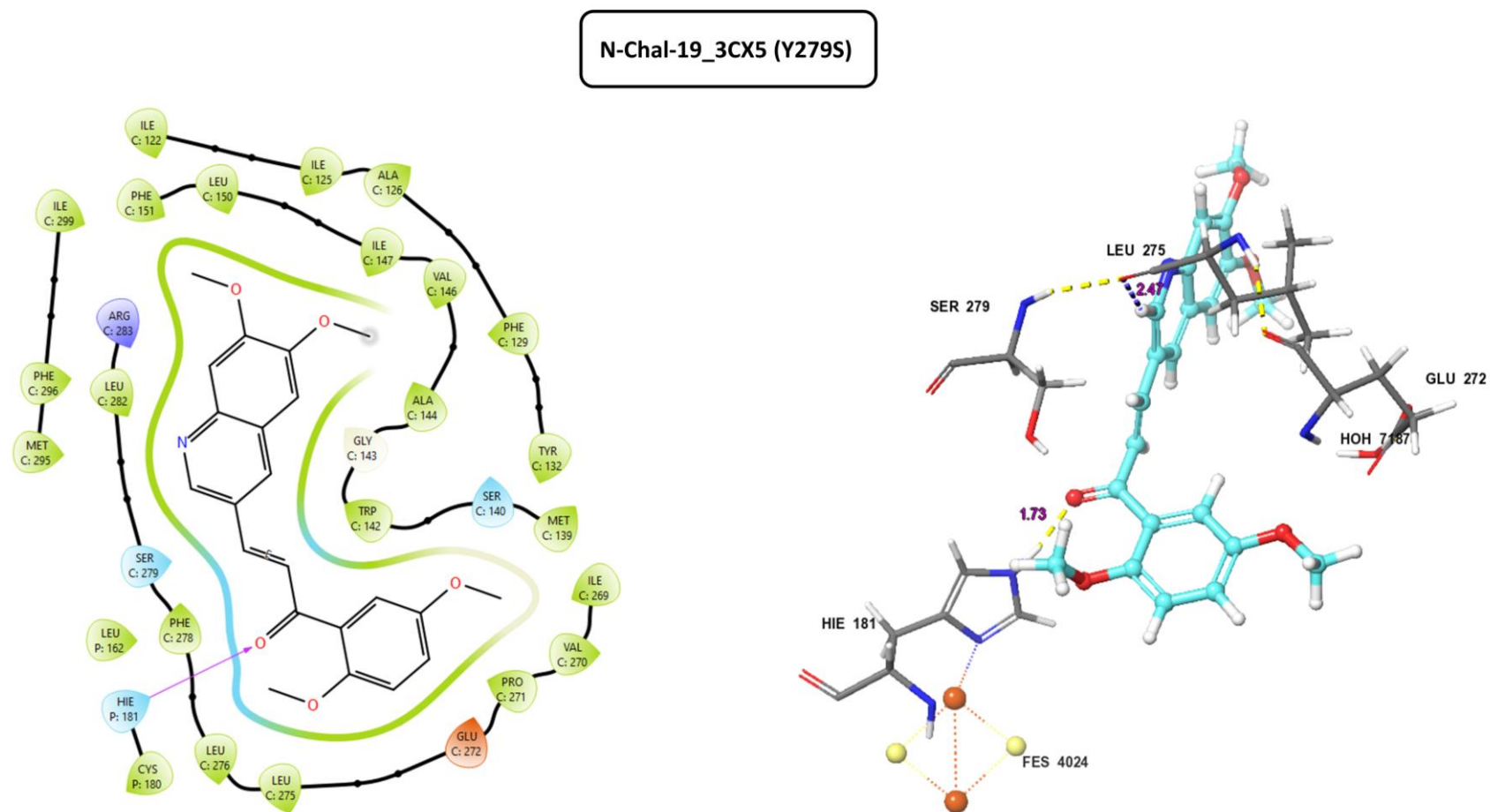


Figure 7.9: 2D and 3D LID for N-Chal-19 in 3CX5 (Y279S) mutant mode.

Supplementary: MD simulation graphs for N-Chal-19 in 3CX5 (Y279S) mutant model

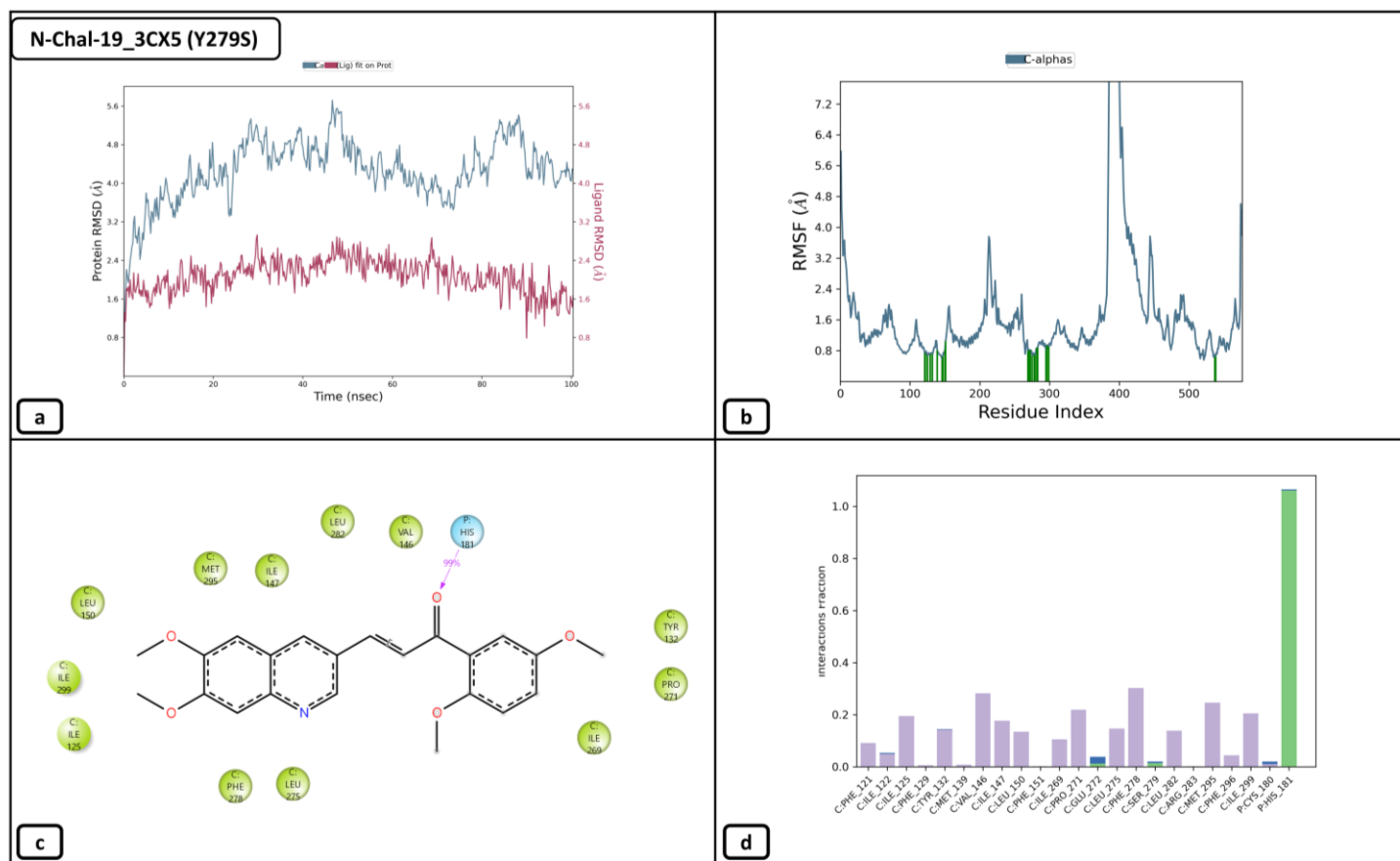


Figure 7.10: MD simulation of ligand **N-Chal-19** in complex with **3CX5 (Y279S)** mutant model. (a) RMSD plot of the protein-ligand complex over 100 ns of MD simulation where the blue curve represents the RMSD of the protein Cα atoms, providing insights into the backbone stability and overall conformational changes during the simulation, the red curve corresponds to the ligand RMSD, showing its movement relative to the protein binding site. (b) RMSF plot for the protein for characterizing local fluctuations along the protein chain, where green vertical bars show the protein residues interacting with the ligand, and peaks are the loop regions that fluctuate the most during simulation. (c) Schematic diagram showing ligand atoms interacting with the protein residues for more than 10% of the simulation. (d) Protein-ligand contact histogram representing the frequency of the interactions categorised into four types: hydrogen bonds, hydrophobic bonds, ionic bonds and water bridges. Residues with higher interaction frequencies indicate key binding site contacts contributing to ligand stability.

Chapter 8

Collective discussion, conclusion and recommendations for future work

8.1 Collective Discussion

Malaria continues to be a pressing global health burden, and the growing incidence of resistance against frontline antimalarials including artemisinin, necessitates the continuous search for new therapeutic strategies. The cytochrome *bc₁* (cyt *bc₁*) complex of *Plasmodium falciparum* is a well-validated drug target for antimalarial therapy, particularly due to its role in the parasite's electron transport chain. This chapter presents the collective findings of this PhD study, spanning from **Chapter 3** (data collation) to **Chapter 7** (designing novel compounds), highlighting the potential to address the increasing challenge of drug resistance, with a specific focus on atovaquone resistance.

8.1.1 DATA COLLATION

The journey started with identifying atovaquone as a known *Plasmodium* cyt *bc₁* complex inhibitor in clinical use (Siregar et al., 2015; Rossiter, 2022) and becoming aware that the parasite has developed resistance against this drug (Fivelman et al., 2002; Gil et al., 2003; Schwartz et al., 2003). The study proceeded with an extensive literature review and compilation of a comprehensive database of *Plasmodium* cyt *bc₁* complex inhibitors, leading to the identification of several chemical scaffolds with potential inhibitory activity against the target *Plasmodium* cyt *bc₁* complex, as discussed in **Chapter 3**. This review further facilitated the selection of compounds that demonstrated activity against the atovaquone-resistant strain and also exhibited *Plasmodium* cyt *bc₁* enzyme inhibitory properties as discussed in **Chapter 4**. A total of 631 compounds, that included 616 distinct chemical compounds and 15 known drugs, were collated for their antimalarial activities against various strains of *Plasmodium* including both ATQ-S and ATQ-R isolates. The different scaffolds included naphthaquinones, tetracyclic benzothiazepines, phthalimides, pyridine-based compounds, methoxyacrylated, methoxyiminoacetates, acridones-acridines, quinolones, chalcones and a set of miscellaneous compounds that chemically did not fit in any of the previous described scaffolds. Known drugs for which the data was collated included atovaquone, artesunate, artemisinin, antimycin A, amodiaquine, chloroquine, clodolol, dihydroartemisinin (DHA), floxacrine, GSK932121A, mefloquine, myxothiazol, piperazine, quinine and WR249685. Of the 631 compounds, 403 compounds (including the known drugs) were observed to be active against at least

one of the *Plasmodium* strains, either sensitive or resistant types. Of these, only 135 compounds exhibited activity against one of the ATQ-R strains and 22 were identified to inhibit *Plasmodium* cytochrome *bc₁* enzyme activity (as discussed in **Chapter 4**). The quinolone scaffold contributed the largest number of potential inhibitors with 130 unique compounds exhibiting activity against the ATQ-R strain, and 11 compounds capable of inhibiting *P. falciparum* cytochrome *bc₁* enzyme activity. In contrast, only three pyridine-based compounds showed activity against the ATQ-R strain, along with one acridone derivative and one compound from the miscellaneous category. The relatively small number of compounds displaying activity against resistant strains and enzyme inhibition, highlights a significant gap in current research. This highlights the necessity of exploring and optimising additional compounds to identify promising lead molecules for further evaluation of their specificity in binding to the *P. falciparum* cytochrome *bc₁* enzyme target site. Additionally, compounds should be analysed for their toxicological profile, along with pharmacokinetics and drug-likeness properties. Gaining insights into the structure-activity relationship and binding affinity of promising compounds could pave the way for new therapeutic strategies to combat emerging atovaquone resistance.

The compilation of previously published compounds is a vital aspect of this study, particularly in identifying diverse chemical scaffolds of active inhibitors and designing novel compounds to combat rising drug resistance. Such collation is highly beneficial, allowing researchers to build upon existing knowledge, minimize redundancy, and accelerate drug discovery by prioritizing the most promising candidates. Additionally, compounds previously dismissed due to inactivity, resistance, or adverse effects can now be revisited and optimised using new insights, potentially leading to the development of innovative therapeutic agents. To support this approach, *in silico* studies, including docking, MD simulations, along with pharmacokinetics and drug-likeness, were conducted as discussed in **Chapter 4**. Further MD simulation studies were performed on two compounds (**I-161** and **H-2**) that displayed activity against atovaquone-resistant strains, to analyse their interactions with the binding pocket residues in the 3CX5 (Y279S) mutant model (**Chapter 4, Figure 4.5**).

8.1.2 MOLECULAR MODELLING

Literature reports the binding modes of atovaquone within the cytochrome *bc₁* binding cavity which greatly helped to understand the key interactions with the binding pocket residues, specifically His¹⁸¹, Glu²⁷², and Tyr²⁷⁹ (Kokhan et al., 2010; Vallières et al., 2013; Akhoun et al., 2014; Birth et al., 2014; Sodero et al., 2017). While doing the data collation it was observed that most chemical synthesis

and *in vitro/in vivo* studies did not include computational *in silico* molecular modelling, more specifically MD simulation studies that could explain the binding interactions with the identified key residues (His¹⁸¹, Glu²⁷², and Tyr²⁷⁹). However, some papers did report the docking studies (Carrasco et al., 2013; Jiménez Villalobos et al., 2013; Borgati et al., 2017; Sodero et al., 2017; Jacobsen et al., 2021). Though docking does offer a quick estimation of binding affinity, MD simulation is more reliable as it provides a realistic and dynamic view of the ligand-protein interactions by simulating the natural environment of the system, making it more accurate for studying ligand stability and interaction mechanism (Sakano et al., 2016). To address this and to use MD simulation to analyse the binding behaviour of the ligand used in the study, two *in silico* models were developed, which played a predominant role throughout this thesis. Since the crystal structure for *Plasmodium* cyt *bc*₁ complex is unavailable, *Saccharomyces cerevisiae* cyt *bc*₁ complex (PDB ID 3CX5) was used to construct the two models: one representing the wild type (WT) and another simulating an atovaquone-resistant Y279S mutation (equivalent to the Y268S mutation in *Plasmodium*) where the residue Tyr²⁷⁹ was substituted with Ser²⁷⁹ to analyze the binding behaviour of compounds (**Methods, Section 2.2**) (Solmaz and Hunte, 2008; Song et al., 2015; Jacobsen et al., 2021; Schrödinger Release Notes - Release 2023-2) .

Initial docking studies on the collated data were performed using the Schrödinger Release 2021-1 version to obtain the docking scores. However, when attempting to correlate these scores with IC₅₀ values, it became evident that docking scores primarily predicted binding affinity and were most useful for compound screening rather than precise activity prediction.

As the study progressed, new challenges emerged. The Schrödinger Release 2021-1 version did not support the MM-GBSA binding energy calculations and MD simulations for the study target, resulting in errors. To overcome this, the models were reconstructed using Schrödinger Release version 2023-2, allowing for advanced studies that included MMGBSA calculations and MD simulation. Given the large size of the 3CX5 crystal structure, computational limitations made it impractical to perform these studies on the full crystal structure. To overcome this hurdle, truncated models were built by removing extraneous chains and ligands, retaining only the chains involved in the cyt *bc*₁ binding pocket, namely chain P and C. This allowed for the construction of refined protein models for both WT and Y279S mutant (**Methods, Section 2.2.8**).

8.1.2.1 Ramachandran Plots

The Ramachandran plots were used to evaluate and display the distribution of the phi (ϕ) and psi (ψ) dihedral angles (in degree) of the amino acid side chains in the 3CX5-WT and 3CX5-Y279S mutant models. Most of the residues were observed in the favoured and allowed regions, marked by red and yellow coloured areas, respectively. Only a few residues fell into the disallowed white regions. The plots in the top and bottom left quadrants indicated that the models contained both β -sheets and α -helices. Further, the plots in the top and bottom right quadrants represented loop structures.

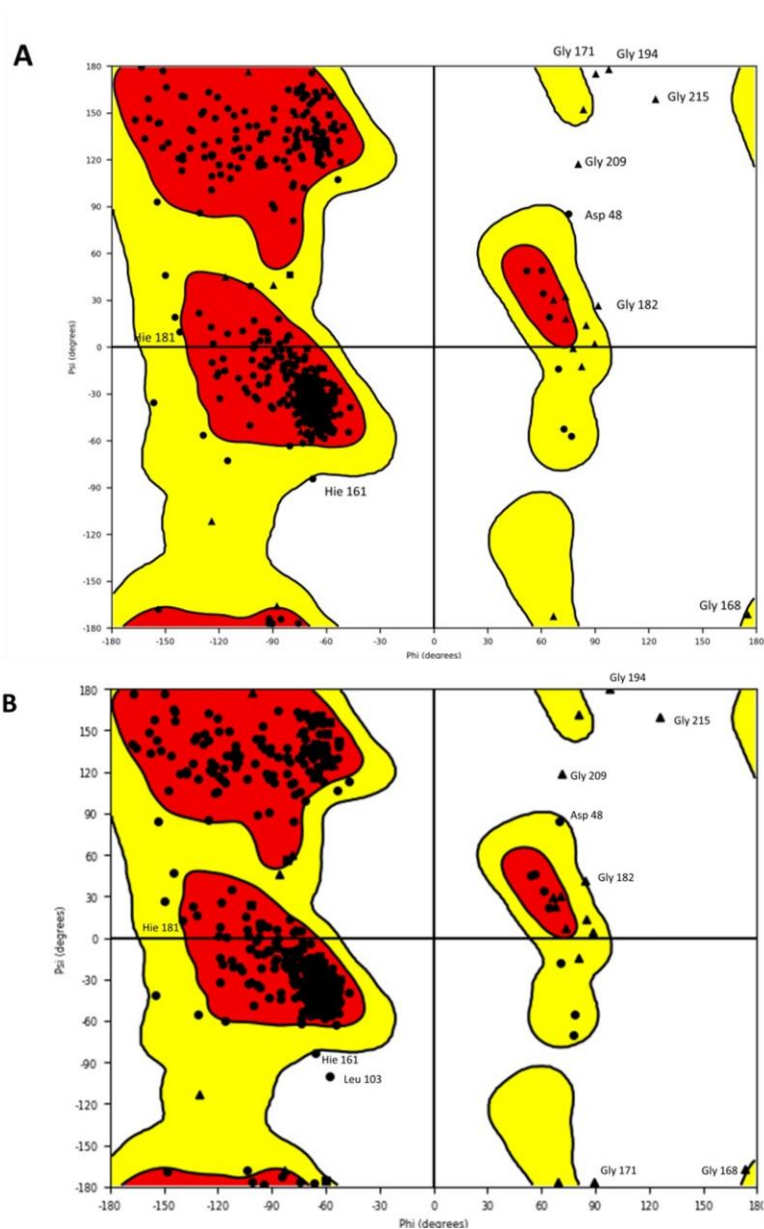


Figure 8.1: Ramachandran plots for 3CX5-WT model (A); and 3CX5-Y279S model (B). Glycine is plotted as triangles, proline as squares, and all other residues are shown as circles. The red regions are the favoured regions, the yellow regions are the allowed regions, and the white regions are disallowed regions.

Table 8.1 shows the residues that were seen in the disallowed (white) region and at the borderline of disallowed and allowed (yellow) regions. No significant change was observed in the number of residues in the disallowed and allowed regions. Asp 48 was seen to have moved inside the allowed (yellow) region and Leu 103 was observed to be added in the disallowed (white) region in the 3CX5-Y279S mutant model (**Figure 8.1 B**). Further, the plots were inspected for key residues Hie 181, Glu 272, Tyr 279 and Ser 279 (in mutant model). All these residues fell in the favoured region in both the models, with only Hie 181 which was seen at the border of the favoured and the allowed region in the 3CX5-WT model (**Figure 8.1 A**).

Table 8.1: Residues identified in the disallowed region of the Ramachandran plots of the 3CX5 (WT) and 3CX5 (Y279S mutant) protein models.

Residues in the disallowed region	
3CX5-WT	3CX5-Y279S mutant
Gly 171	Gly 171
Gly 194	Gly 194
Gly 215	Gly 215
Gly 209	Gly 209
Asp 48 (on the borderline of yellow and white)	Asp 48 (inside yellow region)
Gly 182 (on the borderline of yellow and white)	Gly 182 (on the borderline of yellow and white)
His 161 (on the borderline of yellow and white)	His 161 (on the borderline of yellow and white)
Gly 168 (on the borderline of yellow and white)	Gly 168 (on the borderline of yellow and white)
	Leu 103

Table 8.2: The phi (ϕ) and psi (ψ) dihedral angles of key amino acid side chains in the 3CX5-WT and 3CX5-Y279S mutant models, indicating no significant change.

Residues in 3CX5-WT	Phi (ϕ)	Psi (ψ)
Glu 272	-54.54	138.74
Tyr 279	-65.99	-42.13
Hie 181	-141.87	9.7
Residues in 3CX5-Y279S mutant	Phi	Psi
Glu 272	-53.4	140.5
Ser 279	-69.59	-38.05
Hie 181	-138.3	8.5

8.1.2.2 MD Simulations for Apo-protein

The MD simulation results for WT and Y279S mutant apo-protein models are as discussed below.

Protein-Ligand Root Mean Square Deviation (RMSD): RMSD determination helped examine the apo-protein's conformational changes for a 100ns MD simulation. At the beginning of the simulation, there was a jump in the RMSD as the system equilibrated and reached a stationary state. The RMSD profile of the wild-type apo-protein (**Figure 8.2 A**) stabilized around 4.0 Å upto 40 ns, indicating structural equilibrium. After 40 ns, there was again rise in RMSD, which later came down and started to stabilise from 60 ns onwards. For the Y279S mutant apo-protein (**Figure 8.2 B**), there was initial rise in RMSD values as the system equilibrated and reached stationary state after 20 ns and remained relatively stable until the end of the simulation.

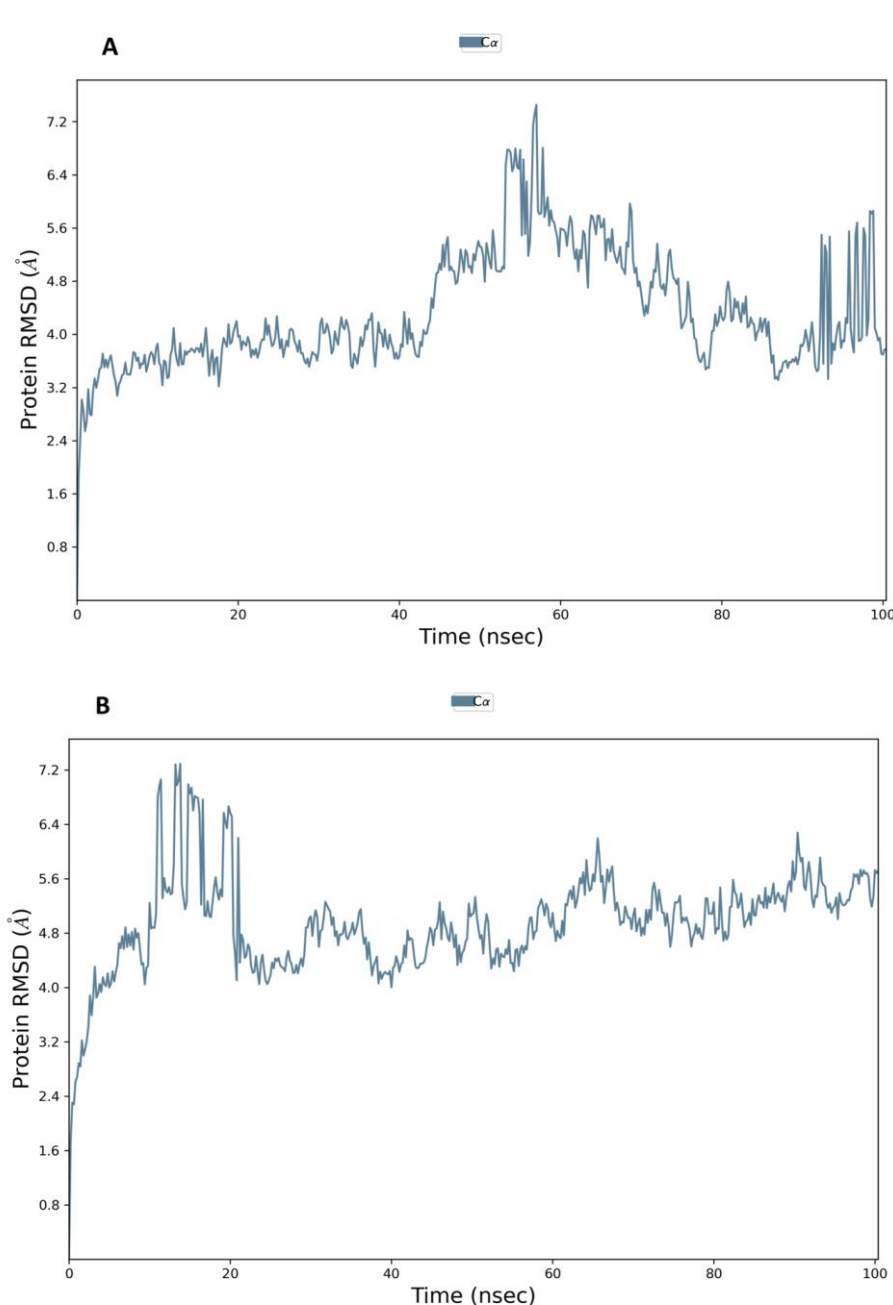


Figure 8.2: Root Mean Square Deviation (RMSD) plots of apo-protein structures for 3CX5 (WT) (**A**) and 3CX5 (Y279S mutant) (**B**) models over 100 ns of MD simulation obtained using Schrödinger Desmond.

Protein Root Mean Square Fluctuation (RMSF): The protein RMSF (**Figure 8.3 A and B**) helped to identify and characterize the local changes that occurred within the 3CX5 (WT) and 3CX5 (Y279S mutant) apo-proteins configurations, more specifically noting the RMSF values for His¹⁸¹, Glu²⁷², and Tyr²⁷⁹ or Ser²⁷⁹. The RMSF was determined to analyse the structural changes in the protein resulting from the Y279S mutations (**Figure 8.3 B**). The highest fluctuations were observed at around residue index 400, suggesting the loop regions which fluctuate the most during simulation.

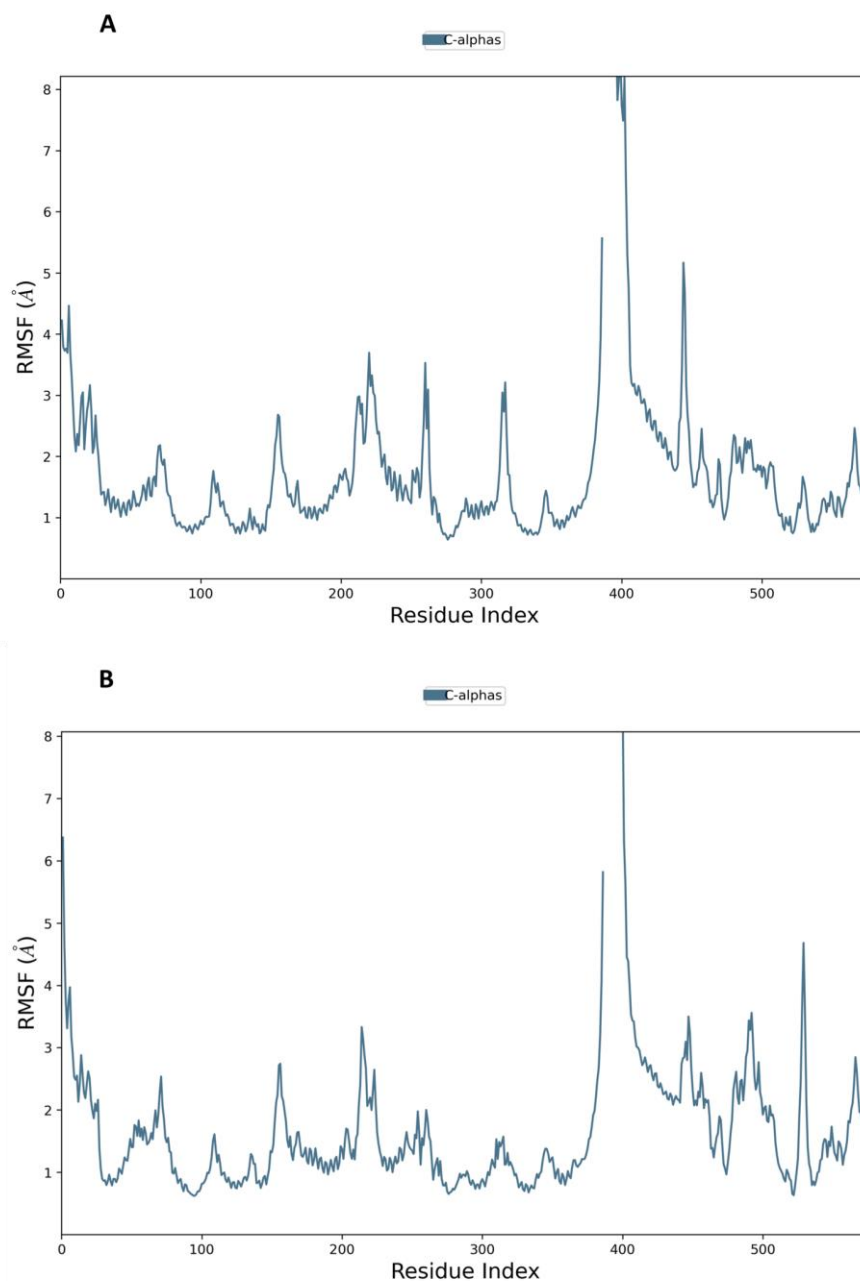


Figure 8.3: RMSF plots for 3CX5 (WT) (**A**) and 3CX% (Y279S mutant) (**B**) apo-protein models.

The core structure of the protein seemed to remain stable, as indicated by localised analysis of key domains in the protein root mean square fluctuation (RMSF) values (**Table 8.3**). The RMSF values for the key residues (His¹⁸¹, Glu²⁷², Tyr²⁷⁹ and Ser²⁷⁹) remained below 1 Å in both the models for apo-proteins.

Table 8.3: RMSF values of key residues in both atovaquone-bound and apo-protein models for WT and mutant proteins.

Residue	Chain	C α -Protein RMSF (Å)			
		3CX5-WT (apo-protein)	3CX5-WT (atovaquone-bound)	3CX5-Y279S (apo-protein)	3CX5-Y279S (atovaquone-bound)
His ¹⁸¹	P	0.81	0.85	0.87	0.92
Glu ²⁷²	C	0.77	0.86	0.79	0.67
Tyr ²⁷⁹	C	0.69	0.72		
Ser ²⁷⁹	C	-		0.74	1.00

Protein secondary structure: Secondary structure analysis indicated that both the wild-type and Y279S mutant apo-proteins retained similar global folding. The wild type maintained 49.32% helical and 5.79% β -strand content (total SSE = 55.12%), whereas the Y279S mutant displayed 49.10% helix and 5.22% strand (total SSE = 54.33%) (**Figure 8.4**). This change was very small and insignificant and suggest no major loss of ordered structure. Overall, the mutation did not compromise the secondary-structure integrity of the apo-protein during the simulation.

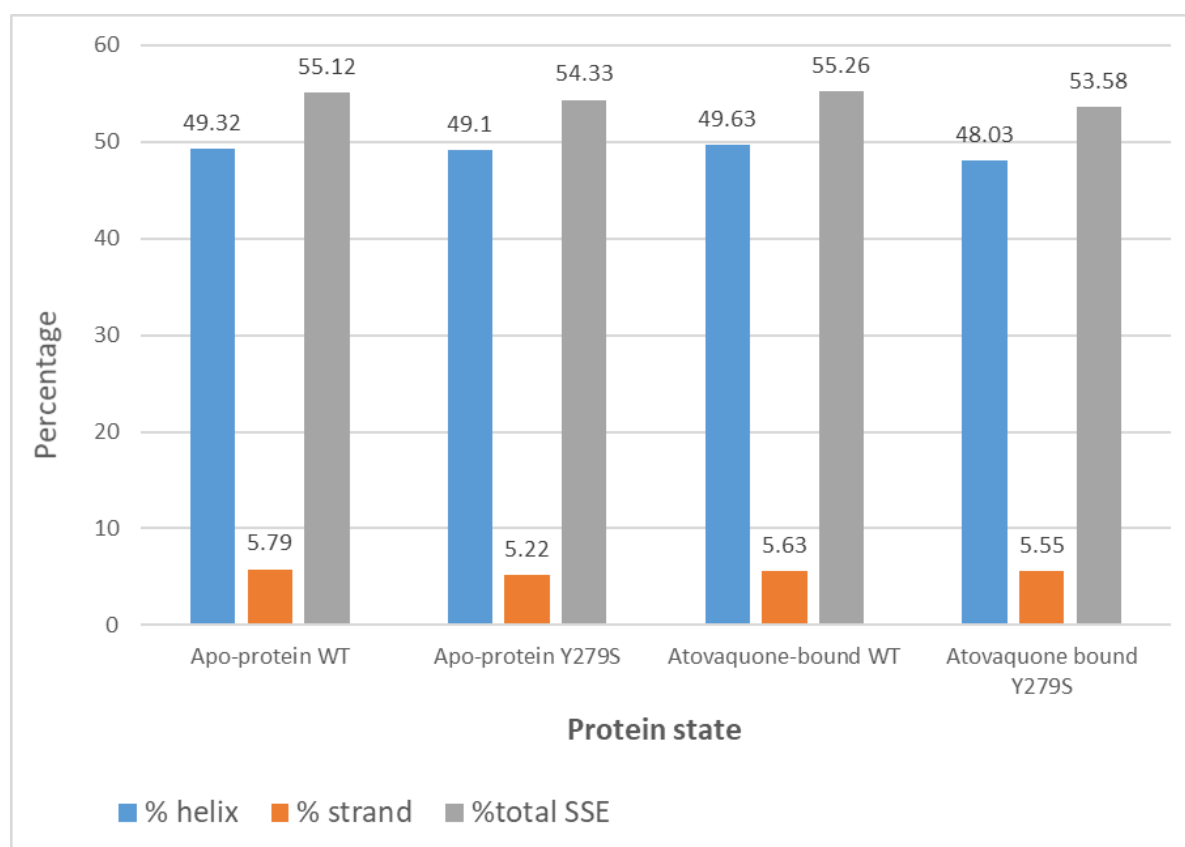


Figure 8.4: Secondary structure analysis of apo-protein and atovaquone-bound proteins for 3CX5 (WT) and 3CX5 (Y279S mutant) models

Analysis of the protein secondary structure during MD simulations provided additional insights into the effect of the Y279S mutation and its impact on ligand binding. The apo-protein forms of both wild-type and mutant models displayed comparable secondary structure composition, with the mutant showing only a marginal reduction in total secondary structural elements (55.12% in WT vs 54.33% in Y279S). This observation suggests that the mutation alone does not grossly disrupt the global fold of the protein but may subtly affect local stability.

Upon atovaquone binding, the wild-type protein maintained its structural integrity, reflected in a total secondary structure content of 55.26%, nearly identical to the apo-WT system. In contrast, binding of atovaquone to the Y279S mutant resulted in a measurable decrease in helicity (48.03% vs 49.63% in WT + atovaquone) and a drop in total secondary structure content to 53.58%. This reduction indicates that atovaquone fails to stabilize the secondary structure of the mutant protein as effectively as it does in the wild type. Such loss of stabilization could translate into weaker binding interactions and impaired ligand accommodation, consistent with the observed resistance of the mutant model to atovaquone.

Atovaquone is known to interact with His¹⁸¹ via H-bond and with Glu²⁷² through a water bridge; interactions with the two key residues are crucial for maintaining its position in the binding pocket and ensuring its inhibitory function (Kessl et al., 2003; Birth et al., 2014; Song et al., 2015). When the bulky residue Tyr²⁷⁹ gets replaced by the smaller Ser²⁷⁹, the essential interaction with His¹⁸¹ is lost, causing atovaquone to shift away from these residues, ultimately leading to a loss of activity in the mutant strain (Carrasco et al., 2013; Akhoun et al., 2014).

The initial docking studies were conducted using atovaquone, to analyse its binding modes. A similar pattern was observed in my model where atovaquone interacted with His¹⁸¹ (via H-bond) and Glu²⁷² (via water bridge), along with Tyr²⁷⁹ (via aromatic H-bonds) when docked in the wild-type model, yielding a docking score -9.98 kcal/mol (**Figure 8.5- A.1 and A.2**). In the mutant model, where Tyr²⁷⁹ was replaced with Ser²⁷⁹, atovaquone lost its key interactions with His¹⁸¹ and Glu²⁷² and shifted away from the binding pocket to the energetically more favourable site within the binding pocket (**Figure 8.5- B.1 and B.2**). This observation was further supported by the MM-GBSA calculations, where the binding energy of atovaquone in the mutant model ($\Delta G_{\text{Bind}} = -21.66$ kcal/mol) was significantly lower than in the WT model ($\Delta G_{\text{Bind}} = -12.63$ kcal/mol). The more negative MM-GBSA value for the mutant complex indicated a lower and more favourable binding energy of the ligand-protein complex.

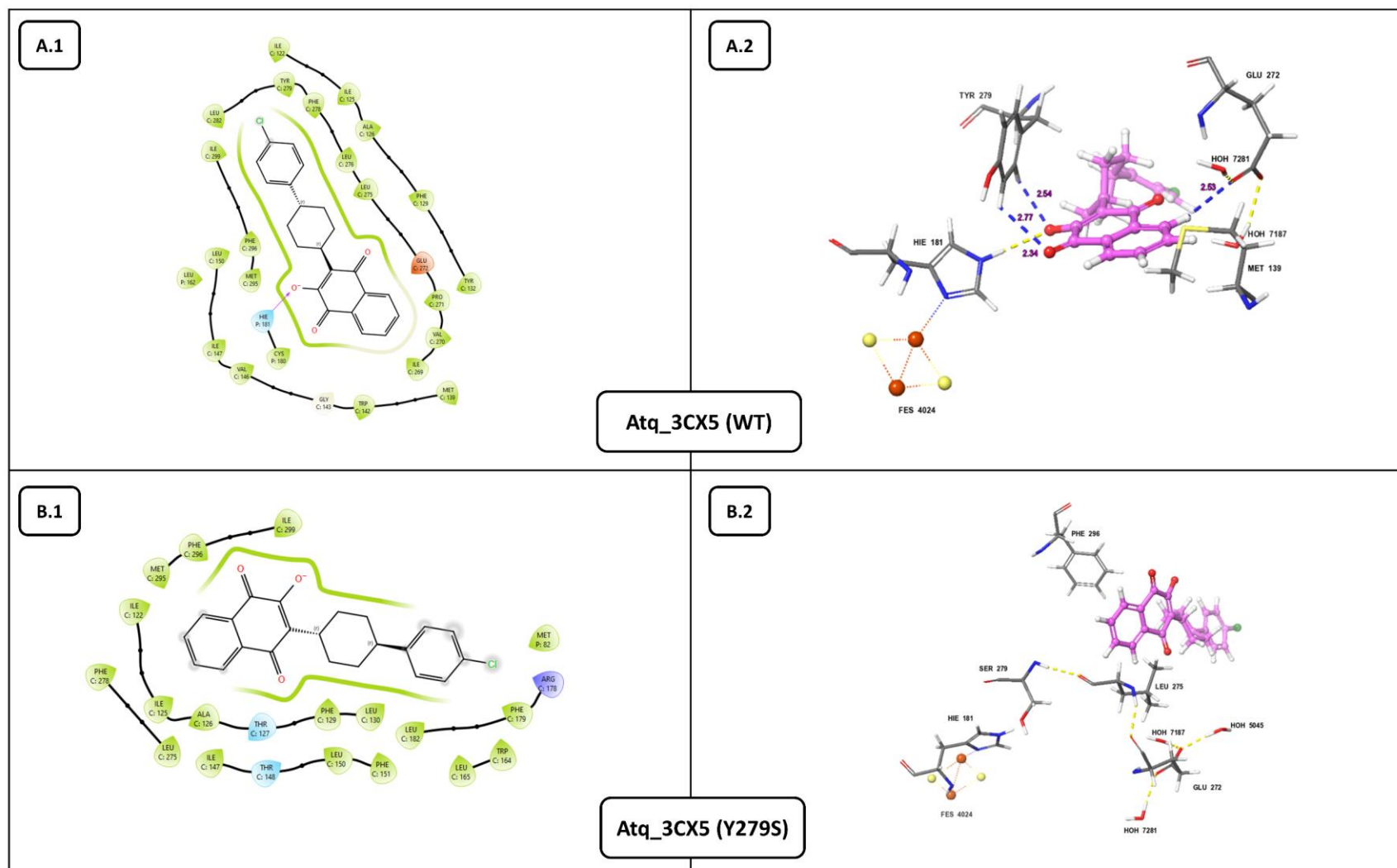
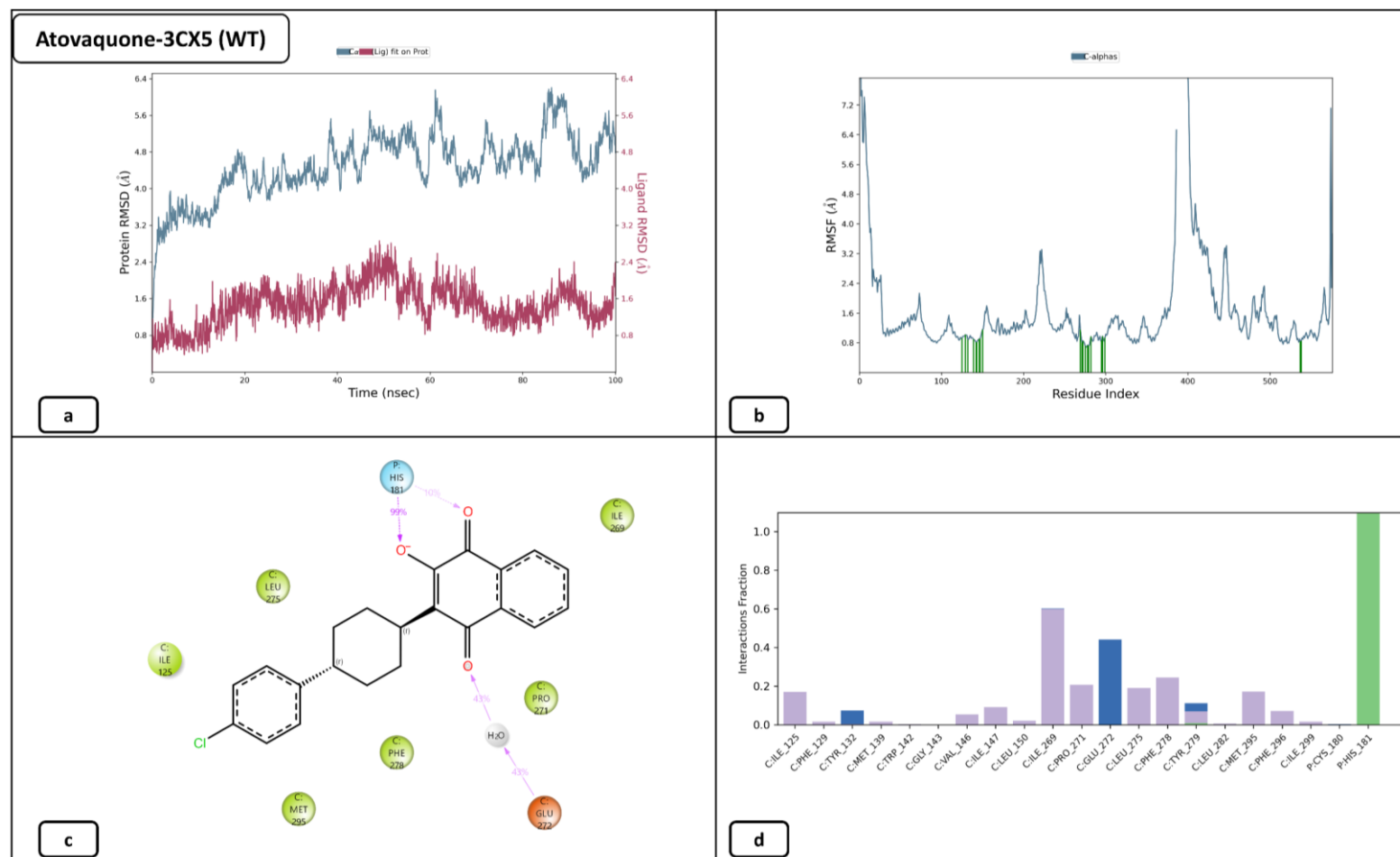


Figure 8.5: 2D and 3D representations to compare binding modes of atovaquone in the **WT (A.1 and A.2)** and **Y279S mutant (B.1 and B.2)** models.

The docking studies were followed by running a 100 ns MD simulations on atovaquone in complex with both, the WT (**Figure 8.6**) and the Y279S mutant (**Figure 8.7**) models (**Methods Section 2.2.8**). Results for the MD simulations were analysed for protein and ligand root mean square deviation (RMSD), protein root mean square fluctuations (RMSF) and ligand-protein (L-P) contacts during the 100 ns simulation time.

From the simulation interaction diagram (SID), it was observed that His¹⁸¹ acted as an H-bond donor and interacted with hydroxyl oxygen for 99% of the simulation time and with carbonyl oxygen for 10% of the simulation time. While, Glu²⁷² acted as an H-bond donor and interacted with the carbonyl oxygen of the naphthoquinone ring via a water bridge for 43% of the simulation time (**Figure 8.6 (c)**). These observations were in agreement with the interactions of atovaquone reported in literature, as these residues play a significant role and have been vital for the stabilisation of atovaquone inside the cavity, and supported that our model worked in the expected manner (Birth et al., 2014; Carrasco et al., 2013; Jacobsen et al., 2021; Kessl et al., 2003; Seddiki et al., 2008).



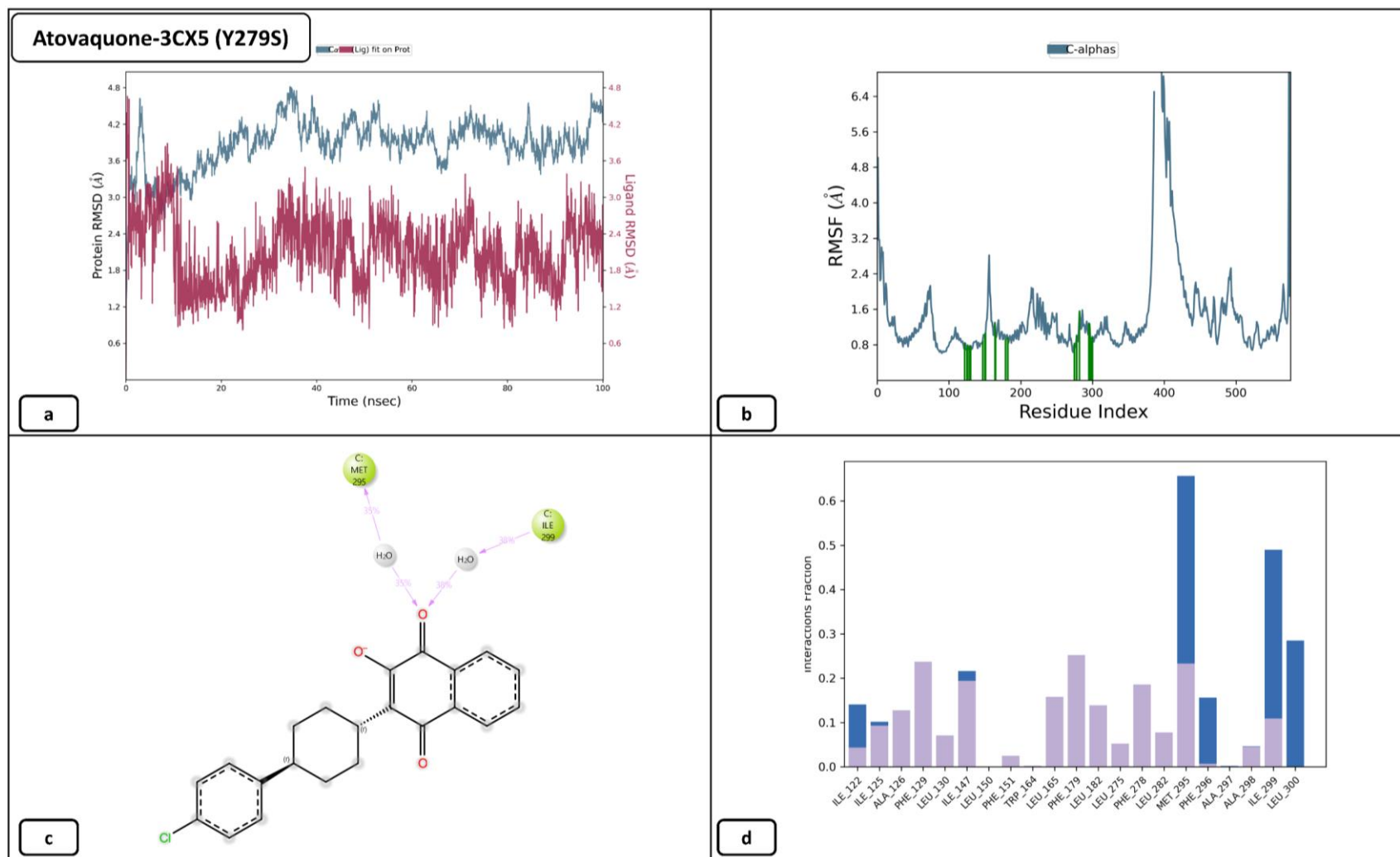


Figure 8.7: The MD simulation of atovaquone in complex with **3CX5 (Y279S)** model. **(a)** RMSD plot, **(b)** RMSF plot, **(c)** Simulation interaction diagram (SID), and **(d)** Protein-ligand contact histogram.

Similarly, when MD simulations on atovaquone were conducted using the 3CX5 (Y279S) mutant model and compared with the results obtained with the WT model, it was clearly observed that due to the mutation of Tyr²⁷⁹ to Ser²⁷⁹, there was a shift in position of atovaquone within the pocket that led to the loss of its interaction with the key residues His¹⁸¹ and Glu²⁷². This finding aligned with previously reported literature, which suggested that the Y268S mutation in *P. falciparum* reduces hydrophobic interactions with atovaquone and alters its H-bonding potential by disrupting the crucial His¹⁸¹ interaction between atovaquone and the iron-sulfur protein (ISP) (Korsinczky et al., 2000; Kessl et al., 2007).

The MD simulation studies were followed by predicting the physicochemical, pharmacokinetic and drug-likeness properties for atovaquone. The pKa value was calculated using the Instant JChem software from Chemaxon (**Method, Section 2.3.1**), followed by using online tools SwissADME (Daina et al., 2017; SwissADME) for pharmacokinetics and drug-likeness (**Methods, Section 2.3.2**) and ProTox3.0 (**Methods, Section 2.3.3**) for toxicity predictions (Drwal et al., 2014; Banerjee et al., 2024; ProTox-3.0).

The pKa values for atovaquone was predicted to be 5.25, which indicated that atovaquone is moderately acidic in nature and would mostly remain in unionised form in the acidic pH environment and cross the cell membrane to bind with the target. At mitochondrial pH value of 7.5 to 8.0, atovaquone should mostly be in the ionic form. This was supported by the literature that atovaquone interacts with the binding pocket in the ionic form where its hydroxyl oxygen gets ionised (Birth et al., 2014).

Atovaquone was also predicted to display favourable pharmacokinetic and drug-likeness properties by following Lipinski's Rule of 5 (Ro5) with high gastro intestinal (GI) absorption. It was also predicted to exhibit safe toxicity profile with being non-carcinogenic, non-immunotoxic, non-mutagenic and non-cytotoxic.

Following the *in silico* studies, *in vitro* experiments were performed to evaluate atovaquone's antimalarial activity against the ATQ-S NF54 and ATQ-R FCR3 *Plasmodium* strains (**Methods, Section 2.4**), along with its toxicity profile in the normalised human embryonic kidney epithelial (HEK293) and human erythroleukemia (K562) cell lines (**Methods, Section 2.5**). According to the results obtained it displayed antiplasmodial activity IC₅₀ values of 0.021 ± 0.011 µM (NF54) and 1.713 ± 0.014 µM (FCR3). Cell growth at 50 µM drug concentration was calculated to be 52.34 ± 7.92 % for HEK293 cell and 11.19 ± 1.75 % for K562 cell line.

The whole set of *in silico* results obtained for atovaquone, including docking scores, MMGBSA scores, binding modes and MD simulation results in the WT and Y279S mutant models, were used as reference points for comparison with other test compounds studied in the thesis to establish a ground if they had a potential to act against ATQ-R strain in the similar manner as atovaquone does in the WT strain by interacting with key residues His¹⁸¹ and Glu²⁷² resulting in inhibition of cyt *bc*₁ activity. Similarly, the *in vitro* results were also used as reference points to compare the antimalarial activity and cell viability of tested compounds. Though all these results obtained for atovaquone are not included as a separate chapter in this thesis, they are used in all chapters (**Chapter 4 to Chapter 7**) to draw comparison with the corresponding results. I plan to publish this entire set of atovaquone results (both *in silico* and *in vitro*) in future.

8.1.3 Analysis of 1,4-naphthoquinone-1,2,3-triazole hybrid compounds against atovaquone-resistant malaria, using *in vitro* and *in silico* methods

Chapter 5 discusses results for the *in vitro* and *in silico* studies performed on the set of 10 compounds belonging to 1,4-naphthoquinone-1,2,3-triazole hybrids (provided generously by Prof David D. N'Da, North West University, South Africa) (Erasmus et al., 2021). This chapter revealed that although atovaquone which itself is a naphthoquinone compound has developed resistance in *Plasmodium* parasite, there are still novel naphthoquinone hybrids that display potential to inhibit atovaquone-resistant strain. The hybrids were tested against atovaquone-resistant FCR3 *Plasmodium* strain, and exhibited antimalarial activities in the sub-micromolar range (IC₅₀ value range 0.028 µM to 0.651 µM) (**Chapter 5, Section 5.3.1, Table 5.1**). The *in vitro* results were supported by *in silico* results where the binding modes and trajectories of compounds were studied using the 100 ns MD simulation, and it was observed that compounds interacted with 3CX5 (Y279S) mutant model by forming bonds with key residues His¹⁸¹ and Glu²⁷² (**Chapter 5, Table 5.1, Figure 5.1**). Two compounds, namely **D12** and **D13** exhibiting the best potency with activities 61-fold and 52-fold, respectively, compared to atovaquone, indicating their strong potential to act against resistance. MD simulation studies conducted to understand the binding mode and the trajectory of compound within the 3CX5 (Y279S) mutant model also revealed that these two compounds maintained interaction with His¹⁸¹ and Glu²⁷² in the Y279S mutant model suggesting favourable binding behaviour and supporting the finding of the *in vitro* results (**Chapter 5, Figure 5.2 and 5.3**). Additionally, except hybrid D7, all hybrids displayed favourable pharmacokinetics and drug-likeness properties and *in silico* toxicity profiles (**Chapter 5, Table 5.2**), highlighting the importance of *in silico*

approaches in early-stage drug discovery. The insights gained from this study provided a rational basis for further structural optimisation and preclinical evaluation of these hybrids. The findings of this chapter have been published in Experimental Parasitology (Agarwal et al., 2025).

8.1.4 Evaluation of naphthyl bearing 1,3,4-thiadiazoleacetamide hybrids

In **Chapter 6**, a series of 2-(4-substituted piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide hybrids, previously reported as *P. falciparum* dihydrofolate reductase (DHFR) inhibitors, were investigated using *in silico* methods to determine their inhibitory potential against the cyt *bc*₁. Compounds were previously tested for their antimalarial activity against atovaquone-sensitive NF54 strain. To determine if cyt *bc*₁ could also be a possible target of these hybrids, *in silico* molecular modelling studies were performed to determine their binding interaction in the 3CX5 (WT) model. Here the WT model was selected so that *in silico* results could be compared with the *in vitro* results (ATQ-S NF54) and suggest if cyt *bc*₁ could be the potential target in addition to the previous reported DHFR (Pal et al., 2023). Docking results (**Chapter 6, Table 6.1**) suggested that these hybrids did not interact with the binding pocket in the expected manner. The docking scores revealed lower binding affinity towards the cyt *bc*₁ target and interactions with the binding pocket residues mostly involved π - π interactions. But since docking results are not always reliable, selective compounds (**T5, T6 and T8**, displaying antimalarial activity against NF54) were analysed further for their interaction with the cyt *bc*₁ complex using MD simulation studies against 3CX5 (WT) model. The results suggested that hybrids T6 and T8 were able to interact with His¹⁸¹ (via H-bond) for 94% and 71% of the simulation time, respectively, in a 50 ns simulation run (**Chapter 6, Figure 6.3 and 6.4**). However, when the simulation runtime was extended to 100 ns, hybrid T6 was observed to interact with His¹⁸¹ for 87% of the simulation time, and hybrid T8 interacted for only 59% of the simulation time with His¹⁸¹. On the contrary, hybrid T5, displaying the best antimalarial activity, did not interact at all with His¹⁸¹ via H-bond. There was only some π - π interaction between His¹⁸¹ and T5 (**Chapter 6, Figure 6.3 (b) and 6.4 (b)**). These observations suggested that cyt *bc*₁ complex is not the main target for the action of hybrid T5, however Pal et al. (2023) indicated that *Pf*DHFR could be the possible target of hybrids **T5, T6 and T8**. In the present study, interactions of **T6 and T8** with the binding pocket suggested cyt *bc*₁ as one of the possible targets, highlighting the possibility of dual mode of action of these hybrids. According to pharmacokinetics and drug-likeness results, hybrid **T8** violated a Lipinski's Ro5 with the molecular weight >500 g/mol and also predicted to be immunotoxic and mutagenic (**Chapter 6, Table 6.2 and 6.3**). On the other hand, hybrid T6

complied with all Lipinski's Ro5 and also predicted to be non-carcinogenic, non-immunotoxic, non-mutagenic and non-cytotoxic. The finding discussed in this chapter identified hybrid **T6** as the lead compound and showed the way for future work to take it for further exploration and perform *cyt bc₁* enzyme inhibition assay to ascertain the target for mechanism of action. Future work may also include analysing the compound against atovaquone-resistant strain using a combination of *in vitro* and *in silico* studies.

8.1.5 Designing novel compounds using computer-aided drug discovery process (CADD)

Chapter 7 focussed on designing novel compounds using the computer-aided drug designing (CADD) approach. From the literature review reported in Chapters 3 and 4, it was observed that chalcones displayed great potential as antimalarial agents against atovaquone sensitive W2 and D6 *Plasmodium* strains (**Chapter 7, Table 7.1**) (Gutteridge et al., 2006; Rodrigues et al., 2010). However, due to the lack of any data for its activity against atovaquone-resistant strains, it seemed that the scaffold was not explored for its inhibitory potential to fight resistance. Hence, it was selected as the template to design novel chalcones derivatives with potential to target ATQ-R strain, along with prediction of their pharmacokinetic and drug-like properties. Given the established significance of interactions with His¹⁸¹ and Glu²⁷², novel derivatives were designed using CADD and R-Group enumeration, aiming to enhance interactions with the binding pocket residues, primarily through hydrogen bonds and water bridges. As finding of this chapter, three ligands, namely **N-Chal-19**, **N-Chal-45** and **N-Chal-46** were identified to exhibit the highest binding affinity against the 3CX5 (Y279S) mutant model (**Chapter 7, Table 7.2**), along with satisfactory *in silico* drug-likeness and ADMET properties with no violations of the Lipinski's Ro5 (**Chapter 7, Table 7.4 and 7.5**). As shown below in **Figure 8.8**, according to the simulation interaction diagrams obtained from a 100 ns MD simulations, all three ligands (N-Chal-19, N-Chal-45 and N-Chal-46) displayed interactions with His¹⁸¹ through hydrogen bonds. Additionally, N-Chal-45 and N-Chal-46 also exhibited interaction with Glu²⁷² through water bridges, suggesting a strong rationale for synthesising and further exploring these compounds through *in vitro* analysis. The *in silico* studies conducted in Chapter 7 laid the foundation for further investigations and optimisation of the newly designed ligands, including antimalarial and cytotoxicity assays, to assess the efficiency and potential as antimalarial agents against resistant strains of *P. falciparum*.

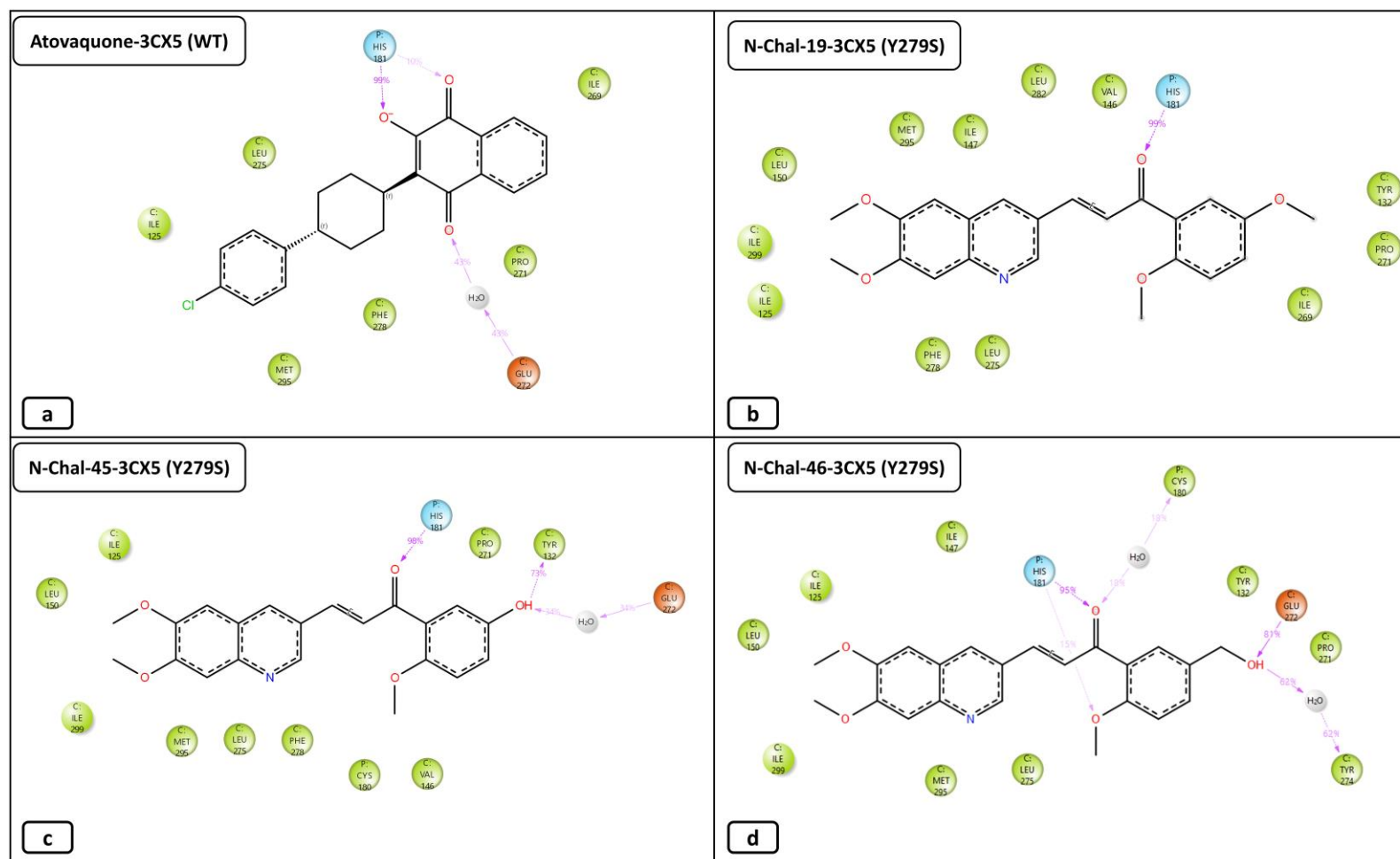


Figure 8.8: Schematic diagram showing ligand atoms interacting with the protein residues for more than 10% of the simulation for **atovaquone (a)** in complex with **3CX5 (WT)** and for **CdId-19 (b)**, **N-Chal-45 (c)**, and **N-Chal-46 (d)** in complex with **3CX5 (Y279S)** models.

8.2 Concluding remarks

Two of the 1,4-naphthoquinone-1,2,3-triazole hybrids, **D12** and **D13**, demonstrated strong activity against the atovaquone-resistant FCR3 strain. It is crucial to investigate their inhibitory effects on the *Plasmodium* cyt *bc*₁ enzyme and understand their mechanism of action. Likewise, it is important to examine hybrid **T6** for its potential as a dual-target inhibitor, specifically evaluating its antimalarial activity against atovaquone-resistant strain and inhibitory effects on the *Plasmodium* cyt *bc*₁ enzyme. Additionally, novel ligands **N-Chal-19**, **N-Chal-45** and **N-Chal-46** were identified as candidates exhibiting inhibitory potential against atovaquone-resistant model.

8.3 Limitations of the current study

Although, the study identified compounds **D12** and **D13** (**Chapter 5**) as promising candidates with inhibitory potential against atovaquone-resistant strains, the mechanism of action remains unconfirmed until further investigation into their inhibitory activity against the *Plasmodium* cyt *bc*₁ enzyme is conducted. Similarly, for compound **T6** (**Chapter 6**), that was identified as a potential candidate to be a dual inhibitor for DHFR and cyt *bc*₁ complex, determining the cyt *bc*₁ enzyme activity is essential. Further, the *in silico* findings of **Chapter 7** offer predictive insights into the binding affinity, drug-likeness, and pharmacokinetic properties of the investigated ligands. However, further *in vitro* validation is necessary to confirm their biological activity and potential as viable drug candidates.

8.4 Recommendations for future work

For future, it will be worth to investigate the cyt *bc*₁ enzyme inhibitory potential of compounds **D12**, **D13** and **T6** to ascertain their mechanism of action and to see if the *in silico* observations match with the *in vitro* potential in the real world. Regarding 2-(4-substituted piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide hybrids (**Chapter 6**), it is recommended to analyse these compounds by running MD simulations on atovaquone-resistant model, and based on the *in silico* results, conduct *in vitro* antimalarial activity against atovaquone-resistant strain, along with investigating their cyt *bc*₁ enzyme inhibitory activity. For newly designed chalcones, it is recommended to synthesize ligands **N-Chal-19**, **N-Chal-45** and **N-Chal-46** and investigate their antimalarial activity *in vitro*, and based on those results take the study forward to analyse the cyt *bc*₁ enzyme inhibitory potential.

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Chapter 9

Appendices

Appendix A: Biosafety Clearance Certificate_Protocol Number: 20190404



Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE (IBC)
(R 14/16)

CLEARANCE CERTIFICATE

PROTOCOL NUMBER: 20190404

BRIEF DESCRIPTION OF APPLICATION:

Chemotherapeutic properties of novel synthetic and natural compounds

APPLICANT: Prof RL van Zyl et al

SUPERVISOR: Pharmacy and Pharmacology

SCHOOL/DEPARTMENT : School of Therapeutic Sciences

DATE CONSIDERED: 24 February 2020

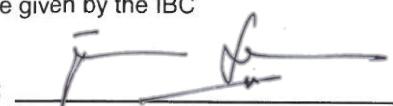
EXPIRY DATE: 24 February 2025

DECISION OF COMMITTEE: Approved unconditionally
Renewal 20090503 Dr R van Zyl

1. This clearance certificate expires on 24 February 2025 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form
4. Unreported changes to the application may invalidate the clearance given by the IBC

DATE: 13 March 2020

CHAIRPERSON:


(Professor J Larkin)

DECLARATION OF APPLICANT:

To be completed in duplicate and **one copy** returned to the Research Office, Phillip Tobias Building, Room 301, 3rd Floor, 29 Princess of Wales Terrace, Parktown, Faculty of Health Sciences, University.

1. I have read, understood and accepted the approval conditions above
2. I agree to submit a yearly progress report to the Committee and to submit an interim report on the form provided, in the event of any significant unforeseen event, e.g. suspension of a drug trial, temporary closure or relocation of my laboratory, etc
3. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
4. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at <http://web.wits.ac.za/Academic/Research/Biosafety.htm>
5. I declare (delete as appropriate) that:
 - a. I have all the approvals required by statute or regulation and by the funding agencies supporting this work, or
 - b. that I will not begin work until such approvals are obtained

Signed: _____

Date: _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendices B

Appendix B.1a: Approval Letter by Deputy Registrar_Protocol_M210121

Appendix B.1b: HREC Clearance Certificate Number_M210121

Appendix B.2: Consent Letter_Blood Donation

Appendix B.3: Patient Information Sheet

22 April 2021

Priyanka Agarwal
Student number (2286575)
PhD in Pharmacology
School of Therapeutic Sciences

TO WHOM IT MAY CONCERN

“Targeting cytochrome bc1 of Plasmodium falciparum”

This letter serves to confirm that the above project has received permission to be conducted on University premises, and/or involving staff and/or students of the University as research participants. In undertaking this research, you agree to abide by all University regulations for conducting research on campus and to respect participants' rights to withdraw from participation at any time.

If you are conducting research on certain student cohorts, year groups or courses within specific Schools and within the teaching term, permission must be sought from Heads of School or individual academics.

Ethical clearance has been obtained. Protocol number: (M210121)

Research Commencement: (As per consent obtained from the relevant HOD's)



Nicoleen Potgieter
University Deputy Registrar



R49 Ms P Agarwal

**HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M210121**

NAME: Ms P Agarwal
(Principal Investigator)

DEPARTMENT: School of Therapeutic Sciences
Department of Pharmacy and Pharmacology
Medical School
University

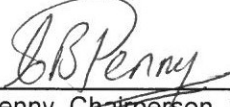
PROJECT TITLE: *Targeting cytochrome bc1 of Plasmodium falciparum*

DATE CONSIDERED: 2021/01/29

DECISION: Approved unconditionally

CONDITIONS: Previous condition satisfied on 2021/05/10

SUPERVISOR: Professor RL van Zyl

APPROVED BY: 
Dr CB Penny, Chairperson, HREC (Medical)

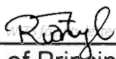
DATE OF APPROVAL: 2021/04/16

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report.** When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **January** and therefore reports and re-certification will be due in the month of **January** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).


Signature of Principal Investigator

Date

Department of Pharmacy and Pharmacology

School of Therapeutic Sciences, Faculty of Health Sciences · Private Bag 3, Wits, 2050, South Africa
Tel: +27 11 717 2542 · E-mail: Priyanka.Agarwal1@students.wits.ac.za · www.wits.ac.za

CONSENT TO DONATE BLOOD

I have discussed the above points with volunteers; it is my opinion that the participant understands the risks, benefit and obligations involved in participating in this project.

Researcher's signature**Date**

I understand that my participation is voluntary and that I may refuse to give permission or can withdraw my consent and stop taking part at any time without penalty.

I hereby freely agree to supply the necessary information such as to donate blood and understand that there is no financial benefit to volunteering.

Volunteer's signature**Date**

**Pharmacology Division / WRIM
Blood Donation Log book**

Surname	
First name	
Age	
Assigned Donor #	

To assess if you meet all the inclusion and exclusion criteria for donating blood, please complete the following two sheets each time you volunteer to donate blood.

Donor #	Donation Date	Blood type	In good health today?	Eaten in the last 4 hours?	Donated blood to blood bank	Approved to donate	Number of tubes drawn	Consent to donate blood	Senior Technician
		Y/N/Specify	Y/N	Y/N	N/Y. When	Y/N	max 8	signature	signature

Pharmacology Division / WRIM
Blood Donation Log book

To assess if you meet all the inclusion and exclusion criteria for donating blood, please complete the following two sheet each time you volunteer to donate blood.

Donor #	Females only: Are you pregnant?	Are you currently on any of the following medications or taken any within the last 2 weeks:						Anaemia/ blood disorders?	Diabetes, thyroid, epilepsy?	Cardio-vascular or lung disorders?	Other chronic illnesses?
		Antimicrobial (antibiotic, antifungal, antiparasitic, antiviral)	Coagulation drugs: aspirin, heparin, indomethacin, warfarin	Anti-epileptics	Vitamins/anti-oxidants	Natural remedies or traditional medicines	Other				
	Y/N/NA	Y/N	Y/N	Y/N	Y/N	Y/N	Y/N/Specify	Y/N	Y/N	Y/N	Y/N/Specify

Appendix C

Appendix C: HREC_Cell Line Waiver_W-CBP-201106-01

UNIVERSITY OF THE
WITWATERSRAND
JOHANNESBURG



HUMAN RESEARCH ETHICS
COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms P Agarwal
School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University
E-mail: 2286575@students.wits.ac.za

CC: Supervisor: Professor R van Zyl <Robyn.vanZyl@wits.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 06/11/2020

REF: R14/49

PROTOCOL NO: W-CBP-201106-01 (This is your ethics application study reference number.
Please quote this reference number in all correspondence relating to this
study)

PROJECT TITLE: *Targeting cytochrome bc1 of Plasmodium falciparum -
commercially-derived cell line aspects only*

Please find attached the Ethics Waiver Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/ClearScanWaiver.wps

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

06/11/2020

Ref: W-CBP-201106-01

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

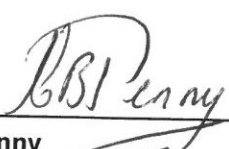
Investigator: Ms P Agarwal
Student No. (if appropriate): 2286575
Staff No. (if appropriate):

Supervisor: Professor R van Zyl

School: Therapeutic Sciences
Department: Pharmancy and Pharmacology
Medical School
University

Project title: *Targeting cytochrome bc1 of Plasmodium falciparum - commercially-derived cell line aspects only*

Reason: In vitro laboratory study.
No human participants will be involved in the study.



Dr CB Penny

Co-Chairperson: Human Research Ethics Committee (Medical)

Research Office Secretariat:
Physical address: Phillip Tobias Building, 3rd Floor, Office 302, Corner York Road and Princess of Wales Terrace, Parktown, Johannesburg 2193.
Postal address: Private Bag 3, Wits 2050
Tel Nos. +27 (0)11-717-1234/2656/2700/1252
Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za
Website: <http://www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/>

Appendix D

Appendix D: Animal Ethics Waivers

Sunday, August 30, 2020

To Professor Candy

re: ANIMAL ETHICS WAIVER FOR PHD STUDENT

I obtained animal ethics waivers for my PhD student to use *Artemia* species and *Anopheles* larvae with the following two reference numbers:

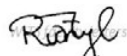
- *R van Zyl Waiver (Artemia) 07-11-2017-O*
- *R van Zyl –Anopheles Waiver 07-11-2017-O*

The following student will be using both *Artemia* and *Anopheles* in their research:

- Ms Priyanka Agarwal [2286575] – “Targeting cytochrome *bc1* of *Plasmodium falciparum*”

Please could this student use the above two animal ethics numbers for her research as she is part of the larger project I am undertaking.

Yours sincerely,



Personal Professor Robyn van Zyl

Pharmacology Division: Head and Main Supervisor
Dept of Pharmacy & Pharmacology: Deputy Head
School of Therapeutic Sciences
WITS Research Institute for Malaria (WRIM)
Pharmacology Research Director
Faculty of Health Sciences



Appendix E

Appendix E: TRREE-Module 1 (2023)_Training Certificate



Zertifikat Certificat

Certificado Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

Priyanka Agarwal

a complété avec succès - has successfully completed

Module 1 (2023) - Introduction to Research Ethics

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

Release Date: 2024/09/01

CID : G6Xd9DPt5K

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator



Programmes de formation continue (2 crédits)
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Appendices: Published Papers

Appendix F: Published Paper 1: Naphthyl bearing 1,3,4-thiadiazoleacetamides targeting the parasitic folate pathway as anti-infectious agents: *in silico*, synthesis, and biological approach

Appendix G: Published Paper 2: Synthesis, Antiprotozoal activity, Molecular Docking and ADME study of 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives

Appendix H.1: Published Paper 3: A Dual *In vitro* and *In silico* Approach to Evaluate 1,4-Naphthoquinone-1,2,3-triazole Hybrids Against Atovaquone-Resistant Malaria

Appendix H.2: Supplementary Information S1_Molecular Dynamics Simulation Report_Hybrid D-12.

Appendix H.3: Supplementary Information S2_Molecular Dynamics Simulation Report_Hybrid D-13.

Appendix H.4: Authors' declarations. Chapter 5.

RESEARCH ARTICLE

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Naphthyl bearing 1,3,4-thiadiazoleacetamides targeting the parasitic folate pathway as anti-infectious agents: *in silico*, synthesis, and biological approach†Kavita Pal,^a Sahil Lala,^{id} ^{bc} Priyanka Agarwal,^{bc} Tarosh S. Patel,^{id} ^d Jenny Legac,^e Md. Ataur Rahman,^f Saiema Ahmed,^g Nida Shahid,^h Sneha Singh,ⁱ Kajal Kumari,ⁱ Hari Madhav,^{id} ^a Abhik Sen,ⁱ Nikhat Manzoor,^g Bharat C. Dixit,^d Robyn Van Zyl,^{bc} Philip J. Rosenthal^e and Nasimul Hoda ^{id} ^{*a}

Malaria is still a complex and lethal parasitic infectious disease, despite the availability of effective antimalarial drugs. Resistance of malaria parasites to current treatments necessitates new antimalarials targeting *P. falciparum* proteins. The present study reported the design and synthesis of a series of a 2-(4-substituted piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide hybrids for the inhibition of *Plasmodium falciparum* dihydrofolate reductase (PfDHFR) using computational biology tools followed by chemical synthesis, structural characterization, and functional analysis. The synthesized compounds were evaluated for their *in vitro* antimalarial activity against CQ-sensitive PfNF54 and CQ-resistant PfW2 strain. Compounds T5 and T6 are the most active compounds having anti-plasmodial activity against PfNF54 with IC₅₀ values of 0.94 and 3.46 μM respectively. Compound T8 is the most active against the PfW2 strain having an IC₅₀ of 3.91 μM. Further, these active hybrids (T5, T6, and T8) were also evaluated for enzyme inhibition assay against PfDHFR. All the tested compounds were non-toxic against the Hek293 cell line with good selectivity indices. Hemolysis assay also showed non-toxicity of these compounds on normal uninfected human RBCs. *In silico* molecular docking studies were carried out in the binding pocket of both the wild-type and quadruple mutant Pf-DHFR-TS to gain further insights into probable modes of action of active compounds. ADME prediction and physiochemical properties support their drug-likeness. Additionally, they were screened for antileishmanial activity against *L. donovani* promastigotes to explore broader applications. Thus, this study provides molecular frameworks for developing potent antimalarials and antileishmanial agents.

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1. Introduction

Malaria, a devastating tropical infectious disease caused by *Plasmodium* parasites, remains a global health concern. Its impact depends on both the *Plasmodium* species and host immunity. The most dangerous form of malaria is caused by *Plasmodium falciparum*. Recent estimates indicate approximately 241 million malaria cases and 627 000 malaria deaths globally, surpassing the previous year's figures.¹ The malaria parasite has developed resistance to several commonly used antimalarials, such as pyrimethamine, chloroquine, and artemisinin primarily due to genetic mutations.^{2,3} However, the emergence of resistance also results in failures of artemisinin combination therapies (ACT) in different parts of the world. This resistance is associated with mutations in the Kelch13 gene, resulting in a longer duration for parasite clearance after artemisinin treatment.^{4–6} The persistence of these resistant

strains poses a significant threat to malaria control and elimination efforts. As a result, there is an urgent need for the discovery and development of new antimalarial drugs with different mechanisms of action to counter resistance and ensure effective malaria treatment.^{7,8} Recently the molecular hybridization approach has been explored in the development of new antimalarials⁹ and inhibitors that target various crucial metabolic enzymes involved in the lifecycle of *P. falciparum*. Across the list of various target enzymes involved in lifecycle of the *P. falciparum*, dihydrofolate reductase thymidylate synthase (*Pf*DHFR-TS) is the putative and one of the prevalent targets for malaria chemotherapy. It is a bifunctional enzyme and catalyst of the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, which is the precursor of the co-factors required for the synthesis of purine nucleotides, thymidylate and many amino acids.¹⁰ Thus, inhibiting the DHFR (dihydrofolate reductase) enzyme disrupts the DNA synthesis pathway resulting in the death of the rapidly multiplying cells. Pyrimethamine (PYR) and cycloguanil (CYC), the active metabolites of proguanil, are potent DHFR inhibitors and were effectively used for *P. falciparum* malaria treatment. Unfortunately, the resistance of the parasite to these drugs occurred rapidly due to point mutation, and hence, their clinical utility has been drastically affected.^{11,12}

Our research focuses on *Pf*DHFR as a target for developing novel antimalarials.^{13,14} We present naphthyl-bearing 1,3,4-thiadiazole-based compounds with potential for a new malaria treatment. These compounds were evaluated for antimalarial efficacy, employing molecular docking and enzymatic inhibition assays. All protozoan parasites, including Plasmodia and Leishmania, have a single bifunctional protein that codes for both DHFR and TS activity which is involved in the folate metabolic pathway, but they have different specific amino acid sequences and modes of interaction in the active site. In contrast, mammalian cells (as well as bacteria and yeast) have distinct genes that code for small monofunctional DHFR and TS proteins.¹⁵ Thus, considering the presence and similarity between malaria and leishmania DHFR enzyme,^{16–18} we assessed the designed compounds against the promastigote stage of *Leishmania donovani* to explore broader applications.

2. Results and discussion

2.1. Design strategy of new 2-(4-substituted piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide hybrids

Encouraged by the structural features of the different active malaria and *Pf*DHFR inhibitors, the present idea was perceived to design new hybrids as promising antimalarials targeting DHFR enzyme. Structure-based drug design and molecular hybridization methods were used to design new hybrids by incorporating the imperative bioactive structural features of the reported malaria and *Pf*DHFR inhibitors. Numerous medicinal chemistry practices, such as bioisosteric replacement, hetero atom replacement, insertion of a flexible

linker, heterocyclic or aromatic ring substitution, and alkyl chain substitution, were used to design the final hybrids as depicted in Fig. 1. We report here a series of new 2-(4-substituted piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide derivatives. Here, we connected naphthyl-bearing 1,3,4-thiadiazole pharmacophore with substituted secondary amine conjugates through a flexible acetamide linker. In medicinal chemistry, naphthalene establishes a versatile and multifaceted platform and appears as a promising moiety in drug design due to its various biological^{19–23} and clinical^{24,25} applications. The naphthyl ring formed π - π interaction with the amino acid residue of the active site^{26–29} ((I) and (II)) (Fig. 1). In our designed series of compounds, we replaced the triazole ring of compound (I) with a 1,3,4-thiadiazole ring without disturbing the position of the naphthyl ring as it is an antimalarial base scaffold for *Pf*DHFR inhibitors.^{26,30} The 1,3,4-thiadiazole-based analogues (III) and (IV) (Fig. 1) have accomplished huge therapeutic implications in the field of parasitic drug development. They are active against various parasitic diseases such as leishmania,^{31–33} trypanosoma,^{34,35} toxoplasma gondii,³⁶ and malaria.³⁷ The parasitic activities of the rigid five-membered 1,3,4-thiadiazole ring may be due to its ability to be non-covalently associated with certain domains of the polypeptide chain through two unique hydrogen bonds. Thus, the preference for selecting a 1,3,4-thiadiazole ring for the designed compounds was due to its ability to retain an anti-parasitic nature. The antimalarial activity of antifolates is suggested to be enhanced by a flexible linker, aromaticity, and hydrophobic groups (III) and (VII) (Fig. 1).^{38,39} By keeping this thought in mind, a flexible acetamide linker was introduced to the designed compounds with the aim to increase their potency for the inhibition of *Pf*DHFR enzyme. Various aromatic and hydrophobic substituents were introduced on the piperazine ring which is connected to the 1,3,4-thiadiazole ring through a flexible acetamide linker to explore the significance of various chemical groups. The piperazine ring and its derivatives enhance the hydrophilicity and antimalarial properties of the compound,^{13,40} (V) & (VI) (Fig. 1).

Thus, in the present investigation, we report the design, synthesis, and antimalarial activity of a novel series of 2-(4-substituted piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide derivatives (T1–T10). All the compounds were further evaluated for antimalarial activity against CQ-resistant W2 and CQ-sensitive NF-54 strains of *P. falciparum*, hemolytic assay against human RBC, and *in vitro* cytotoxicity assay against the normalized human embryonic kidney epithelial (HEK-293) cell line. Some active compounds (T5, T6, and T8) were also evaluated for *Pf*DHFR enzymatic inhibition assay. Also, we investigated the interaction of these hybrids in the binding site of both wild and mutant forms of *Pf*DHFR protein using molecular docking studies to determine the probable mechanism of their action. The physicochemical and ADMET properties of the best active compounds were also calculated to study their

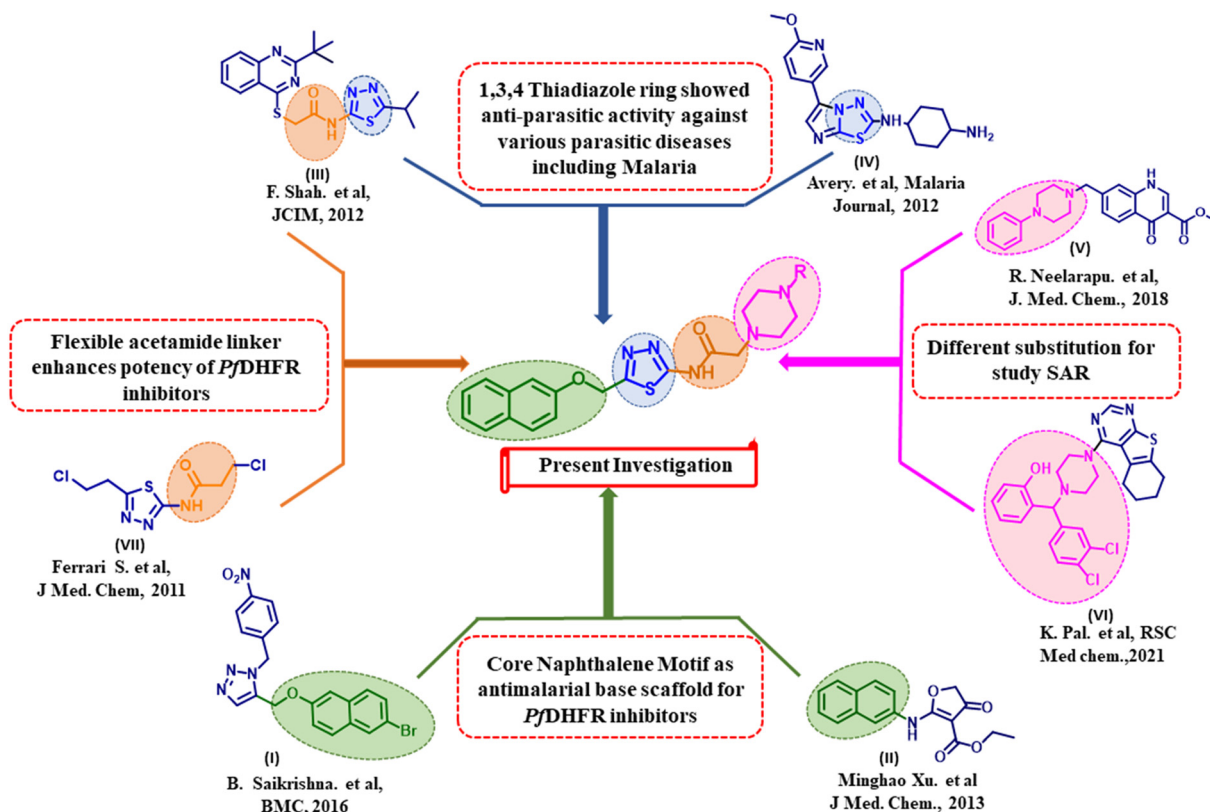


Fig. 1 Rationale for the design of naphthyl bearing 1,3,4-thiadiazole and substituted secondary amine conjugates linked through the acetamide linker.

pharmacokinetic behaviour. Additionally, considering the presence and similarity between malaria and leishmania DHFR enzyme, these compounds were also evaluated for the anti-leishmanial activity to explore their further pharmacological properties.

2.2. Chemistry

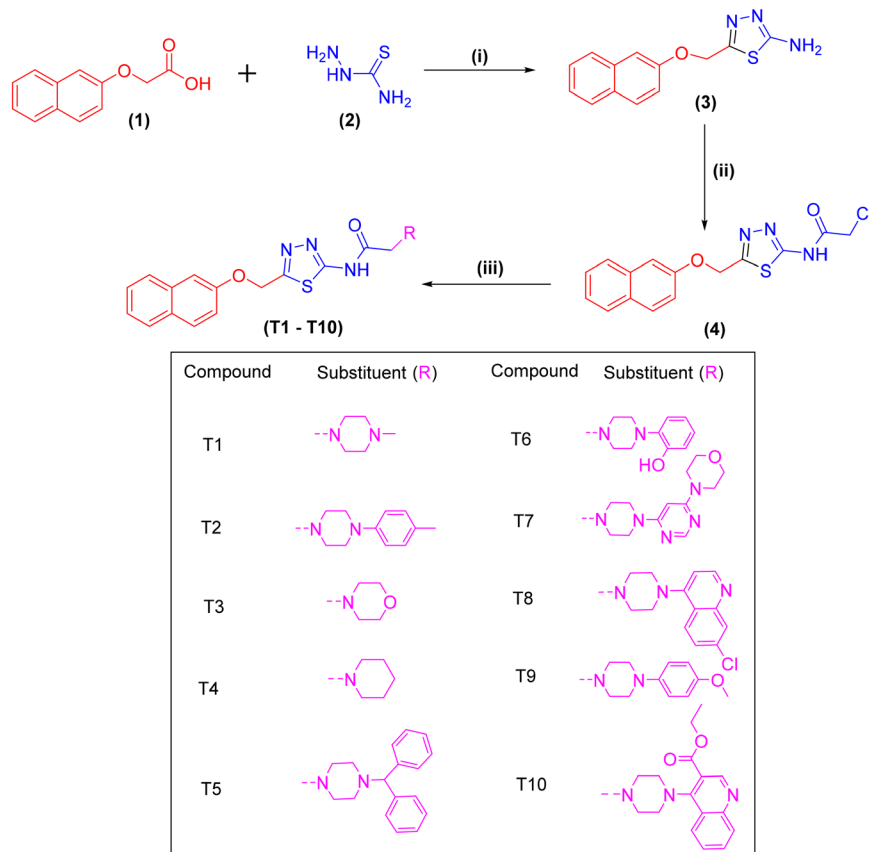
The compounds of interest were synthesized by the reaction steps outlined in Scheme 1. The intermediate 5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-amine (**3**) was prepared through a cyclization reaction between 2-naphthoxy acetic acid and thiosemicarbazide in the presence of POCl_3 . The compound (**3**) was further reacted with chloroacetyl chloride in the presence of triethylamine to obtain (5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)carbamic chloride (**4**). Finally, compound (**4**) was reacted with various substituted piperazines to obtain target compounds (**T1–T10**). All the compounds were purified by column chromatography and stable in a solid state at room temperature. All the synthesized compounds are characterized by various spectroscopic (^1H NMR, ^{13}C NMR, and mass spectra) data and were found to be in good compliance with their represented structures.

2.3. Pharmacological evaluation

2.3.1. *In vitro* antimalarial and antileishmanial evaluation of the compounds. All the synthesized compounds were

evaluated for *in vitro* antimalarial activity against both chloroquine (CQ)-sensitive (NF54) and chloroquine (CQ)-resistant (W2) strains of *P. falciparum*. The screened data (IC_{50} values) are tabulated in Table 1. The IC_{50} values presented indicate the concentration of the tested compound in $\mu\text{g mL}^{-1}$ that is necessary to inhibit 50% of the parasite growth. The displayed numbers are the average values collected from three replicate experiments.

All the compounds showed good to moderate anti-plasmodial activity against the NF54 CQ-sensitive strain of *P. falciparum*. Compounds **T5**, **T6**, and **T8** were the most active against the NF54 CQ-sensitive strain. Compound **T5** showed promising activity having an IC_{50} of $0.940 \mu\text{M}$ which was approximately 10-fold more active than compounds **T6** and **T8** having IC_{50} values of 3.466 and $3.926 \mu\text{M}$ respectively, but were significantly different from the activity of chloroquine and quinine. Compounds **T1** and **T3** displayed moderate antimalarial activity with IC_{50} values of 7.828 and $10.68 \mu\text{M}$ respectively. Compounds **T2**, **T4**, **T7**, and **T10** displayed low antimalarial activity having IC_{50} values of 63.670 , 21.480 , 61.330 , and $28.860 \mu\text{M}$ respectively. For the CQ-resistant W2 strain, compound **T8** was the most active compound having an IC_{50} of $3.919 \mu\text{M}$. The rest of the compounds showed anti-plasmodial activity with IC_{50} above $10 \mu\text{M}$. The resistance index (RI), which represents the ratio of the IC_{50} value against the CQ-resistant strain to the IC_{50} value against the CQ-sensitive strain, for compound **T8** was near the ideal value (RI



Scheme 1 Synthesis of 2-(4-substituted piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide derivatives (**T1–T10**). Reagents and conditions: (i) POCl_3 , reflux at 75°C (yield 80–90%); (ii) chloroacetyl chloride, dioxane, triethylamine, RT, 2 h (yield 70–80%); (iii) various substituted piperazine, or morpholine or piperidine and triethylamine, DMF, reflux at $70\text{--}80^\circ\text{C}$ (yield 80–90%).

Table 1 The *in vitro* antimalarial and antileishmanial activity of synthesized compounds (**T1–T10**) against CQ-sensitive (NF54) and CQ-resistant (W2) strains of *P. falciparum* and *L. donovani* respectively

Compound	<i>Pf</i> W2 strain (IC_{50}) (μM)	<i>Pf</i> NF54 strain (IC_{50}) (μM)	<i>L. donovani</i> (IC_{50}) (μM)	Resistance index ^a (RI)
T1	>10.00	7.828 ± 1.780	7.09 ± 3.54	—
T2	>10.00	63.670 ± 1.691	6.19 ± 1.85	—
T3	>10.00	10.69 ± 1.124	5.94 ± 2.53	—
T4	>10.00	21.480 ± 1.332	6.71 ± 3.52	—
T5	>10.00	0.940 ± 2.028	3.55 ± 1.82	—
T6	>10.00	3.466 ± 1.251	4.34 ± 2.25	—
T7	>10.00	61.330 ± 3.659	6.61 ± 2.62	—
T8	3.919 ± 0.594	3.926 ± 1.246	7.20 ± 4.50	0.99
T9	>10.00	>200	5.53 ± 1.12	—
T10	>10.00	28.860 ± 1.613	5.32 ± 2.30	—
Chloroquine	0.121 ± 0.003	0.012 ± 0.002	—	10.08
Quinine	—	0.108 ± 0.003	—	—
Miltefosine	—	—	0.19	—

^a Resistance index (RI) = IC_{50} (W2 strain)/ IC_{50} (NF54 strain).

$\cong 1$) and better than CQ (RI = 10.08), indicating that the **T8** hybrid was potent against both the sensitive and resistant *P. falciparum* strains. A lower RI is suggestive of a promising lead antimalarial as this demonstrates its equipotent nature regardless of the susceptibility of the strain, which further implies its utility in solving the drug resistance issue. Examination of the bioactivity data for CQ-sensitive (NF54)

disclosed interesting structure–activity relationships (SARs) among the set of tested compounds. It has been observed that the anti-plasmodial activity depends on the electron-rich or electron-deficient nature of the carbon of the substituent attached to the piperazine. Substituents having electron-deficient carbon (sp^3 hybridized carbon) attached to the piperazine ring showed better activity than those having

electron-rich carbon (sp^2 hybridized carbon). Compound **T5** exhibited the highest activity having an IC_{50} of 0.940 μM . In **T5**, the carbon of the substituent attached to piperazine was electron deficient as it is sp^3 hybridized. The further activity also depended on the steric factor of the group attached to the substituent. Compounds **T1**, **T3**, and **T4** also contained sp^3 hybridized atoms but the groups are arranged in space in such a way that they were not able to form interaction with the amino acid residue of the active site, resulting in a decrease in activity. The biphenyl groups in **T5** oriented in space in such a way that they were able to form strong interaction with the residue of the active site of the target protein and to induce inhibition. This might be the possible reason for their better activity. This result also showed the importance of the piperazine ring along with the sp^3 hybridized atom for antimalarial activity. Compounds **T6** and **T8** were the next compounds that showed good activity having IC_{50} values of 3.466 and 3.926 μM respectively. In these molecules, the carbon of the substituent attached to the piperazine ring was electron-rich, as it is sp^2 hybridized. In the **T8** molecule the carbon was more electron-rich as the carbon of the quinoline ring results in lower activity than **T6** which has less electron-rich carbon due to the presence of the $-OH$ group in adjacent carbon. Compounds **T10**, **T2**, and **T9** exhibited the lowest antimalarial activity with IC_{50} values of 28.860, 63.670, and >200 μM respectively as they also contain electron-rich sp^2 hybridized carbon. Further, their electron-rich character was enhanced by the methyl and methoxy group attached to the phenyl ring in **T2** and **T9** respectively, but the ester group attached to the quinoline ring in **T10** decreased the electron density resulting in better activity than **T2** and **T9**. Hence from the above discussion, we concluded that **T5**, **T6**, and **T8** are the best compounds which showed promising antimalarial activity and further studies can be done to discover the insight.

The synthesized compounds (**T1–T10**) were also evaluated for *in vitro* antileishmanial activity against the promastigote form of *L. donovani* using miltefosine as the standard drug and results were calculated in the form of IC_{50} which are summarized in Table 1. All the compounds showed good to

moderate antileishmanial activity but lower potential than standard drugs. Compounds **T5** and **T6** are the most promising compounds among other compounds having IC_{50} values of 3.55 and 4.34 μM . The rest of the compounds showed antileishmanial activity in the range of 5.32–7.20 μM . The most interesting feature of these compounds as revealed from the bioactivity data is that these compounds showed similar trends for antimalarial and antileishmanial activity.

2.3.2. Cytotoxicity evaluation. All the synthesized compounds were also evaluated for cytotoxicity assay against the normalized human embryonic kidney epithelial (HEK293) cell line to determine whether the mentioned activities are due to their anti-plasmodial efficacy or cytotoxicity (Table 2).

As evident, compound **T5** was the most active as an antimalarial with a low cytotoxic effect (94.4% at 50 μM) against the human kidney epithelial (HEK294) cell line, resulting in a high safety index and a high selectivity to the malaria parasite over the human cells. Similarly, compound **T6** has a safety index of 14.43 as a result of the low antimalarial activity and low cytotoxic activity. The rest of the compounds had low to moderate cytotoxicity (Table 2).

2.3.3. Hemolysis assay. To establish the biocompatibility of the investigated compounds with uninfected RBCs (red blood cells), the hemolytic activity of compounds (**T1–T10**) at varied concentrations (12.5 M to 200 M) against human erythrocytes was determined. RBCs were incubated in the presence of the tested compounds, where chloroquine was used as a positive control. It was observed that the lysis of erythrocytes has occurred. At 12.5 μM concentration, all the compounds showed cell lysis of less than 10%. **T8**, **T6**, **T5**, and **T1** are the least toxic as compared to the rest of the compounds. At the highest concentration of 200 μM , **T4** showed the least toxicity followed by **T5**, **T8**, and **T6**. Fig. 2 shows the hemolysis data of all the test compounds.

These results suggest that the anti-plasmodial action of the tested compounds is not due to RBC lysis but real inhibitory action on the *Plasmodium* parasite. The investigated compounds did not have any antagonistic effect on erythrocytes at the concentrations at which they kill the

Table 2 Cytotoxicity of compounds (**T1–T10**) against the human kidney epithelial (HEK293) cell line and their selectivity index

Samples	Antimalarial activity		Cytotoxicity – HEK293					Safety index
	IC_{50} (μM)	S.D.	% cell growth @50 μM	S.D.	IC_{50} (μM)	S.D.	Toxicity	
T1	7.83	1.78	42.75	4.82	69.25	0.14	Moderate	8.85
T2	63.67	1.69	66.40	2.71	ND		Moderate	0.79
T3	10.69	1.12	69.99	3.29	ND		Moderate	6.54
T4	21.48	1.33	86.90	4.02	ND		Low	2.33
T5	0.94	2.03	94.38	8.44	ND		Low	53.22
T6	3.47	1.25	92.90	5.34	ND		Low	14.43
T7	61.33	3.66	87.41	9.46	ND		Low	0.82
T8	3.93	1.25	43.26	1.75	70.37	1.10	Moderate	9.44
T9	>200		57.99	5.30	ND		Moderate	0.25
T10	28.86	1.61	59.76	9.80	ND		Moderate	1.73
CQ	0.012	0.002	58.52	1.50	17.21	3.75	Moderate	4166.67
QN	0.108	0.003	ND					ND

CQ: chloroquine, QN: quinine, ND: not determined.

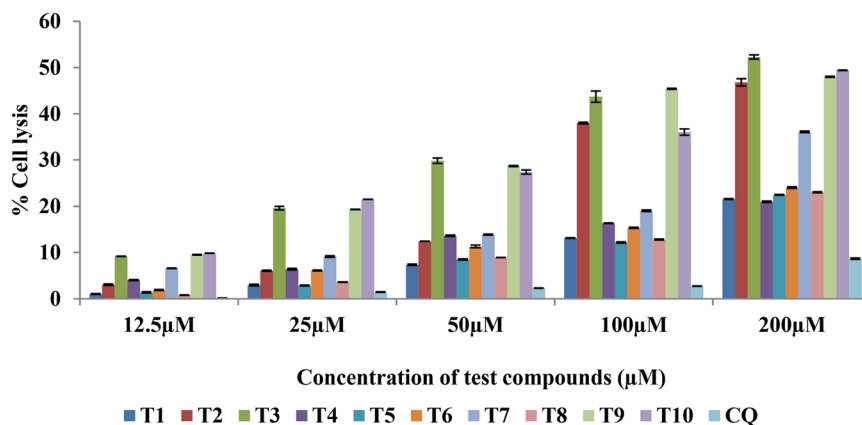


Fig. 2 Hemolysis assay of CQ and test compounds (T1–T10). Hemolysis was determined by recording absorption at 450 nm and comparing it to hemolysis achieved with 1% Triton X-100 (reference for 100% hemolysis). This data is a mean of triplicate experiments.

parasite. This study shows the non-toxicity of the antimalarial compound on normal uninfected human RBCs.

2.3.4. Enzyme inhibition assays against *Pf*-DHFR. The active scaffolds reproduced out of *in vitro* antimalarial screening were further screened for enzyme inhibitory efficacy against bovine liver DHFR enzyme which provides a higher resemblance to *Pf*-DHFR and cytotoxicity against Vero cells. As enumerated in Table 3, the active entities demonstrated a good inhibitory potency against the enzymes as compared to the standard inhibitors. The inhibitory concentrations of the compounds were obtained as IC_{50} values which indicated that among the tested molecules (T5, T6, and T8), compounds T5 and T6 were found to be the most potent inhibitors. In the cytotoxicity assay, the test molecules induced no toxicity against Vero cells even at a concentration of 1000 μ M and thus showed a nontoxic behavior. The selective index calculated was found to be more than 100 which supports the fact that utilizing the scaffolds as active antimalarial compounds inhibits dihydrofolate reductase enzyme. Hence, these results indicated that T5 and T6 having IC_{50} values of 1.8683 and 2.4945 μ M could be promising antimalarial lead compounds against the *Plasmodium falciparum* parasite.

2.4. Computational studies

2.4.1. Docking study. To gain further insights into probable modes of action of the synthesized hybrids to act as antimalarials as well as to predict molecular positioning of

the synthesized hybrids at the active site, molecular docking studies of the best active compounds were accomplished in the binding pocket of both the wild type *Pf*-DHFR-TS (PDB ID: 3QGT) and quadruple mutant *Pf*-DHFR-TS (N51I, C59R, S108 N, I164L; PDB ID: 3QG2) structures using Glide (Schrodinger, LLC, New York, NY, 2021-2). The binding energies of naphthyl bearing 1,3,4-thiadiazole hybrids toward the wild and quadruple mutant *Pf*-DHFR-TS structures were similar to the standard *Pf*-DHFR inhibitor (pyrimethamine) and the native substrate, dihydrofolate (Table 4).

To validate our docking model, we used pyrimethamine as a control ligand and docked with both target proteins along with our experimental ligands. Pyrimethamine was also available as co-crystallized in the crystal structures of wild-type DHFR (PDB: 3QGT) and mutant-type DHFR (PDB: 3QG2); therefore we superimposed the docked pose of pyrimethamine with the original position of pyrimethamine co-crystallised with wild and mutated *Pf*-DHFR and the results are shown in Fig. 3.

The results clearly demonstrated that the docked pose of pyrimethamine was significantly similar to the crystalized structure. Further, the calculated RMSD values with wild *Pf*-DHFR (3QGT) and mutated *Pf*-DHFR (3QG2) were 0.546 and 0.165, respectively which validated our docking protocol. The docked conformations of the best-scoring ligands (T5 and T6) in the binding pocket of quadruple mutant and wild-type *Pf*-DHFR-TS are illustrated in Fig. 4 and 5. Compound T5 binds strongly in the mutant *Pf*-DHFR's binding site

Table 3 *Pf*-DHFR enzyme inhibition and cytotoxicity assay of potent scaffolds^a

Compounds	<i>Pf</i> -DHFR inhibition (IC_{50} μ M)	Cytotoxicity Vero cells (IC_{50} μ M)	S.I.
T5	1.8683 $\pm$ 0.039	N.C.	535.245
T6	2.4945 $\pm$ 0.073	N.C.	400.881
T8	2.9367 $\pm$ 0.057	N.C.	340.518
PYM	0.1407 $\pm$ 0.009	N.T.	
CQ	0.0312 $\pm$ 0.007	N.T.	

^a The results indicated are average of triplicate, N.C.: no cytotoxicity observed up to a concentration of 1000 μ M, N.T.: not tested, S.I.: selective index (IC_{50} value of cytotoxicity assay/ IC_{50} value of enzyme inhibition assay).

Table 4 Glide docking energies and docking scores of active compounds, along with the reference compounds, in wild-type *Pf*-DHFR (PDB ID: 3QGT) and quadruple mutant *Pf*-DHFR (PDB ID: 3QG2)

Compounds	Docking results with wild type <i>Pf</i> -DHFR-TS		Docking results with quadruple mutant <i>Pf</i> -DHFR-TS	
	Glide energy	XP Gscore	Glide energy	XP Gscore
T1	-51.08	-6.26	-51.94	-6.78
T3	-53.52	-6.98	-58.37	-8.53
T4	-55.57	-7.50	-56.41	-7.10
T5	-66.76	-9.46	-68.76	-9.70
T6	-57.59	-8.05	-58.59	8.96
T8	-56.60	-7.80	-57.84	-7.90
Dihydrofolate	-65.15	-9.34	69.79	-10.59
Pyrimethamine	-57.70	-8.80	-59.48	-8.60

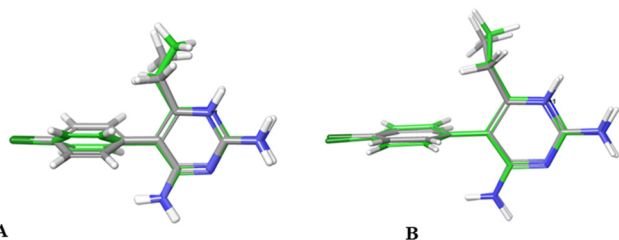
forming a major hydrogen bond interaction between the Leu164 residue of the plasmodial DHFR and the linker -NH group between the thiadiazole ring and biphenyl piperazine of **T5**. The nitrogen of the thiadiazole ring also forms a hydrogen bond with the oxygen atom of the side chain of Tyr170. Compound **T6** also binds with mutant *Pf*-DHFR's binding site by forming a hydrogen bond with Leu164.

The hydrogen bond was formed with the linker -NH group of the thiadiazole ring and 2-hydroxyphenyl piperazine of compound **T6**. Further, the aromatic phenyl ring of Phe58 contributes to forming π - π interactions with the thiadiazole ring which is important as this amino acid residue is known to cause steric hindrance to standard *Pf*-DHFR inhibitors such as pyrimethamine in the mutant enzyme. With wild-type *Pf*-DHFR, the carbonyl group of **T5** forms two hydrogen bond interactions with Tyr170 and Ala16 amino acid residues. Compound **T6** formed one hydrogen bond between the Ile164 residue of wild-type *Pf*-DHFR and the linker -NH group between the thiadiazole ring and 2-hydroxyphenyl piperazine.

Resistance against classical *Pf*-DHFR inhibitors is due to a quadruple mutation (N51I, C59R, S108N, and I164L) in the binding site of *Pf*-DHFR which, in turn, obstructs the effective binding of the folate inhibitors with the enzyme. The N51I and C59R mutations do not arouse any major structural changes in the protein's binding site but, the I164L mutation leads to a spatial shifting of the amino acid residues 164–167 and affects the cavity of the binding site which leads to the steric clashing of Phe58 with rigid

inhibitors such as pyrimethamine and cycloguanil.¹² In the current docking study, we have observed that the highest scoring ligand **T5** is at a suitable distance from Phe58, thus evading steric interaction with Phe58's aromatic side chain while with **T6**, Phe58 forms π - π interaction.

2.4.2. *In silico* physicochemical and ADMET prediction. To further check the drug ability of the synthesized hybrids, *in silico* physicochemical and pharmacokinetics ADMET predictions of the best active compounds (**T5**, **T6** and **T8**) were carried out using QikProp (Schrodinger, LLC, New York, NY, 2021-2) and the results are presented in Tables 5 and 6. The active compounds were tested for Lipinski's rule of five (Ro5), which is a set of *in silico* strategies applied to predict the probability of high oral absorption. An orally active compound must have less than 2 violations of Lipinski's rule. All the tested compounds approved the rule of five demonstrating their potential to become orally active drugs. The molecular weight of some tested compounds was higher than 500 Da, thus violating one of the Ro5. In medicinal chemistry, an effort to increase the selectivity and potency of lead compounds is by increasing molecular weight but several oral bio-available drugs exist far beyond Ro5. Physicochemical properties like size, polarity, lipophilicity, and conformational dynamics affect the oral bioavailability which can be assessed by additional *in silico* properties, the number of rotatable bonds (<10), and the polar surface area ($\leq 140 \text{ \AA}^2$). The test compounds displayed rotatable bonds of less than 10 and the polar surface area was in the tolerable range ($\leq 140 \text{ \AA}^2$) (Table 5). The prediction of ADME parameters for these compounds was also performed, and the results are presented in Table 6. Except for the QPlogHERG descriptor (prediction for HERG-K⁺ channel inhibition), all other ADME parameters were found to lie within the acceptable range. Thus, overall, these compounds were found to have significant predicted absorption, distribution, metabolism, and excretion descriptors, indicative of their expansion as orally-active lead hybrids.

**Fig. 3** Superimposed docked pose of pyrimethamine (green and blue colour) with the original position of pyrimethamine co-crystallised (grey colour) with (A) wild *Pf*DHFR (PDB: 3QGT) and (B) mutated *Pf*DHFR (PDB: 3QG2).

3. Conclusion

In summary, the present investigation unveiled a new series of naphthyl-bearing 1,3,4-thiadiazole conjugates as dual

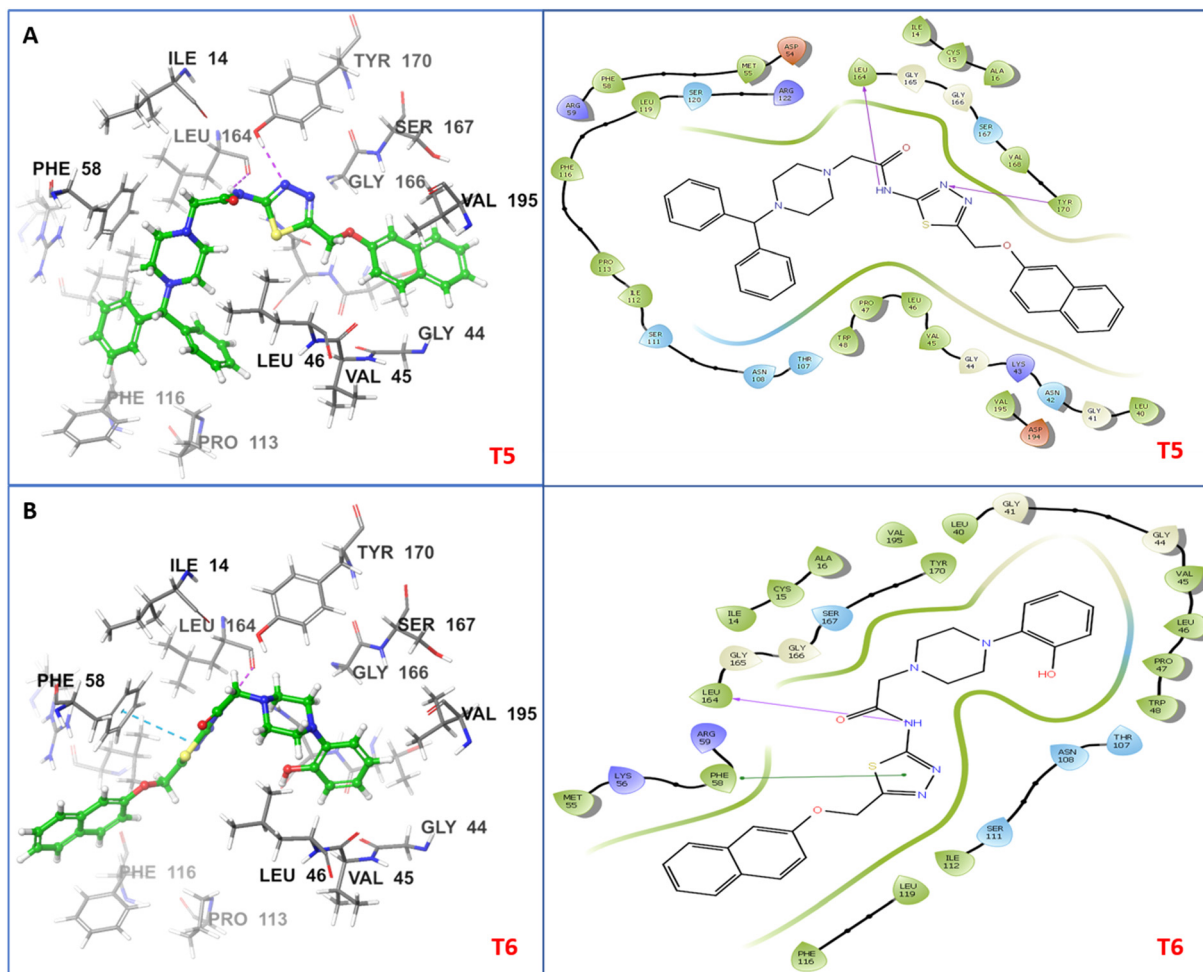


Fig. 4 3D and 2D docking poses showing the interactions of compounds **T5** (A) and **T6** (B) in the binding sites of quadruple mutant *Pf*-DHFR-TS (PDB ID: 3QG2). H-bonds and π - π interaction are indicated with pink and blue dashed lines in the 3-D view and 2-D view of binding interaction respectively.

inhibitors of malaria and leishmania for the development of anti-protozoal agents. The structure of the synthesized hybrids was validated by ^1H NMR, ^{13}C NMR, and HRMS. These compounds were evaluated against chloroquine-resistant (W2) and chloroquine-sensitive (NF54) strains of *Plasmodium falciparum* and the promastigote form of *L. donovani* to discover their antimalarial antileishmanial efficacy respectively. Compounds **T5**, **T6**, and **T8** were the most active against the NF54 CQ-sensitive strain. Compound **T5** showed promising activity having an IC_{50} of $0.940\ \mu\text{M}$ which was approximately 10-fold more active than compounds **T6** and **T8** having IC_{50} values of 3.466 and $3.926\ \mu\text{M}$ respectively, but less active than standard drugs chloroquine and quinine. For the CQ-resistant W2 strain, compound **T8** was the most active compound having an IC_{50} of $3.919\ \mu\text{M}$. The resistance index (RI) of the synthesized compounds was lower than for chloroquine (CQ), signifying that the synthesized hybrids were active against both the sensitive and resistant *P. falciparum* strains. A lower RI demonstrates the

equipotent nature of conjugates irrespective of the susceptibility of the strain. These compounds were also screened for hemolysis assay against uninfected RBCs to check their biocompatibility, suggesting that the antimalarial action of the tested compounds is not due to RBC lysis but real inhibitory action on the *Plasmodium* parasite. Further non-cytotoxicity of compounds was determined through *in vitro* cytotoxic evaluation against the mammalian (Hek293) cell line with good safety indices. The antimalarial potency was further demonstrated by the hybrids exhibiting an active inhibition of enzyme proving their efficacy as dihydrofolate reductase inhibitors and thus intruding the malarial folate pathway. The molecular docking study has been directed to predict their interaction and inhibition of *Pf*DHFR enzyme occupying the active binding pocket with great ease. These compounds were also evaluated for their antileishmanial efficacy. The calculated physicochemical and pharmacokinetic properties of the active compounds predicted good oral bioavailability. Based on the collective efforts from the structure–activity relationships, molecular

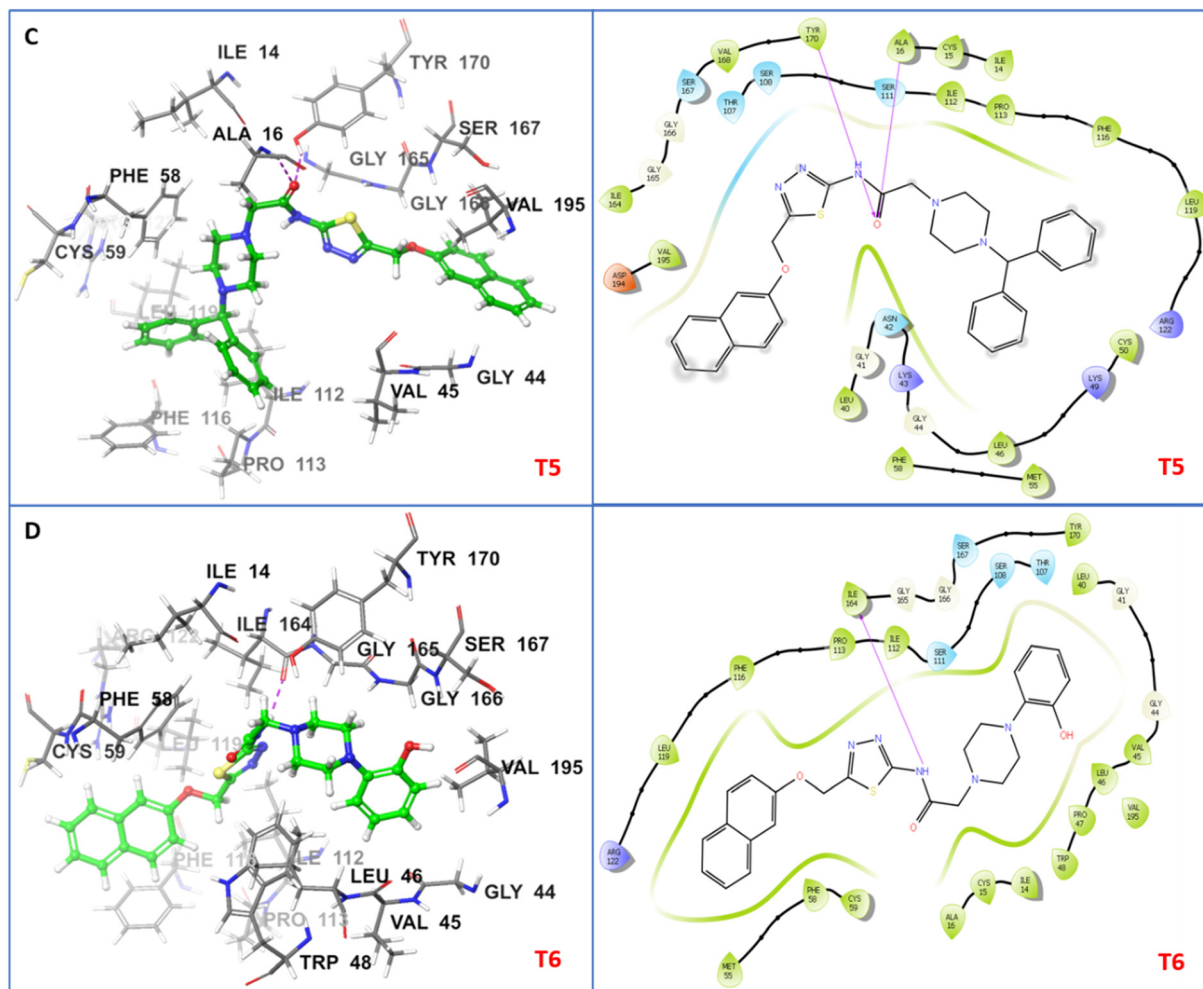


Fig. 5 3D and 2D docking poses showing the interactions of compounds **T5** (C) and **T6** (D) in the binding sites of wild-type *Pf*-DHFR-TS (PDB ID: 3QGT). H-bonds and π - π interaction are indicated with pink and blue dashed lines in the 3-D view and 2-D view of binding interaction respectively.

Table 5 *In silico* prediction of Lipinski's rule of 5 for the most active compounds (**T5**, **T6**, & **T8**)

Compounds	MW (≤ 500)	HBA (≤ 10)	HBD (≤ 5)	clog <i>P</i> (2–5)	No. of violations to rule of five
T5	549.69	6	1	3.24	1
T6	475.56	6	2	3.93	0
T8	545.06	6	1	2.72	1

MW = molecular weight; HBD = number of hydrogen donors; HBA = number of hydrogen acceptors; clog *P* = log octanol/water partition coefficient.

Table 6 *In silico* prediction of ADMET properties of most active compounds (**T5**, **T6**, & **T8**)

Compounds	% human oral absorption ($>80\%$ high, $<25\%$ poor)	QPPCaco (nm s) (<25 poor, >500 great)	QplogBB (-3.0 to 1.2)	QPPMDCK (<25 poor, >500 great)	Qplog _{K_ha} (-1.5 to 1.5)	Qplog ^{HERG} (>-5 causes concern)	PSA (7.0 – 200.0)	#rotor (0 – 15)
T5	97.49	267.00	−0.339	227.803	1.03	−10.30	80.42	9
T6	84.17	161.11	−1.13	129.86	0.49	−8.39	102.16	7
T8	70.60	162.40	−0.658	326.56	0.854	−8.81	92.82	6

docking studies on *Pf*-DHFR, anti-plasmodial assay, enzyme inhibition assay, and ADME predictions, this work will promote further studies of compounds **T5**, **T6**, and **T8**. They

have the potential for further structural modifications to optimize their properties to generate antimalarial lead entities.

4. Experimental

4.1. Chemistry

All required chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), Merck (Germany), Alfa Aesar (Massachusetts), and Spectrochem Pvt. Ltd. (India) and used as received. The progress of reactions was monitored and R_f values which were determined using analytical thin-layer chromatography (TLC) with Merck silica gel 60–120 and F254 pre-coated plates having 0.25 mm thickness. Spots on the TLC plates were visualised using ultraviolet light both at short (254 nm) and longwave (365 nm) UV light. The final products of the series were purified by flash chromatography on silica gel (mesh size 200–400). The ^1H NMR and ^{13}C NMR spectroscopic evaluations were recorded on Bruker 500 MHz and 126 MHz spectrometers with deuterated solvent. The chemical shifts (δ) are described in ppm using tetramethylsilane (TMS) as the internal standard (δ 0.00 ppm) and multiplicities of NMR signals are designated as s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), br (broad coupling), and m (multiplet, for unresolved lines). Mass spectrometric analyses (LC-HRMS) were carried out on the microTOF-Q instrument. The melting points were recorded using Tanco PLT-276 equipment and are uncorrected.

4.1.1. Procedure for the synthesis of 5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-amine (3). A mixture of 2-naphthoxy acetic acid (5 g, 24.7 mmol) and thiosemicarbazide (2.25 g, 24.7 mmol) was refluxed in phosphorous oxychloride (13 ml) for half an hour at 75 °C. After cooling down to room temperature, water was added. The reaction mixture was refluxed for 4 hours at 70–80 °C. After cooling, the reaction mixture was basified to pH 8 by the dropwise addition of 50% NaOH solution under stirring. The precipitate was filtered and recrystallized from ethanol (yield 80–90%).

4.1.2. Procedure for the synthesis of 2-chloro-*N*-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide (4). To a 250 mL flask equipped with a stir bar, 5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-amine (3) (5 g, 19.43 mmol) in 30 ml dioxane was added, followed by triethylamine (5.89 g, 58.2 mmol) and the reaction mixture was stirred at room temperature for 5 minutes. A solution of chloro acetyl chloride (2.19 g, 19.43 mmol) in dioxane (30 mL) was added dropwise to the reaction mixture over a period of 20–30 minutes at 0 °C. Then the reaction mixture was continuously stirred to reach room temperature for 1–2 h. The progress of the reaction was monitored through TLC. After completion of the reaction, the reaction mixture was poured into ice-cold water with continuous stirring. The obtained precipitate was filtered, washed with cold water (3 × 50 ml), and dried under vacuum to afford pure intermediate 5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)carbamic chloride (4) as a white solid in 70–80% yield and used as such without further purification.

4.1.3. General procedure for the synthesis of 2-(4-substituted piperazin-1-yl)-*N*-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide (T1–T10). To the stirred

solution of 5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)carbamic chloride (4) (0.4 g, 1.19 mmol) in 10 ml DMF, trimethylamine (0.18 g, 1.78 mmol) and various substituted piperazines (1.78 mmol) were added and the reaction mixture was refluxed at 70–80 °C for 5–6 h. The progress of the reaction was monitored through TLC. After completion of the reaction, the reaction mixture was cooled to room temperature and poured into ice-cooled water with continuous stirring. The precipitate formed was filtered and washed 2–3 times with water and dried under a vacuum to obtain a solid compound. These crude products were purified by column chromatography using ethyl acetate/hexane as a mobile phase to obtain pure compounds (T1–T10) in 80–90% yield.

2-(4-Methylpiperazin-1-yl)-*N*-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide (T1). White solid, yield 85.0%; m.p. 160–164 °C; ^1H NMR (500 MHz, CDCl_3) δ 7.70–7.66 (m, 3H), 7.40–7.36 (m, 1H), 7.31–7.27 (m, 1H), 7.19 (t, J = 1.5 Hz, 1H), 7.15 (dd, J = 9.0, 2.5 Hz, 1H), 5.49 (s, 2H), 3.22 (s, 2H), 2.61 (s, 4H), 2.48 (s, 4H), 2.27 (s, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 168.61, 162.55, 159.17, 155.40, 134.22, 129.86, 129.48, 127.69, 127.01, 126.63, 124.26, 118.19, 107.09, 64.88, 60.73, 54.81, 53.44, 45.82. Mass in MeOH for $\text{C}_{20}\text{H}_{23}\text{N}_5\text{O}_2\text{S}$ (Mol. wt = 397.4970 g mol $^{-1}$) (ESI, m/z): observed $[\text{M} + \text{H}]^+$: 398.1655; calculated $[\text{M} + \text{H}]^+$: 398.1645.

***N*-(5-((Naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)-2-(4-(*p*-tolyl)piperazin-1-yl)acetamide (T2).** White solid, yield 85.0%; m.p. 160–164 °C; ^1H NMR (500 MHz, CDCl_3) δ 7.70–7.66 (m, 3H), 7.40–7.35 (m, 1H), 7.31–7.27 (m, 1H), 7.20–7.18 (m, 1H), 7.15 (dd, J = 9.0, 2.6 Hz, 1H), 7.03 (d, J = 8.0 Hz, 2H), 6.80 (d, J = 8.0 Hz, 2H), 5.49 (s, 2H), 3.32 (s, 2H), 3.17 (t, J = 4.9 Hz, 4H), 2.75 (s, 4H), 2.21 (s, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 168.33, 162.61, 159.18, 155.40, 134.23, 129.87, 129.82, 129.49, 127.70, 127.02, 126.64, 124.28, 118.60, 116.97, 107.64, 64.88, 60.79, 53.60, 49.94, 20.48. Mass in MeOH for $\text{C}_{26}\text{H}_{27}\text{N}_5\text{O}_2\text{S}$ (Mol. wt = 473.1885 g mol $^{-1}$) (ESI, m/z): observed $[\text{M} + \text{H}]^+$: 474.1969; calculated $[\text{M} + \text{H}]^+$: 474.1958.

2-Morpholino-*N*-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide (T3). White solid, yield 90.0%; m.p. 170–174 °C; ^1H NMR (500 MHz, CDCl_3) δ 7.70–7.66 (m, 3H), 7.40–7.35 (m, 1H), 7.31–7.27 (m, 1H), 7.20–7.18 (m, 1H), 7.15 (dd, J = 9.0, 2.6 Hz, 1H), 7.03 (d, J = 8.0 Hz, 2H), 6.80 (d, J = 8.0 Hz, 2H), 5.49 (s, 2H), 3.32 (s, 2H), 3.17 (t, J = 4.9 Hz, 4H), 2.75 (s, 4H), 2.21 (s, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 168.33, 162.61, 159.18, 155.40, 134.23, 129.87, 129.82, 129.49, 127.70, 127.02, 126.64, 124.28, 118.60, 116.97, 107.64, 64.88, 60.79, 53.60, 49.94, 20.49. NMR (500 MHz, CDCl_3) δ 10.51 (s, 1H), 7.71–7.66 (m, 3H), 7.39–7.36 (m, 1H), 7.31–7.28 (m, 1H), 7.19 (m, 1H), 7.15 (dd, J = 9.0, 2.5 Hz, 1H), 5.49 (s, 2H), 3.71 (t, J = 4.5 Hz, 4H), 3.22 (s, 2H), 2.55 (t, J = 4.5 Hz, 4H). ^{13}C NMR (126 MHz, CDCl_3) δ 168.25, 162.63, 159.08, 155.39, 134.22, 129.87, 129.48, 127.69, 127.01, 126.64, 124.28, 118.59, 107.63, 66.75, 64.88, 61.35, 53.88. Mass in MeOH for $\text{C}_{19}\text{H}_{20}\text{N}_4\text{O}_3\text{S}$ (Mol. wt = 384.4540 g mol $^{-1}$) (ESI, m/z): observed $[\text{M} + \text{H}]^+$: 385.1386; calculated $[\text{M} + \text{H}]^+$: 385.1329.

***N*-(5-((Naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)-2-(piperidin-1-yl)acetamide (T4).** White solid, yield 95.0%; m.p.

176–180 °C; ^1H NMR (500 MHz, CDCl_3) δ 7.70–7.65 (m, 3H), 7.39–7.35 (m, 1H), 7.29–7.27 (m, 1H), 7.19 (d, J = 2.5 Hz, 1H), 7.14 (dd, J = 9.0, 2.5 Hz, 0H), 5.48 (s, 2H), 3.17 (s, 2H), 2.48 (t, J = 5.0 Hz, 4H), 1.59 (p, J = 5.7 Hz, 4H), 1.41 (m, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ 169.15, 162.41, 159.22, 155.43, 134.24, 129.84, 129.47, 127.68, 127.02, 126.61, 124.24, 118.61, 107.63, 64.91, 61.51, 55.08, 25.88, 23.37. Mass in MeOH for $\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_2\text{S}$ (Mol. wt = 382.4820 g mol $^{-1}$) (ESI, m/z): observed $[\text{M} + \text{H}]^+$: 383.1548; calculated $[\text{M} + \text{H}]^+$: 398.1536.

2-(4-Benzhydrylpiperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide (T5). White solid, yield 93.0%; m.p. 182–184 °C; ^1H NMR (500 MHz, CDCl_3) δ 7.70–7.64 (m, 3H), 7.38–7.26 (m, 6H), 7.22–7.17 (m, 5H), 7.14–7.09 (m, 3H), 5.47 (s, 2H), 4.20 (s, 1H), 3.20 (s, 2H), 2.56 (s, 4H), 2.40 (s, 4H). ^{13}C NMR (126 MHz, CDCl_3) δ 168.77, 162.48, 159.13, 155.42, 142.29, 134.23, 129.86, 129.48, 128.60, 127.92, 127.69, 127.13, 127.01, 126.63, 124.26, 118.60, 107.62, 75.98, 64.90, 60.74, 53.96, 51.70. Mass in MeOH for $\text{C}_{32}\text{H}_{31}\text{N}_5\text{O}_2\text{S}$ (Mol. wt = 549.6930 g mol $^{-1}$) (ESI, m/z): observed $[\text{M} + \text{H}]^+$: 550.2267; calculated $[\text{M} + \text{H}]^+$: 550.2271.

2-(4-(2-Hydroxyphenyl)piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide (T6). White solid, yield 92.0%; m.p. 152–154 °C; ^1H NMR (500 MHz, CDCl_3) δ 7.70–7.65 (m, 3H), 7.40–7.36 (m, 1H), 7.31–7.27 (m, 1H), 7.19–7.12 (m, 4H), 7.05–7.01 (m, 1H), 6.89–6.87 (m, 1H), 6.84–6.81 (m, 1H), 5.49 (s, 2H), 3.39 (s, 2H), 2.94 (s, 4H), 2.80 (s, 4H). ^{13}C NMR (126 MHz, CDCl_3) δ 161.66, 158.18, 154.34, 150.32, 137.12, 133.18, 128.85, 128.45, 126.66, 125.97, 125.62, 123.26, 120.47, 119.31, 117.54, 112.00, 106.13, 63.82, 59.68, 53.24, 51.20. Mass in MeOH for $\text{C}_{25}\text{H}_{25}\text{N}_5\text{O}_3\text{S}$ (Mol. wt = 475.5670 g mol $^{-1}$) (ESI, m/z): observed $[\text{M} + \text{H}]^+$: 476.1766; calculated $[\text{M} + \text{H}]^+$: 476.1751.

2-(4-(6-Morpholinopyrimidin-4-yl)piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide (T7). White solid, yield 90.0%; m.p. 158–160 °C; ^1H NMR (500 MHz, CDCl_3) δ 8.22 (s, 1H), 7.72–7.66 (m, 3H), 7.39 (t, J = 7.5 Hz, 1H), 7.30 (t, J = 7.5 Hz, 1H), 7.19 (s, 1H), 7.15 (d, J = 9.0 Hz, 2H), 5.50 (s, 2H), 3.72–3.64 (m, 8H), 3.52 (s, 4H), 3.29 (s, 2H), 2.65 (s, 4H). ^{13}C NMR (126 MHz, CDCl_3) δ 175.43, 155.57, 134.41, 131.54, 129.90, 127.85, 127.00, 126.67, 124.43, 118.57, 107.63, 102.41, 95.20, 86.61. Mass in MeOH for $\text{C}_{27}\text{H}_{30}\text{N}_8\text{O}_3\text{S}$ (Mol. wt = 546.6500 g mol $^{-1}$) (ESI, m/z): observed $[\text{M} + \text{H}]^+$: 547.2282; calculated $[\text{M} + \text{H}]^+$: 547.2234.

2-(4-(7-Chloroquinolin-4-yl)piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide (T8). White solid, yield 87.0%; m.p. 167–169 °C; ^1H NMR (500 MHz, CDCl_3) δ 8.68 (d, J = 5.1 Hz, 1H), 8.03 (d, J = 2.0 Hz, 1H), 7.84 (d, J = 9.0 Hz, 1H), 7.71–7.66 (m, 3H), 7.40–7.35 (m, 2H), 7.31–7.27 (m, 1H), 7.19 (d, J = 3.5 Hz, 1H), 7.15 (dd, J = 9.0, 2.6 Hz, 1H), 6.81 (d, J = 5.2 Hz, 1H), 5.50 (s, 2H), 3.39 (s, 2H), 3.28 (t, J = 4.5 Hz, 4H), 2.87 (t, J = 5.0 Hz, 4H). ^{13}C NMR (126 MHz, CDCl_3) δ 167.09, 161.64, 157.70, 155.78, 154.34, 149.69, 134.50, 133.18, 128.86, 128.45, 127.43, 126.67, 125.96, 125.61, 123.91, 123.28, 120.56, 117.53, 108.04, 106.59, 63.83, 59.85, 52.48, 50.96. Mass in MeOH for $\text{C}_{28}\text{H}_{25}\text{ClN}_6\text{O}_2\text{S}$ (Mol. wt = 544.1448 g mol $^{-1}$) (ESI, m/z): observed $[\text{M} + \text{H}]^+$: 545.1507; calculated $[\text{M} + \text{H}]^+$: 545.1521.

2-(4-(4-Methoxyphenyl)piperazin-1-yl)-N-(5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)acetamide (T9). White solid, yield 80.0%; m.p. 165–168 °C; ^1H NMR (500 MHz, CDCl_3) δ 7.70–7.66 (m, 3H), 7.40–7.35 (m, 1H), 7.31–7.27 (m, 1H), 7.20–7.18 (m, 1H), 7.15 (dd, J = 9.0, 2.6 Hz, 1H), 7.03 (d, J = 8.0 Hz, 2H), 6.80 (d, J = 8.0 Hz, 2H), 5.49 (s, 2H), 3.83 (s, 3H), 3.32 (s, 2H), 3.17 (t, J = 4.9 Hz, 4H), 2.75 (s, 4H). ^{13}C NMR (126 MHz, CDCl_3) δ 168.33, 162.61, 159.18, 155.40, 134.23, 129.87, 129.82, 129.49, 127.70, 127.02, 126.64, 124.28, 118.60, 116.97, 107.64, 64.88, 60.79, 55.30, 53.60, 49.94. Mass in MeOH for $\text{C}_{26}\text{H}_{27}\text{N}_5\text{O}_3\text{S}$ (Mol. wt = 489.5940 g mol $^{-1}$) (ESI, m/z): observed $[\text{M} + \text{H}]^+$: 490.1912; calculated $[\text{M} + \text{H}]^+$: 490.1907.

Ethyl 4-(4-(2-((5-((naphthalen-2-yloxy)methyl)-1,3,4-thiadiazol-2-yl)amino)-2-oxoethyl)piperazin-1-yl)quinoline-3-carboxylate (T10). White solid, yield 95.0%; m.p. 175–178 °C; ^1H NMR (500 MHz, CDCl_3) δ 8.86 (s, 1H), 8.10–8.02 (m, 1H), 7.70–7.66 (m, 4H), 7.51–7.48 (s, 1H), 7.38–7.35 (m, 1H), 7.30–7.27 (m, 1H), 7.21–7.19 (m, 3H), 7.15 (dd, J = 8.9, 2.6 Hz, 1H), 5.50 (s, 2H), 4.43 (q, J = 7.5 Hz, 2H), 3.39 (s, 2H), 2.85 (t, J = 5.0 Hz, 8H), 1.40 (t, J = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 168.35, 166.94, 162.64, 159.21, 155.95, 155.39, 150.54, 149.51, 134.22, 131.00, 129.87, 129.67, 129.48, 127.69, 127.00, 126.79, 126.65, 124.29, 124.22, 118.58, 107.64, 64.88, 62.04, 61.17, 54.14, 51.35, 14.88. Mass in MeOH for $\text{C}_{31}\text{H}_{30}\text{N}_6\text{O}_4\text{S}$ (Mol. wt = 582.6790 g mol $^{-1}$) (ESI, m/z): observed $[\text{M} + \text{H}]^+$: 583.2131; calculated $[\text{M} + \text{H}]^+$: 583.2122.

5. Pharmacological evaluation

5.1. *In vitro* anti-plasmodial activity

The CQ-sensitive strain NF-54 and CQ-resistant strain W2 of *P. falciparum* were maintained continuously *in vitro* in supplemented RPMI-1640 culture media at 37 °C and gassed with a mixture of 5% CO_2 , 3% O_2 , and 92% N_2 .⁴¹ 5% D-sorbitol was used to synchronize the culture in the ring stage.⁴² To evaluate the antimalarial activity of the hybrids, the synchronized ring-stage parasites were adjusted to a final parasitemia of 2 percent and 2% hematocrit, to which serial dilutions of the compounds and positive control, quinine, were added after 24 hours of incubation. Uninfected erythrocytes and drug-free parasitized erythrocytes are used as negative control. Following 48 hours of incubation period, the plates were frozen at –70 °C for 1 hour and thawed for another hour. The lysate (25 ml) was transferred to a second plate, to which 100 ml Malstat™ and 20 ml nitro blue tetrazolium–phenazine thiosulphate (1:1) mixture were added to each well and incubated for 40 min at 37 °C to compute the parasite lactate dehydrogenase (pLDH) activity.⁴³ Thereafter, 5% acetic acid was added to each well, and the absorbance of the formazan products formed a record at 620 nm, as an indicator of parasite viability. The percentage of parasite growth, taking the appropriate controls into account, was calculated and used to calculate the concentration required to inhibit parasite growth by 50% (IC_{50} value) from

log sigmoid dose–response curves using the GraphPad Prism® 5.0.0 software. Each experiment was repeated in triplicate.⁴⁴

5.2. Cytotoxicity assay

The normalized human embryonic kidney epithelial (HEK-293) and human erythroleukemia (K562) cell lines were preserved at 37 °C in a humidified environment with 5% CO₂ as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.⁴⁴ A cell suspension (10 000 cells per well) was incubated at 37 °C for 48 h with serial dilutions of compounds/positive control. A final concentration of less than 1% DMSO did not affect the viability of the cells. Thereafter, 40 µl of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; 5 mg ml⁻¹ in phosphate buffer saline (pH 7.3)) was added to each well and incubated for a further 4 hours. DMSO was used to dissolve the formazan crystals and then quantified spectrophotometrically at an absorbance of 540 nm with a reference wavelength of 690 nm.⁴⁵ Percent cellular viability was determined using the appropriate controls and used to calculate the IC₅₀ values which were compared to the positive control, cisplatin. The experiment was repeated in triplicate.

5.3. Hemolysis assay

The toxicity of compounds to host cells was determined by the *in vitro* hemolytic assay as described earlier.⁴⁶ RBCs from a healthy individual were collected in tubes containing EDTA, an anticoagulant. The erythrocytes were harvested by centrifugation for 10 min at 2000 rpm at 20 °C and washed three times in PBS. To the pellet, PBS was added to yield a 10% (v/v) erythrocytes/PBS suspension. The 10% suspension was then diluted 1/10 in PBS. From each suspension, 100 µl was taken in triplicate and added to Eppendorf tubes. Complete hemolysis was achieved with 1% Triton X-100. Tubes were incubated for 1 h at 37 °C and then centrifuged for 10 min at 2000 rpm and 20 °C. Supernatant absorbance was measured spectrophotometrically at 450 nm.⁴⁷ The hemolysis percentage was calculated using the following equation:

% Hemolysis

$$= \frac{[(A_{450} \text{ of test compound treated sample} - A_{450} \text{ of buffer treated sample}) / (A_{450} \text{ of 1\% Triton X-100 treated samples} - A_{450} \text{ of buffer treated sample})] \times 100}$$

5.4. Enzyme inhibition assays against Pf-DHFR

The enzyme inhibition strength was ascertained using bovine liver DHFR as a model enzyme which renders a higher resemblance to Pf-DHFR.^{48,49} The mixture used for assay contained 50 µM Tris-HCl buffer (pH 7.4), 50 µM NADPH, 20 µL DMSO or the same volume of DMSO solution containing the test compounds to a final concentration of 10⁻¹¹–10⁻⁵ M, and 0.02 units of bovine liver DHFR, in a final volume of 2.0 ml. The mixture was incubated at room temperature for 2.0

min after the addition of the enzyme and further addition of 25 µM FH2 initiated the reaction; the change in absorbance (ΔA/min) was measured at 340 nm. The activity under these conditions was linear for 10 min, and each duplicate experiment was repeated three times.^{50,51} Results are reported as % inhibition of enzymatic activity calculated using the following formula:

$$\% \text{ Inhibition} = 100$$

The % inhibition values were plotted *versus* drug concentration (log scale). The 50% inhibitory concentration (IC₅₀) of each compound was obtained using the Graph Pad Prism program, version 3 (San Diego, CA).

5.5. *In vitro* antileishmanial assay against promastigote stage forms of *L. donovani*

Promastigote stage forms of *L. donovani* were cultured in M199 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) at 22.5 °C. Cultures were allowed to reach the metacyclic phase (5–6 days post inoculation) as determined by the stationary behavior of parasite number and rosette formation. Parasites at different time points were counted in a Neubauer haemocytometer in a solution of 5% Giemsa and 2% formaldehyde in 0.14 M NaCl, under light microscopy. To evaluate the 50% inhibitory concentration (IC₅₀), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used with modifications. Compounds were dissolved in DMSO and then diluted with a medium to achieve the required concentrations. Parasites were diluted in complete medium to 5 × 10⁶ parasites per mL and 100 µL of the suspension was seeded into each well of 96 well plates. The plates were incubated at 25 °C. Several serial dilutions of each drug were added to the culture in 96 wells of microplates. Miltefosine was used as the reference anti-leishmanial drug. Following incubation for 24 hours, 20 µL of MTT solution (5 mg mL⁻¹) was added to each well over 3–4 hours. The plates were then centrifuged, the supernatants were discarded and the resulting pellets were dissolved in 100 µL of lysing buffer consisting of 20% (w/v) of a solution of SDS (Sigma) and 40% of *N,N*-dimethylformamide (Merck) in water. The absorbance was measured spectrophotometrically at 550 nm and a reference wavelength of 650 nm. The results are expressed as IC₅₀; each IC₅₀ value is the mean ± standard deviation of at least three separate experiments performed in duplicate.

6. Protocol for computational studies

6.1. Docking study

The 2D structures of the target compounds were generated using Chem Bio Draw Ultra 12.0 (<https://www.cambridgesoft.com>) and the energy-minimized 3D structures of test compounds were generated through the Ligprep module in Schrodinger. Partial atomic charges were computed using the OPLS_2005 force field. The correct Lewis structure for each of these ligands was

generated and optimized with default settings (Ligprep 2.5, Schrodinger, LLC, New York, NY, 2021-2). The 3D crystal structures of wild-type *Pf*DHFR-TS (PDB ID: 3QGT; resolution 2.30 Å) and quadruple mutant (N51I + C59R + S108N + I164L) *Pf*DHFR-TS (PDB ID: 3QG2; resolution: 2.30 Å) were recovered from the protein data bank (<https://www.rcsb.org>). The proteins were ready for docking using the Protein Preparation Wizard (Maestro 12.8 Schrodinger, LLC, New York, NY, 2021-2). The water molecules within 5 Å of the protein structures were taken into account. The bond order and formal charges were allotted and hydrogen atoms were added to the enzyme crystal structure. The position of co-crystallized ligand pyrimethamine in both wild and mutant protein structures was used to select the center and size of the receptor grid, which was produced using Glide (Schrodinger, LLC, New York, NY, 2021-2) with default settings for all properties. The grid size was preferred to be sufficiently large to comprise all active site residues engaged in substrate binding. The cofactor NADH in the *Pf*DHFR-TS wild and mutant structures was also taken as part of the receptor protein. All compound conformers were docked to each of the receptor grid files (*Pf*DHFR-TS wild and mutant structures) using Glide extra precision (XP)-mode. For the refinement and scoring, default settings were used.

6.2. *In silico* physicochemical and ADME prediction

All the ligands were exposed to energy minimization and geometry optimization using the MOPAC2016 module. These structures of the target compounds were used to predict the pharmacokinetic and drug-likeness parameters by Schrodinger suit's QikProp module (Schrodinger, LLC, New York, NY, 2021-2). The program QikProp employs the method of Jorgensen⁵² to compute pharmacokinetic properties and descriptors such as octanol/water partitioning coefficient, aqueous solubility, brain/blood partition coefficient, intestinal wall permeability, plasma protein binding, and others.

Abbreviations used

<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
SAR	Structure-activity relationship
NMR	Nuclear magnetic resonance
ADMET	Absorption, distribution, metabolism, excretion, and toxicity
CQ	Chloroquine
ACT	Artemisinin-based combination therapy
<i>Pf</i> DHFR	<i>Plasmodium falciparum</i> dihydrofolate reductase
POCl ₃	Phosphorous oxychloride
HEK293	Human embryonic kidney epithelial cell lines
rt	Room temperature
m.p.	Melting point
TLC	Thin layer chromatography
ppm	Parts per million
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium

Conflicts of interest

There are no conflicts of interest to declare.

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Research Paper

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Synthesis, Antiprotozoal activity, Molecular Docking and ADME study of 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives

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Abstract

A series of chemotherapeutics comprising 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives (**4a -4i**) was synthesized, characterized and evaluated for antiprotozoal activity. These compounds were screened for the anti-amoebic potential for which HM1:IMSS strain of *Entamoeba histolytica* was taken and metronidazole (MTZ) was used as reference amoebicidal drug. Among the nine dihydropyrimidine-phenylacetamide derivatives, five were recorded with better anti-amoebic activity i.e. lower IC₅₀ (0.12 µM to 0.82 µM) values than MTZ (IC₅₀ = 1.8 µM). All the nine compounds displayed minimal antimalarial activity with more than 43% inhibition, with a low toxicity profile against three cell lines; human cell lines, Artemia and host red blood cell, but these compounds were more toxic than quinine. These compounds did not exhibit any significant inhibition against the viability of *Anopheles arabiensis* (KGB) larvae or ova. Docking study provided good binding affinity when interacted with the enzyme *O*-acetyl-L-serine sulfohydrolase (*EhOASS*) of *E. histolytica* (PDB id: 3BM5). These studies demonstrated the dihydropyrimidin-acetamide derivatives to be better anti-amoebic agents.

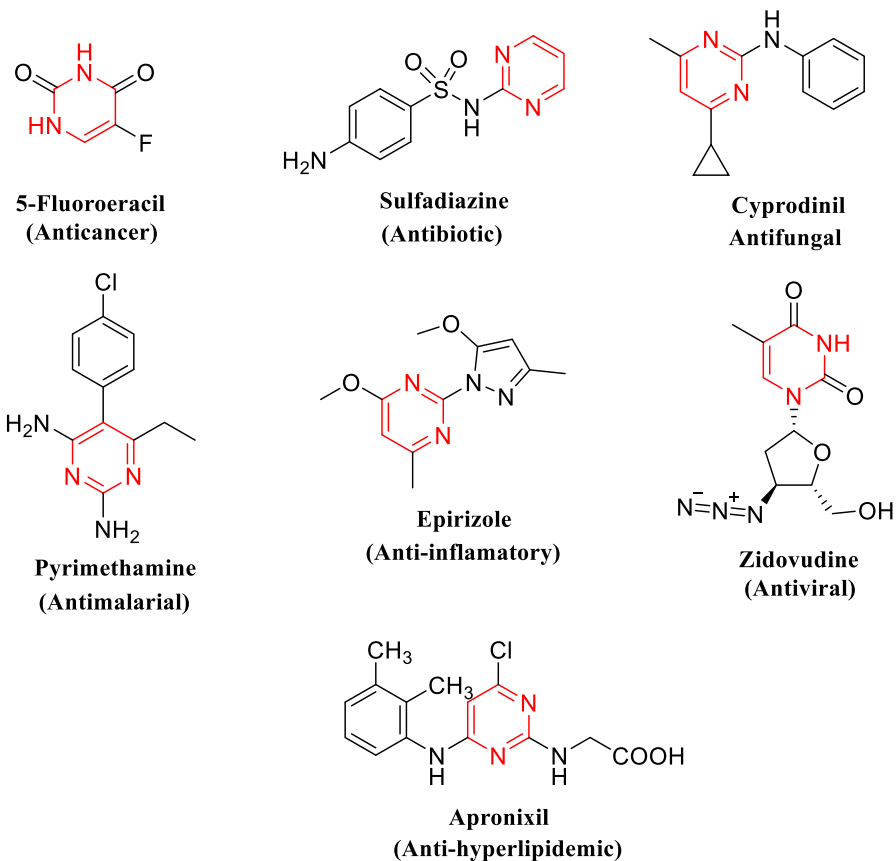
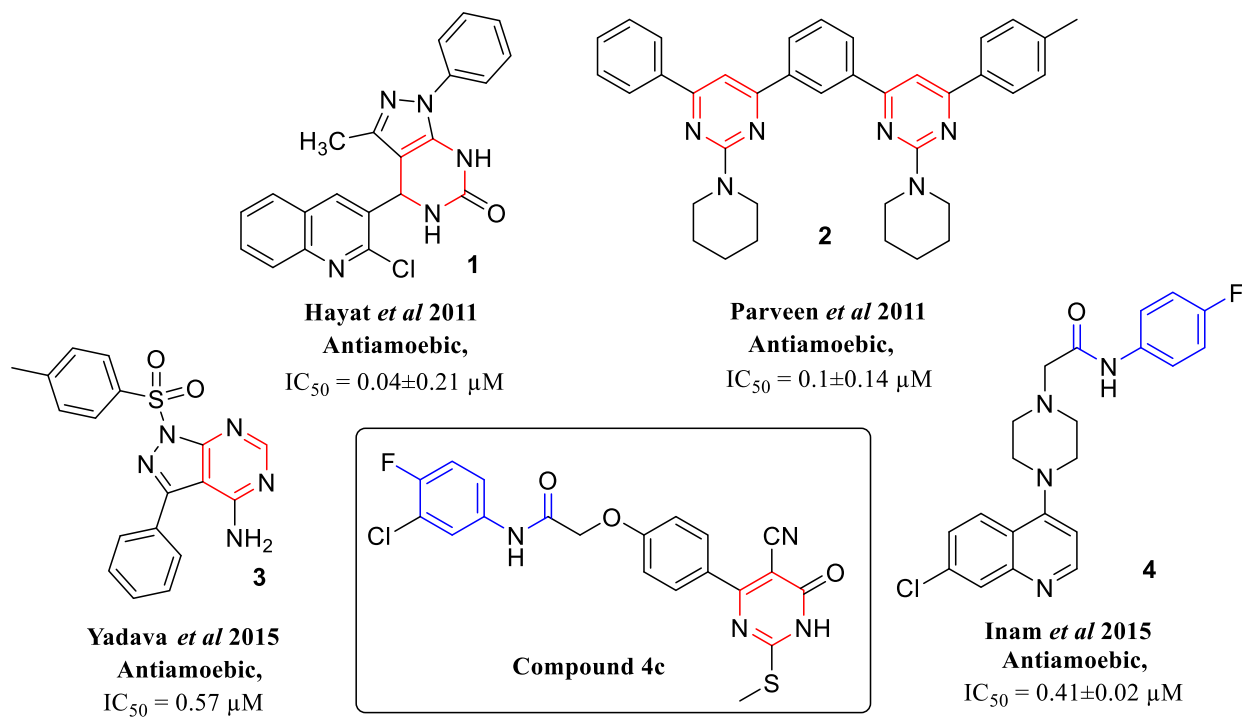
Keywords; Dihydropyrimidine; anti-amoebic, *Entamoeba histolytica*, antimalarial; cytotoxicity.

1. Introduction

Parasitic protozoan infections affect more than 500 million individuals in the world that is huge proportion of the world's population.[1] The widespread distribution of protozoan diseases poses significant challenges for public health. Factors such as poverty, lack of access to clean water, inadequate healthcare facilities, and climate change exacerbate the prevalence and impact of these infections. The two major examples of protozoan diseases are malaria and amoebiasis that have significant impact on global health.[2] Amoebiasis disease is caused by *Entamoeba histolytica* that continues to be a common but major infection of the human gastro-intestinal tract. The transmission mode of *E. histolytica* is carried out by the fecal-oral route, typically through the ingestion of cysts while consuming contaminated food or water.[3] The cysts are highly resistant and can survive harsh environmental conditions, allowing the parasite to spread easily in areas with poor sanitation. It primarily infects the large intestine, where it adheres to and invades the mucosal lining. The infection can lead to ulcer formation in the colon, resulting in ulcerative colitis. Symptoms may include abdominal pain, diarrhea, and the presence of blood and mucus in the stool. In severe cases, the infection can cause fulminant colitis, which is life-threatening and may require surgical intervention, but it can disseminate to other organs through the hematogenous (blood) route. The most common extraintestinal site of infection is the liver, where the parasite can cause an amoebic liver abscess.[4] The worldwide distribution of this infection is recorded about 50 million and deaths tolls about 110000 every year.[5] This infection is recorded high in Sub Saharan Africa (SSA), Sudan, Cote D'Ivoire, Nigeria, Egypt and South Africa and in some developing countries as India, Mexico, and Japan.[6] On the other hand, malaria, being the life-threatening disease, is also a parasitic infection. The causative agent *Plasmodium* species, especially *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*, are causing malaria in human.[7] This infection was recorded with 249 million new clinical cases and approximately 6.08 lakhs deaths worldwide in 2022 according to WHO recent data.[8] Heterocyclic motifs such as nitroimidazoles and chloroquinolines are core moieties, having potential against amoebiasis and malaria, respectively.[9] It was a major setback in the effective control of these disease when parasites showed resistance against their first line drugs.[10] Metronidazole, being the main drug for the treatment of ameobiasis[11], found to be carcinogenic to human and having serious side effect such as neurological alterations and cardiac rhythm impairment.[12] According to literature survey, cross resistance also developed towards

metronidazole.[13] Chloroquine (CQ) also lost its efficiency as a first-line anti-malarial drug as a result of resistant parasites.[14] Thus, the lack of an effective drug is complicating the treatment and threatening the global health. Therefore, it is a requirement to investigate newer compounds for the development of drugs with improved biological activity and lesser toxicity. Considering the need for a novel therapeutic agent to treat protozoal disease, we had developed a series of pharmacophores containing pyrimidine as a core moiety.

Pyrimidines, being an essential constituent of all cells and thus of all living matter, are also pharmaceutically important heterocycles having nitrogen atoms at 1 and 3 positions in the ring.[15] Pyrimidine have played an important role in medicinal chemistry due to their variegated biological activities including anti-viral, antibacterial, antifungal, antioxidant, anticancer, antimalarial, analgesic and anti-inflammatory and antihypertensive activity.[16] Here are some pyrimidine-based scaffolds that are available in market and used in clinical treatment. 5-Fluorouracil is an oral drug to treat cancer disease.[17] While sulfadiazine is used to treat bacterial infections.[18] Flucytosine (5-FC) is an antifungal drug having pyrimidine analogue that interferes with DNA and RNA synthesis in fungal cells.[19] Particularly, the pyrimidine ring, as a core moiety can be found in pyrimethamine, a well-known anti-malarial and anti-toxoplasmosis drug.[20] Zidovudine (AZT) is pyrimidine nucleus analogue that is prescribed to prevent HIV/AIDS.[21] Epirizole[22], a pyrimidine-pyrazole hybrid, and Aronixil[23] are used to reduce inflammation and high lipid levels respectively. **(Fig-1)** Previously, some other pyrimidine-based derivatives were also reported by researchers for their anti-amoebic activity that possessed good potential. Parveen *et al* synthesized a new series of 2,4,6-trisubstituted bis-pyrimidines as anti-amoebic ranging their IC₅₀ values from 0.10 to 9.57 μ M.[24] Whilst, Hayat *et al* reported a series of 10 compounds, pyrazolo[3,4-d]pyrimidine-6-one derivatives.[25] Yadava *et al* synthesized pyrazolo[3,4-d]pyrimidine derivatives possessing good potential as anti-amoebic agent.[26] **(Fig-2)**

**Fig-1:** Clinically available drugs containing pyrimidine motif**Fig-2:** Structural similarity of dihydropyrimidine-phenylacetamide derivative **4c**

2. Materials and methods

2.1. Experimental protocol

All necessary chemicals were obtained from Merck and Aldrich Chemical Company (USA). Thin-layer chromatography (TLC) was performed using precoated aluminum sheets (Silica gel 60 F254, Merck, Germany), and spots were visualized under UV light. Melting points were determined using a Veego REC-22038 A2 instrument and were uncorrected. Elemental analyses were conducted on an Elementar Vario analyzer, with results within $\pm 0.4\%$ of theoretical values. IR spectra were recorded on a Bruker FT-IR spectrophotometer. ^1H NMR spectra was obtained on Bruker Spectrospin DPX spectrometers at 300 MHz using DMSO- d_6 as a solvent and TMS as the internal standard. Chemical shifts are reported in ppm with splitting patterns noted as s (singlet), d (doublet), t (triplet), and m (multiplet). Mass spectra were acquired using ESI-MS (AB-Sciex 2000, Applied Biosystems).

General procedure for the synthesis of chloroacetamide derivatives (2a-2i)

Chloroacetamide derivatives were synthesized by a method reported in the literature.[27]

Synthesis of aldehyde intermediates (3a-3i)

The aldehyde intermediates which contained 2-(4-formyl-phenoxy)-*N*-substituted-phenyl acetamides were synthesized by a reported method.[28]

Synthesis of 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives (4a-4i)

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-*N*-phenylacetamide derivatives were synthesized by a reported method.[29]

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide (4a)

Yield 81.3 %; mp: 290°C; FT-IR ν_{max} (cm^{-1}): 3330 (NH), 2212 (C=N), 1666 (C=O); ^1H NMR (300 MHz, DMSO- d_6) δ (ppm): 10.30(s, 1H, NH), 7.84 (d, 1H, $J=7.8$ Hz, Ar-H), 7.80(d, 2H, $J=8.4$ Hz, Ar-H), 7.65(d, 2H, $J=7.8$ Hz, Ar-H), 7.39-7.23(m, 2H, Ar-H), 7.08(d, 2H, $J=7.5$ Hz, Ar-H), 6.97(s, 1H, NH), 4.86 (s, 2H, CH_2), 2.34(s, 3H, CH_3); Anal. Calc. (%) for $\text{C}_{20}\text{H}_{16}\text{N}_4\text{O}_3\text{S}$: C, 61.21; H, 4.11; N, 14.28; O, 12.23; S, 8.17; found: C, 61.20; H, 4.10; N, 14.29; O, 12.24; S, 8.16; ESI-MS: $m/z = 393$ (M+1).

N-(3-chlorophenyl)-2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)acetamide (4b):

Yield 79.3 %; mp: 275°C; FT-IR $\nu_{\max}$ (cm⁻¹): 3335 (NH), 2214 (C=N), 1660(C=O); ¹H NMR (300 MHz, DMSO-*d*₆) δ (ppm): 10.33(s, 1H, NH), 7.85 (d, 3H, *J*=9.0 Hz, Ar-H), 7.58(d,1H, *J*=7.8 Hz, Ar-H), 7.38-7.31(m, 2H, Ar-H), 7.24(d, 2H, *J*=8.4 Hz, Ar-H), 6.95(s, 1H, NH), 4.78 (s,2H,CH₂), 2.35(s,3H,CH₃); Anal. Calc. (%) for C₂₀H₁₅ClN₄O₃S : C, 56.27; H, 3.54; Cl, 8.31; N, 13.12; O, 11.24; S, 7.51; found: C, 56.26; H, 3.53; Cl, 8.32; N, 13.11; O, 11.25; S, 7.52; ESI-MS: *m/z* = 428 (M+1).

***N*-(3-chloro-4-fluorophenyl)-2-(4-(5-cyano-2-(methylthio-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)acetamide (4c)**

Yield 82.5 %; mp: 287°C; FT-IR $\nu_{\max}$ (cm⁻¹): 3310 (NH), 2215(C=N), 1655(C=O); ¹H NMR (300 MHz, DMSO-*d*₆) δ (ppm): 10.37(s, 1H, NH), 7.77 (d, 1H, *J*=8.7 Hz, Ar-H), 7.47 (d, 1H, *J*=8.1 Hz, Ar-H), 7.35 (t, 1H, *J*=7.8 Hz, Ar-H), 7.30-7.22 (m, 2H, Ar-H), 7.07 (d, 2H, *J*=7.8 Hz, Ar-H), 6.97 (s, 1H, NH), 4.81 (s,2H,CH₂), 2.34 (s,3H,CH₃); Anal. Calc. (%) for C₂₀H₁₄ClFN₄O₃S : C, 54.00; H, 3.17; Cl, 7.97; F, 4.27; N, 12.59; O, 10.79; S, 7.21; found: C, 53.91; H, 3.16; Cl, 7.98; F, 4.25; N, 12.58; O, 10.77; S, 7.22; ESI-MS: *m/z* = 446 (M+1).

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-(2,6-dimethylphenyl)acetamide (4d): Yield 79%; mp: 278°C; FT-IR $\nu_{\max}$ (cm⁻¹): 3330 (NH), 2212 (C=N), 1668(C=O); ¹H NMR (300 MHz, 300 MHz, DMSO-*d*₆) δ (ppm): 10.14(s, 1H, NH), 7.84 (d, 1H, *J*=9.0 Hz, Ar-H), 7.64 (d, 1H, *J*=7.8 Hz, Ar-H), 7.41-7.28 (m, 1H, Ar-H), 7.20 (d, 2H, *J*=7.8 Hz, Ar-H), 7.08 (d, 2H, *J*=8.1 Hz, Ar-H), 6.95 (s, 1H, NH), 4.80 (s, 2H, CH₂), 2.35 (s, 3H, CH₃), 2.06 (s, 6H, CH₃); Anal. Calc. (%) for C₂₂H₂₀N₄O₃S: C, 62.84; H, 4.79; N, 13.32; O, 11.41; S, 7.63; found: C, 62.85; H, 4.78; N, 13.31; O, 11.42; S, 7.64; ESI-MS: *m/z* = 421 (M+1).

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-4-fluorophenyl)acetamide (4e):

Yield 86.1 %; mp: 265°C; FT-IR $\nu_{\max}$ (cm⁻¹): 3347 (NH), 2216 (C=N), 1664(C=O); ¹H NMR (300 MHz, DMSO-*d*₆) δ (ppm): 10.39 (s, 1H, NH), 7.88 (d, 2H, *J*=8.7 Hz, Ar-H), 7.74 (d, 2H, *J*=8.7 Hz, Ar-H), 7.52 (d, 2H, *J*=9.0 Hz, Ar-H), 7.07 (d, 2H, *J*=8.7 Hz, Ar-H), 6.88 (s, 1H, NH), 4.77 (s, 2H, CH₂) , 2.50 (s, 3H, CH₃); Anal. Calc. (%) for C₂₀H₁₅FN₄O₃S: C, 58.53; H, 3.68; F, 4.63; N, 13.65; O, 11.69; S, 7.81; found: C, 58.52; H, 3.69; F, 4.62; N, 13.64; O, 11.68; S, 7.82; ESI-MS: *m/z* = 411 (M+1).

2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-(naphthalen-1-yl)acetamide (4f):

Yield 86.3 %; mp: 295°C; FT-IR $\nu_{\max}$ (cm⁻¹): 3320 (NH), 2212 (C=N), 1668(C=O); ¹H NMR (300 MHz, DMSO-*d*₆) δ (ppm): 10.19 (s, 1H, NH), 7.94 (d, 2H, *J*=7.8 Hz, Ar-H), 7.48 (t, 2H, *J*=8.1 Hz, Ar-H), 7.74 (d, 2H, *J*=7.2 Hz, Ar-H), 7.64 (d, 2H, *J*=7.2 Hz, Ar-H), 7.54-7.49 (m, 1H, Ar-H), 7.49 (d, 2H, *J*=8.4 Hz, Ar-H), 6.53 (s, 1H, NH), 4.91 (s, 2H, CH₂), 2.50 (s, 3H, CH₃); Anal. Calc. (%) for C₂₄H₁₈N₄O₃S: C, 65.14; H, 4.10; N, 12.66; O, 10.85; S, 7.25; found: C, 65.15; H, 4.11; N, 12.65; O, 10.84; S, 7.26; ESI-MS: *m/z* = 443 (M+1)

***N*-(4-acetylphenyl)-2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-**

yl)phenoxy)acetamide (4g): Yield 84.5 %; mp: 298°C; FT-IR $\nu_{\max}$ (cm⁻¹): 3343 (NH), 2212 (C=N), 1660(C=O); ¹H NMR (300 MHz, DMSO-*d*₆) δ (ppm): 10.36 (s, 1H, NH), 8.28 (m, 1H, Ar-H), 7.93-7.83 (m, 2H, Ar-H), 7.71 (d, 1H, *J*=6.9 Hz, Ar-H), 7.25 (d, 2H, *J*=7.5 Hz, Ar-H), 7.16-7.09 (m, 2H, Ar-H), 6.55 (s, 1H, NH), 4.80 (s, 2H, CH₂), 2.56 (s, 3H, CH₃), 2.50 (s, 3H, CH₃); Anal. Calc. (%) for C₂₂H₁₈N₄O₄S: C, 60.82; H, 4.18; N, 12.90; O, 14.73; S, 7.38; found: C, 60.81; H, 4.19; N, 12.91; O, 14.72; S, 7.39; ESI-MS: *m/z* = 435 (M+1).

***N*-(4-bromophenyl)-2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-**

yl)phenoxy)acetamide (4h):

Yield 82.6 %; mp: 274°C; FT-IR $\nu_{\max}$ (cm⁻¹): 3310 (NH), 2212 (C=N), 1658(C=O); ¹H NMR (300 MHz, DMSO-*d*₆) δ (ppm): 10.40 (s, 1H, NH), 7.79 (d, 2H, *J*=8.7 Hz, Ar-H), 7.65 (d, 2H, *J*=8.7 Hz, Ar-H), 7.52 (d, 2H, *J*=9.0 Hz, Ar-H), 7.07 (d, 2H, *J*=8.7 Hz, Ar-H), 6.88 (s, 1H, NH), 4.77 (s, 2H, CH₂), 2.50 (s, 3H, CH₃); Anal. Calc. (%) for C₂₀H₁₅BrN₄O₃S: C, 50.97; H, 3.21; Br, 16.95; N, 11.89; O, 10.18; S, 6.80; found: C, 50.98; H, 3.20; Br, 16.96; N, 11.88; O, 10.17; S, 6.81; ESI-MS: *m/z* = 472 (M+1).

***2*-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-*N*-*p*-tolylacetamide (4i):**

Yield 88.1 %; mp: 292°C; FT-IR $\nu_{\max}$ (cm⁻¹): 3350 (NH), 2218 (C=N), 1655(C=O); ¹H NMR (300 MHz, DMSO-*d*₆) δ (ppm): 10.05 (s, 1H, NH), 7.80 (d, 2H, *J*=8.4 Hz, Ar-H), 7.48-7.41 (m, 2H, Ar-H), 7.20 (d, 2H, *J*=7.8 Hz, Ar-H), 7.08 (d, 2H, *J*=8.7 Hz, Ar-H), 6.96 (s, 1H, NH), 4.74 (s, 2H, CH₂), 2.76 (s, 3H, CH₃), 2.28 (s, 3H, CH₃); Anal. Calc. (%) for C₂₁H₁₈N₄O₃S: C, 62.05; H, 4.46; N, 13.78; O, 11.81; S, 7.89; found: C, 62.04; H, 4.45; N, 13.79; O, 11.82; S, 7.88; ESI-MS: *m/z* = 407 (M+1).

2.2. Biological Evaluation

2.2.1. Anti-amoebic activity

Examination of *in vitro* anti-amoebic activity of all dihydropyrimidine-phenylacetamide derivatives (**4a** -**4i**) against HM1:IMSS strain of *E. histolytica* was performed using the microdilution method, a standard procedure for assessing the efficacy of potential antimicrobial agents.[30] For per ml in Dimethyl sulfoxide (DMSO) 1 mg of each compounds was used to prepared the fresh stock solutions to ensure stability and effectiveness during the testing process.[31] For each experiment, a 96-well microtiter plate was used, which included several controls: Metronidazole (positive control), a well-established anti-amoebic drug, was used as the standard reference to compare the efficacy of the test compounds, negative control Included the parasite (*E. histolytica*) in culture medium without any treatment to monitor the natural growth of the amoeba, and a blank contained only the culture medium without amoeba to serve as a baseline for absorbance readings. The parasite suspension was prepared by adjusting the amoeba concentration to 10^5 cells per mL for with fresh culture medium was taken. This suspension was then distributed evenly into the wells of the 96-well microtiter plate (Corning). To test the compounds' ability to suppress the amoeba growth, various concentrations of compounds under research added to the assigned wells to assess their inhibitory effects. The plate was sealed to keep out contamination after compounds were added, then nitrogen gas was flushed through in for 10 minutes that created an anaerobic environment, an optimal condition for *E. histolytica* growth. After 72 hours incubation period of plate, the amoeba growth was assessed under a microscope to ensure the presence of viable cells. Once the incubation was completed, the culture medium was removed carefully and gently shaking it to discard the supernatant so that unadhered cells or residual compounds get removed. Then, the plate was cleaned with 0.9% sodium chloride solution at 37°C to remove debris. After washing, the plate was dried and methanol was used to maintain cell integrity and adhere them to the plate surface followed by straining with 0.5% (w/v) aqueous eosin for 15 minutes. Using a microplate reader, the optical density (OD) of each well was determined at 490 nm (wavelength allowed for the detection of stained cells) upon straining. The anti-amoebic activity of each compound was determined by calculating the percentage inhibition of parasite growth relative to the negative control. The point was fitted to a curve using a non-linear regression technique, which enabled the determination of the half-maximal inhibitory concentration (IC₅₀) values. At least two independent experiments were conducted for each drug to ensure reproducibility of the results.

2.2.2. Antimalarial activity

The *P. falciparum* (NF54) strain that was susceptible to chloroquine, was continuously cultured *in vitro*. The parasites were cultured in supplement RPMI-1640 culture media for providing the necessary nutrients and conditions for parasite growth. The culture was kept at 37°C in a controlled atmosphere consisting of 5% CO₂, 3% O₂ and 92% N₂. To synchronize the parasite culture to the ring stage, a critical developmental stage of *P. falciparum*, 5% D-sorbitol was used. D-sorbitol selectively lyses mature parasites, leaving only the ring stage intact.[32] After synchronization, titration of ring stage parasites to a final parasitemia (the percentage of infected red blood cells) and hematocrit (the percentage of red blood cells in the culture) of 2%. These parameters were chosen to standardize the starting conditions across all experimental wells. The test compounds, along with the positive control drug quinine, were added to the cultures after 24 hours. After the addition of the compounds, additional culture incubation period was 48 hours at 37°C that allowed the compounds to exert their potential antimalarial effects on the synchronized parasites. Next, the plates were frozen for 1 hour at -70°C. After thawing for 2 hours at room temperature, 25 µL of the lysate was transferred from each well to a non-sterile plate. 100 µL of Malstat™ reagent (a commercially available reagent that reacts with pLDH to produce a colored product) was added to each well followed by addition of 20 µL of a nitroblue tetrazolium (NBT) and phenazine ethosulfate (PES) mixture (1:1). Then, plate was again incubated for 40 minutes at 37°C, allowing the reaction to proceed. After the incubation, addition of 5% acetic acid to each well ended the reaction. The amount of pLDH activity which correlates with parasite viability, is directly proportional to the presence of formazan, a purple-colored product.[33] The 620 nm wavelength was preferred for absorbance of the formazan product using a microplate reader. Percentage inhibition was computed by comparing the absorbance values of the wells treated with the test compounds to the appropriate controls (untreated and quinine-treated wells).

2.2.3. Larvicidal activity

To evaluate the potential larvicidal effects of the synthesized compounds, *Anopheles arabiensis* (KGB) mosquito larvae were obtained from a permanent colony housed at the Botha de Meillon Insectary at the National Institute for Communicable Diseases/WITS Research Institute for Malaria, Johannesburg. The larvae were reared and fed according to established WHO protocols for mosquito maintenance.[34] Fourth instar larvae, the last larval stage before pupation, were chosen for the experiment. 25 larvae were placed in each experimental group and incubated with

the test compounds. The tested concentration of compounds (50 μ M), dissolved in single distilled water. The larvae were incubated for 24 hours at a temperature between 25-28°C, a condition suitable for *Anopheles* mosquito larvae. A well-established positive control, DDT (dichlorodiphenyltrichloroethane), was used. After 24 hours, percentage mortality was recorded for each cohort. The mortality rate was determined by counting the number of dead larvae in each group and comparing it to the total number of larvae exposed to the compounds. The morphological appearance of both the dead and surviving larvae was recorded at 3x magnification using a photographic setup. The morphological traits of the treated larvae were contrasted with those of the untreated controls and the DDT-treated group to identify any unique characteristics caused by the compounds.[35]

2.2.4. *Anopheles ovoidal* assay

Anopheles arabiensis (KGB) eggs were incubated with dihydropyrimidine-phenylacetamide derivatives and controls (DDT and 1% formalin) to determine the interference with the ability of the larvae to hatch. The morphological appearance of the eggs and developing larvae were noted for abnormalities.[36] Each derivative was incubated at 25-28°C with 20-30 eggs at 50 μ M concentration. At 48- and 72-hours' time duration, the number of hatched larvae were recorded. At 3x magnification, the % of hatched *Anopheles* eggs were counted and then expressed as a percentage of the untreated water control. The experiment was performed in triplicate for the data accuracy.

2.2.5. Cytotoxicity assays

Two cancer cell lines: myelogenous leukemia (K562) and human neuroblastoma (SH-SY5Y) were used to assess the anticancer property of targeted compounds, both cultured under conditions similar to the HEK-293 cell line. To evaluate the cytotoxicity of these compounds, these cells were cultured as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM), (a nutrient-rich medium commonly used for the development of variety of mammalian cells). The medium was supplemented with 10% fetal bovine serum (FBS) for essential growth factors and nutrients followed by addition of penicillin (100 IU/mL) and streptomycin (100 μ g/mL) to prevent bacterial contamination. The cells maintenance was done at 37°C in a humidified atmosphere containing 5% CO₂ to promote optimal cell growth. To start the assay, a suspension of 15,000 for K562 cells and 20,000 for HEK-293 and SH-SY5Y cells per well was prepared

followed by addition of serial dilutions of the test compounds or positive control to each well. DMSO was used at a final concentration of less than 1%, as it had no impact on cell viability. After incubating the cells at 37°C for 48 hours, each well was treated 40 µL of MTT reagent (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; 5 mg/mL in phosphate-buffered saline, pH 7.3). After the additional 2 hours of incubation period, the culture medium containing the unreacted MTT was carefully removed. The formazan crystals were dissolved by adding DMSO to each well. The absorbance was measured at 540 nm, with a reference wavelength of 690 nm, using a microplate reader (Labsystems Multiskan RC).[36] Then calculation of percent cellular viability by comparing the absorbance readings from treated wells to the control wells, was done and IC₅₀ values were determined for each compound and compared with the positive control, camptothecin, a known anticancer agent. The entire experiment was conducted in triplicate for reproducible results.

2.2.6. Haemolysis assay

Fresh human red blood cells (RBCs) were collected and prepared to a 1% hematocrit in RPMI-1640 culture medium supplemented with necessary nutrients. The RBC suspension was then incubated with 25 µL of each test compound or control solution. The concentration of the test compounds was 50 µM, chosen to evaluate the potential hemolytic effects at a standardized concentration. The incubation period lasted for 48 hours to allow sufficient time for any potential hemolytic effects to occur.[25] The supernatant was then analyzed for its absorbance at 412 nm. The % haemolysis was achieved using 0.2% (v/v) Triton X-100, a detergent known to completely lyse red blood cells. Triton X-100 serves as a positive control for maximum hemolysis.

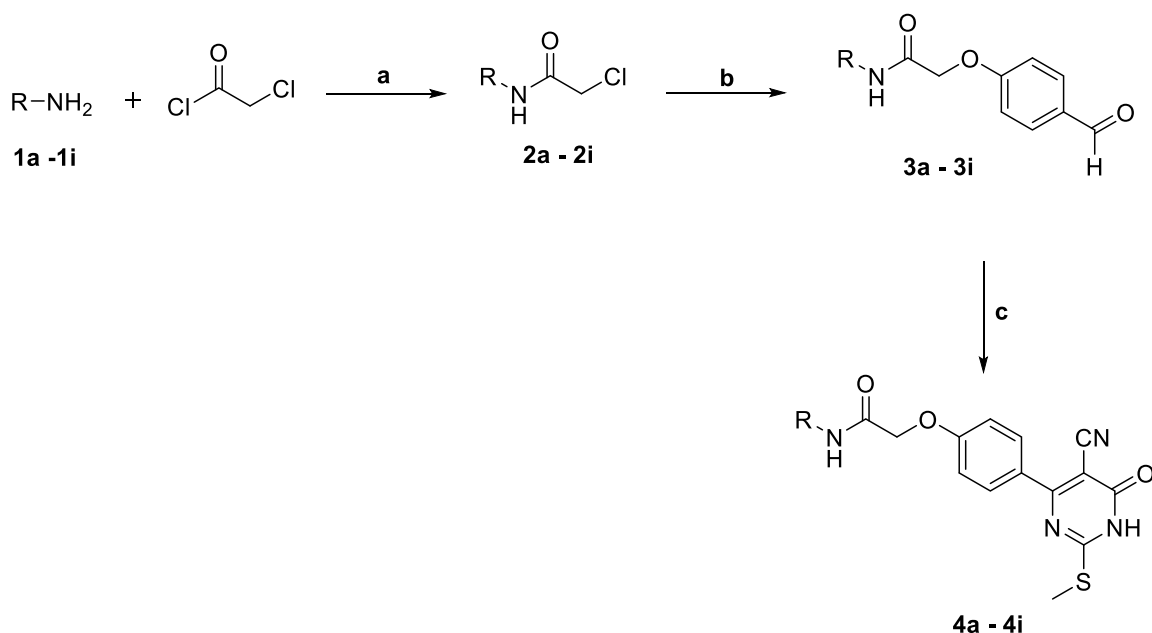
2.2.7. Artemia lethality assay

Artemia franciscana eggs placed in sea salt (38g/L) were hatched in an aerated environment at an optimal temperature for 18 hrs.[37] To each well, 40-60 live *Artemia* in 400 µL of water were added with the test/control drugs and the plates left over night at room temperature. Dead *Artemia* nauplii were then counted at 0, 24 and 48 hr. All *Artemia* were killed following the addition of 25% acetic acid and total number of *Artemia* per well counted. Percentage mortality was then calculated taking the controls into account, where potassium dichromate was used as the positive control. The experiment was repeated in triplicate.

3. Results and Discussion

3.1. Chemistry

The synthetic route leading to the titled compounds, dihydropyrimidine-phenylacetamide derivatives (**4a -4i**), is given in **Scheme 1**. In the first step, different chloro-acetamides (**2a -2i**) were synthesized by substitution of the amine with chloro acetyl chloride by the proposed method. In the second step, the formylphenoxy acetamide intermediates (**3a -3i**) were formed by the reaction of chloro-acetamides and 4-hydroxybenzaldehyde using K_2CO_3 in acetone by a reported method. Then, in the final step, heating a mixture of ethyl cyanoacetate with the formylphenoxy acetamide intermediate and S-methylisothiurea hemisulfate gave the corresponding dihydropyrimidin-phenylacetamide derivatives (**4a -4i**) as the final product with good yield.



Scheme 1; Synthesis of dihydropyrimidine-phenylacetamide derivatives. **Reagents and conditions:** (a) TEA, CH_2Cl_2 (b) KI, K_2CO_3 , 4-hydroxybenzaldehyde, ethanol, reflux, 24 h; (c) $CNCH_2COOC_2H_5$, S-methylisothiurea hemisulfate, ethanol, reflux.

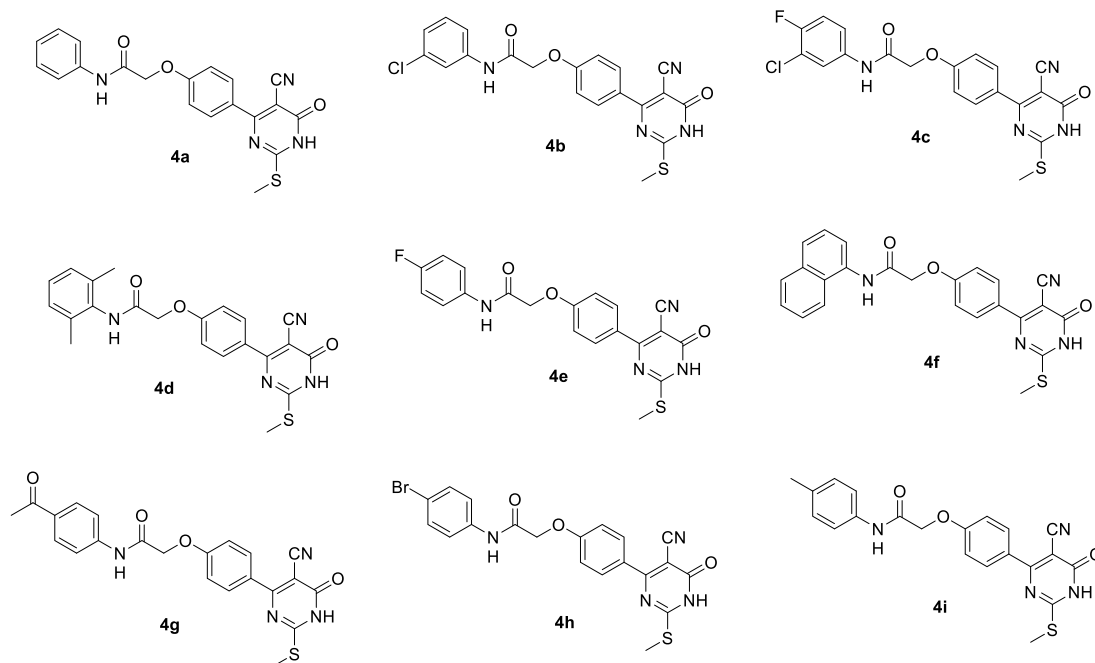


Fig-3: Structure of dihydropyrimidine-phenylacetamide derivatives

All the dihydropyrimidine-phenylacetamide derivatives (**4a -4i**) (**Fig-3**) were stable in solid state at room temperature. A satisfactory spectral data was observed for the proposed structures of these derivatives. Characteristic IR spectra showed CN absorption band around 2212 -2218 cm^{-1} in dihydropyrimidine-phenylacetamide derivatives (**4a -4i**). The ^1H NMR indicated that the disappearance of the peak of aldehyde proton (a singlet in the range δ 9.94-9.96 ppm) and the appearance of the peak of NH proton of pyrimidine ring as a broad singlet in the range δ 6.53-6.97 ppm along with the three protons of S-CH₃ shows a sharp singlet in range δ 2.34-2.76 ppm confirms the formation of dihydropyrimidine. The signals due to the aromatic protons appeared in the expected region. The CN carbon showed peak around δ 115 ppm and S-CH₃ around 13.7 ppm in the ^{13}C NMR of these compounds. The structure of the synthesized compounds was further elucidated by mass spectral studies with M+1 peak. The purity of the compounds was confirmed by elemental analysis.

3.2. Biological evaluation

3.2.1 Anti-amoebic activity

In order to examine the effectiveness of dihydropyrimidine-phenylacetamide derivatives (**4a -4i**) as the anti-amoebic potential against *E. histolytica*, HM1: IMSS strain was taken and

metronidazole (MTZ) ($IC_{50} = 1.80 \mu M$) was utilized as reference drug. The experiments were done in triplicate at each concentration level with three repetitions. The results of anti-amoebic activity are summarized in **Table 1**. All the dihydropyrimidine-phenylacetamide derivatives had IC_{50} values in the range 0.12-7.16 μM . Among these derivatives, five compounds: **4b** ($IC_{50} = 0.82 \mu M$), **4c** ($IC_{50} = 0.12 \mu M$), **4e** ($IC_{50} = 0.34 \mu M$), **4g** ($IC_{50} = 0.51 \mu M$), and **4h** ($IC_{50} = 1.66 \mu M$) were found to exhibit better anti-amoebic activity than MTZ ($IC_{50} = 1.80 \mu M$). Compound **4c**, having weak deactivating chloro group at meta position and fluoro group at the para position of phenyl ring, exhibited most promising activity ($IC_{50} = 0.12 \mu M$) in the series. Compound **4e** ($IC_{50} = 0.34 \mu M$) having strong electronegative fluoro group at para position exhibited better activity. Introduction of strong electron withdrawing *para* substituted acetyl group in compound **4g** ($IC_{50} = 0.51 \mu M$), exerts significant inhibitory activity. Compound **4b** ($IC_{50} = 0.82 \mu M$) having chloro group at meta position and compound **4h** ($IC_{50} = 1.66 \mu M$) with bromo group at the para position also had better activity. Whereas the presence of weak activating methyl group at both *ortho* positions in compound **4d** ($IC_{50} = 5.91 \mu M$) exhibited less activity. Compound **4a** ($IC_{50} = 3.25 \mu M$) without any substitution had better activity than compound **4d**. The substitution of the phenyl ring with bulky naphthyl group in compound **4f** ($IC_{50} = 7.16 \mu M$) reduced the activity. Thus, from the above discussion, all the dihydropyrimidine-phenylacetamide derivatives (**4a -4i**) were good to potent anti-amoebic, with compound **4c** that was the most potent.

3.2.2 Antimalarial activity and cytotoxicity profile

The pharmacological properties of the dihydropyrimidine-phenylacetamide derivatives (**4a -4i**) were varied through the various assays undertaken to investigate their antimalarial, insecticidal and toxicological properties. Of the nine derivatives, the naphthalene derivative, **4f** ($IC_{50} = 56.78 \pm 7.79 \mu M$) was the most active inhibiting 43% parasite growth (**Table 1**), but was not comparable to quinine which is used to successfully eliminate infections.[4] This antiplasmodial inhibition by **4f** appears to be directed against the intra-erythrocytic parasite where the derivative did not induce haemolysis, thereby not affecting the host's cell membrane by increasing permeability properties or interfering with metabolism [haemolysis: 0.12%]. The lack of toxicity of this derivative is replicated in the other toxicity assays which displayed minimal toxicity to the HEK-293 kidney epithelial cells, K562 erythroleukemia cells, and SH-SY5Y neuroblastoma cell

line (% inhibition >65%). The minimal inhibitory effect was also observed in the larvicidal and ovicidal assays (**Table 1**), with only 12.7% of the *Artemia* killed (**Table 2**).

The relative lack of activity of all the derivatives is complemented with the observation that none of the derivatives induced a significant amount of haemolysis [0.08 – 1.77%] compared to quinine [1.76%]. The SH-SY5Y neuroblastoma cell line was the most sensitive to the derivatives with an overall average of 33.8% inhibition compared to 29.12 and 0% for the K562 erythroleukemia and HEK-293 kidney epithelial cells, respectively. The most toxic of the derivatives to *Artemia* was the 4-fluoro, 5-chloro-benzyl derivative (**4c**); where 98.7% of the nauplii were killed compared to 99.6% by the positive control, potassium dichromate. This inhibitory effect was also observed in the ovicidal assay where only 66.8% of the *Anopheles* eggs hatched when treated with **4c**, which was more effective than DDT (91.2% hatched), but not as effective as 1% (v/v) formalin which prevented all eggs from hatching over a 72 hr period (**Table 1**). The effects of **4c** appear to be more selective for more complex multicellular organisms (*Anopheles* larvae, 45%; eggs, 33%; *Artemia*, 99%)(**Table 1**) than the single cells and plasmodia (0, 28, 28 and 28% for the HEK-293 kidney epithelial, K562 erythroleukemia, SH-SY5Y neuroblastoma (**Table 2**) and the NF54 *P. falciparum* parasite (**Table 1**)). When compared to **4e**, which contains a 4-fluoro-benzyl side chain and **4d**, with a 3-chloro benzyl side chain, the chlorine appears to contribute towards some of the toxicity of **4c** with 44.2% death of *Artemia* nauplii in comparison to 25.6% of the fluorine (**Table 2**). However, their singular contribution to the effect on human cell growth (**Table 2**) and the hatching of *Anopheles* larvae (**Table 1**) was minimal.

Table 1: *In vitro* anti-amoebic, antimalarial and mosquito-cidal activity of dihydropyrimidine-phenylacetamide derivatives (4a -4i)

S.No.	Compounds code	Anti-amoebic activity (IC ₅₀ ; μ M)	Antimalarial activity (% growth at 50 μ M)	Larvicidal activity at 50 μ M*	% Ovicidal activity (hatch rate at 50 μ M)*
1	4a	3.25 $\pm$ 0.01	74.85 $\pm$ 6.66	50	94.11
2	4b	0.82$\pm$0.03	74.46 $\pm$ 7.26	55	97.85

3	4c	0.12±0.01	71.56±4.72	45	66.80
4	4d	5.91±0.03	72.58±2.00	48	96.78
5	4e	0.34±0.03	77.04±13.61	50	103.22
6	4f	7.16±0.08	56.78±7.79	45	107.80
7	4g	0.51±0.01	74.69±14.31	48	102.56
8	4h	1.66 ± 0.05	84.68±3.07	48	86.45
9	4i	4.16 ± 0.01	73.64±7.53	55	92.81
10	MTZ	1.8±0.01	n.t	n.t	n.t
11	Quinine	n.t.	20.51±4.50	n.t	n.t
12	DDT	n.t	n.t	100.00	91.20
13	Formalin	n.t.	n.t	n.t	0.00

n.t.: not tested; *: at 72 hours

Table 2: *In vitro* toxicological activity of dihydropyrimidine-phenylacetamide derivatives (4a - 4i)

S.No.	Compounds code	Cytotoxicity profile (% Cell growth at 100µM)			% <i>Artemia</i> inhibitory activity at 50µM
		HEK-293	K562	SH-SY5Y	
1	4a	93.14±8.16	63.02±2.41	54.24±1.73	28.89
2	4b	117.19±14.51	92.18±1.30	86.84±4.67	44.17
3	4c	108.21±10.55	72.02±6.18	71.63±4.94	98.67
4	4d	82.41±4.34	73.89±3.75	53.34±2.15	17.40
5	4e	103.04±6.69	61.08±4.38	58.47±12.91	25.58
6	4f	120.21±3.74	72.96±3.82	68.36±3.64	12.67
7	4g	80.87±8.94	68.85±1.30	58.68±2.57	51.30
8	4h	107.14±4.63	72.74±5.03	93.48±5.80	11.22
9	4i	79.22±5.44	70.91±3.09	60.15±2.02	39.13
10	Camptothecin	0.13±0.11	0.13±0.04	0.53±0.40	n.t
11	Potassium dichromate	n.t	n.t	n.t	99.44

3.3. Molecular docking study of 4-aryl-5-cyano-2-methylthio-6-oxo-1,6-dihydropyrimidine derivatives

Molecular docking is a computational study to check the interaction between ligand and protein. These compounds were interacted with the enzyme *O-acetyl-L-serine sulfohydrolase (EhOASS)* of *E.histolytica*. PDB id of this enzyme is 3BM5 that the PDB format was directly downloaded from protein data bank (www.rcsb.org/pdb). Heteroatoms and water molecules, present in the crystal structure of protein were deleted, and followed by energy minimization in AutoDock tools then saved in PDBQT format. The structure of compounds was drawn in ChemDraw 16.0 and then 3D structure was prepared in chem 3D. PDBQT file of the ligand was prepared in through AutoDock tool. Docking was performed by AutoDock vina 4.2. The docking interaction of output file was visualized in PyMOL.

The molecular docking results indicate that all the dihydropyrimidine-phenylacetamide derivatives (**4a -4i**) had binding free energy in range of -6.3 to -8.0. Compounds **4c** and **4f** show the strongest binding affinity with binding free energies of -8.0 kcal/mol, suggesting to form the most stable ligand-protein complex. Compounds **4a**, **4b**, and **4g** also demonstrate strong binding affinities (ranging from -7.9 to -7.5 kcal/mol) with multiple hydrogen bonds and moderate bond distances. Compound **4i** has the weakest binding affinity with a binding free energy of -6.3 kcal/mol, possibly due to weaker hydrogen bonds as indicated by the longer distances. Docking images of compounds **4c** and **4f** showed the bonding interactions. The 3D images A and B indicated donor and acceptor regions. In 2D structure, Both the compounds **4c** and **4f** showed **two** hydrogen bonding, interacting with amino acids GLU 28 and ILE 44 in case of **4c**, and SER 7 and SER 8 amino acids in case of compounds **4f**. (**Table 3**) (**Fig-4**) Overall, compounds **4c** and **4f** are the most promising candidates for further development based on their strong binding interactions.

Table 3: Binding affinity of dihydropyrimidine-phenylacetamide derivatives (**4a -4i**)

S.No	Compound Code	Binding Free Energy (kcal/mol)	Hydrogen bonds	
			Amino acids residues	Distance in Å
1	4a	-7.5	LYS 58, ASN 88	2.25, 2.59

2	4b	-7.5	TYR 319, LYS 271	2.09, 2.90
3	4c	-8.0	GLU 28, ILE 44	2.77, 2.33
4	4d	-7.4	ASN 158	2.02
5	4e	-7.3	ARG 10	2.65
6	4f	-8.0	SER 7, SER 8	2.68, 2.54
7	4g	-7.9	TYR 14, SER 8, ARG 12, ARG 10, SER 7	2.30, 2.41, 2.67, 2.74, 2.85, 3.08
8	4h	-7.3	SER 8, SER 7, TYR 14	2.10, 2.69, 2.90
9	4i	-6.3	ASP 244, SER 84	2.77, 3.36

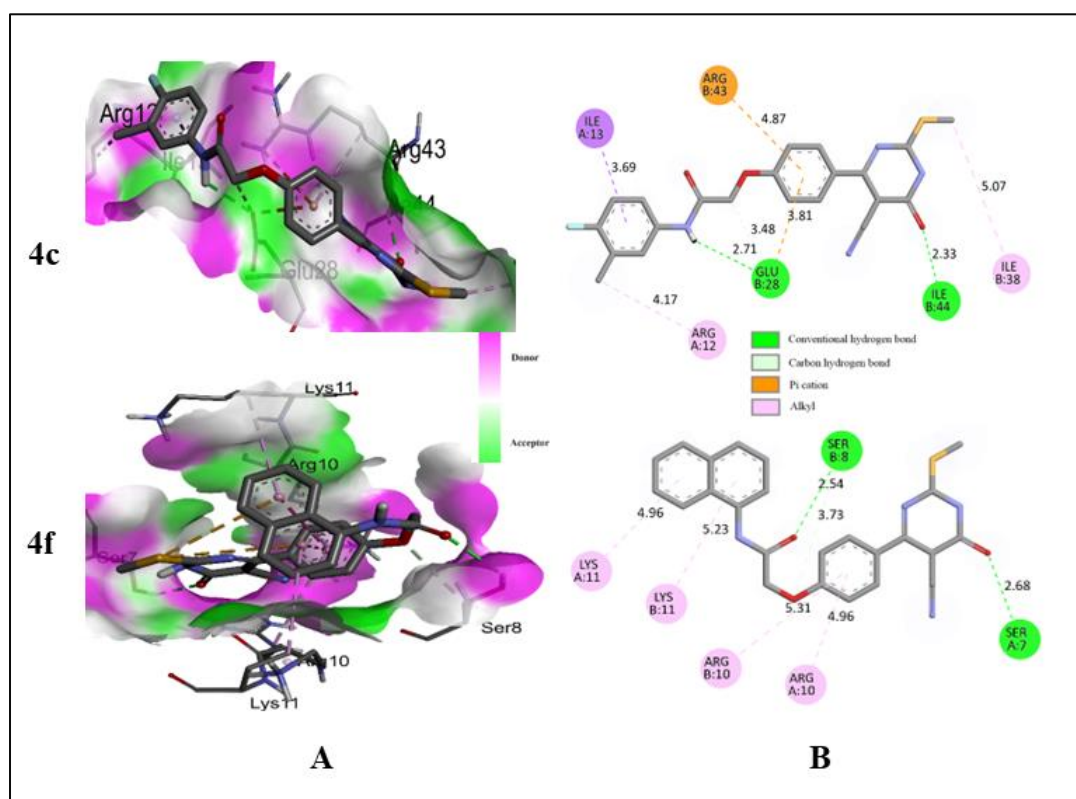


Figure 4: image A indicating H bond-donor (pink) and H-bond acceptor (green) region of **4c** and **4f**. image B shows 2D structure of **4c** and **4f**.

ADME Study of final dihydropyrimidine-phenylacetamide derivatives (4a -4i)

To evaluate the physiochemical properties of these compounds, we need to consider several key factors based on the data provided, such as molecular weight, rotatable bonds, hydrogen bond

acceptors and donors, and TPSA (Topological Polar Surface Area). All the compounds have molecular weights between 392.43 and 471.33 which is less than 500. The compounds have 7 or 8 rotatable bonds, 5 to 6 hydrogen bond acceptors and 2 hydrogen bond donors. The TPSA values range from 133.17 to 150.24. Given that these compounds have TPSA values slightly below from 140 (except for **4g**, which is even higher with 150.24), they might have good oral bioavailability. (**Table 4**) So, these compounds are considered favorable for drug-like properties according to Lipinski's rule of five and better for oral bioavailability. Further, through the application of a decision tree, Toxtree online version (<https://toxtree.sourceforge.net/>) and Chemicalize (<https://chemicalize.com>), *In silico* toxic hazards characteristics predicted that all the compounds were unlikely to permeate through the blood-brain-barrier and the negative Kp values indicated a reduced skin permeation potential (**Table 5**) In contrast, the predictive toxicological profile of the derivatives indicated that all the compounds have a high potential risk of being toxic in humans (Class III) as determined by the revised Cramer rules. In addition, there is a high risk of multiple drug interactions where all the compounds were predicted to be CYP3A4 inhibitors, a hepatic metabolic pathway commonly involved in the metabolism of clinically administered drugs. Similarly, all the compounds were predicted to be CYP2C9 inhibitors, but not CYP2D6 (**Table 5**).

Table 4. Physiochemical properties of target compounds.

S. No	Code	Mol. Weight	Rotatable Bonds	H-bond acceptors	H-bond donors	TPSA	Lipinski violations
1	4a	392.43	7	5	2	133.17	0
2	4b	426.88	7	5	2	133.17	0
3	4c	444.87	7	6	2	133.17	0
4	4d	420.48	7	5	2	133.17	0
5	4e	410.42	7	6	2	133.17	0
6	4f	442.49	7	5	2	133.17	0
7	4g	420.44	8	6	2	150.24	0
8	4h	471.33	7	5	2	133.17	0
9	4i	406.46	7	5	2	133.17	0

Table 5: *In silico* predicative toxicological properties of 2-(4-(5-cyano-2-(methylthio)-6-oxo-1,6-dihydropyrimidin-4-yl)phenoxy)-N-phenylacetamide derivatives.

S.No.	Compound code	Log Kp (cm/s)	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	CYP1A2 inhibitor
1	4a	-6.81	Yes	Yes	Yes	No	Yes	Yes
2	4b	-6.58	Yes	Yes	Yes	No	Yes	Yes
3	4c	-6.61	No	Yes	Yes	No	Yes	No
4	4d	-6.46	No	Yes	Yes	No	Yes	No
5	4e	-6.84	Yes	No	Yes	No	Yes	Yes
6	4f	-6.22	No	Yes	Yes	No	Yes	No
7	4g	-7.29	No	Yes	Yes	No	Yes	No
8	4h	-6.8	Yes	Yes	Yes	No	Yes	Yes
9	4i	-6.64	Yes	Yes	Yes	No	Yes	Yes

Conclusion

The newly synthesized dihydropyrimidine-phenylacetamide derivatives (**4a -4i**) were synthesized and characterized by different spectroscopic and spectrometric techniques like IR, NMR and mass. Further, all these derivatives were tasted for their biological potential. The anti-amoebic screening study indicated that dihydropyrimidine-phenylacetamide derivatives had greater potential against *E. histolytica*, used strain HM1: IMSS. Where, five compounds: **4b** ($IC_{50} = 0.82 \mu M$), **4c** ($IC_{50} = 0.12 \mu M$), **4e** ($IC_{50} = 0.34 \mu M$), **4g** ($IC_{50} = 0.51 \mu M$), and **4h** ($IC_{50} = 1.66 \mu M$) were exhibited better anti-amoebic activity that was less than the reference MNZ ($IC_{50} = 1.80 \mu M$). Compound **4f** had lowest activity with IC_{50} of $7.16 \pm 0.08 \mu M$. Antimalarial screening against *P. falciparum* of these compounds showed low activity but compared to compound **4f** with IC_{50} value of $56.78 \pm 7.79 \mu M$ as the most active against the malaria parasite among all these compounds, followed by **4c** having IC_{50} value $71.56 \pm 4.72 \mu M$. Further, *in silico* docking study indicated that these compounds had good binding affinity in range of -6.3 to -8.0 kcal/mol when interacted with the enzyme *O*-acetyl-L-serine sulfohydrolase (*EhOASS*) of *E. histolytica* (PDB

id: 3BM5). Pharmacological and pharmacokinetic study also showed good oral bioavailability of these derivatives. The above study concluded that compound **4f** which was least active as anti-amoebic, was found to have some efficacy against malaria strain among all the derivatives. Compound **4c** showed lowest IC_{50} (**0.12 μ M**) against amoebic strain HM1: IMSS. Overall, dihydropyrimidine-phenylacetamide derivatives (**4a -4i**) exerted promising anti-amoebic activity and could be used for further development.

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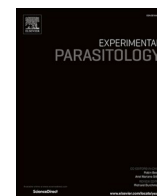
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A dual *In vitro* and *In silico* approach to evaluate 1,4-naphthoquinone-1,2,3-triazole hybrids against atovaquone-resistant malaria

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ABSTRACT

Malaria continues to pose a major global health burden affecting millions annually. Despite advancements in treatment, the emergence of drug-resistant *Plasmodium* strains has undermined current treatment strategies, including atovaquone. Atovaquone is a key mitochondrial inhibitor targeting the cytochrome *bc*₁ (cyt *bc*₁) complex, with resistance primarily driven by mutation in the cytochrome *b* gene. Moreover, atovaquone's reliance on a single target site and its pharmacokinetic limitations further underscore the urgent need for alternative drugs. To address these challenges, this dual *in vitro* and *in silico* study evaluated ten 1,4-naphthoquinone-1,2,3-triazole hybrids targeting atovaquone-resistant (FCR3) *P. falciparum*. Molecular modelling studies were performed on *Saccharomyces cerevisiae* (PDB ID 3CX5), involving the building of a mutant model to simulate the Y279S mutation (equivalent to Y268S mutation in *P. falciparum*), in order to rationalise the observed results. Additionally, pharmacokinetic properties and drug-likeness of these hybrids were predicted *in silico*. Hybrids **D12** and **D13** exhibited strong antiplasmodial activities, 61- and 52-fold, respectively, more than atovaquone. Molecular modelling studies indicated a strong correlation between *in silico* and *in vitro* activities by displaying binding interactions between the ligand and the mutant model. Structure-activity relationships (SAR) analysis identified key structural features essential for favourable binding interactions with target binding site residues. Furthermore, *in silico* evaluations of these hybrids suggested good oral bioavailability and high gastrointestinal absorption, with no significant risk of severe toxicity. Hybrids **D12** and **D13** exhibit potential as lead candidates, with their strong *in vitro* efficacy well-supported by *in silico* data, warranting further optimisation and development.

1. Introduction

Malaria is a severe protozoal disease prevalent in 83 endemic countries with 263 million cases and 597,000 deaths reported in 2023. Over half of this burden is concentrated in WHO African regions (WHO, 2025). A major contributor to the rising incidences is the growing resistance of *Plasmodium falciparum* to current antimalarial drugs and insecticides. This highlights the urgent need for new therapies that are effective against resistant strains, have improved pharmacokinetic profile and are based on validated molecular targets.

One such pathway is the *Plasmodium* mitochondrial electron transport chain (PmtETC) containing five key enzymes: NADH:ubiquinone

oxidoreductase (*Pf*NDH2), succinate dehydrogenase (Complex II or SDH), glycerol-3-phosphate dehydrogenase (G3PDH), malate:quinone oxidoreductase (MQO), and dihydroorotate dehydrogenase (DHODH) (Nixon et al., 2013b). The PmtETC regenerates ubiquinone, a crucial electron acceptor for DHODH, which is essential for pyrimidine biosynthesis (Gutteridge et al., 1979; Painter et al., 2007). Along with this, cytochrome *bc*₁ complex (cyt *bc*₁), cytochrome *c*, *aa3* cytochrome *c* oxidase, and F1Fo-ATPase (complex V) are also components of the ETC (Fisher et al., 2020, 2021).

The cyt *bc*₁ complex (complex III; EC 1.10.2.2) plays an important role in mitochondrial respiration by oxidizing ubiquinol to ubiquinone and transferring electrons to maintain the mitochondrial membrane

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potential (Vaidya and Mather, 2005, 2009). This dimeric complex consists of up to 11 subunits in eukaryotes, with three core catalytic subunits: cytochrome *b*, cytochrome *c*₁ and Rieske iron-sulfur protein (Gardner et al., 2002; Hikosaka et al., 2015; Nixon et al., 2013b). The electron transfer process, known as Q-cycle, is coupled with proton translocation, generating an electrochemical gradient across the mitochondrial membrane (Mitchell, 1975).

Atovaquone, a hydroxynaphthoquinone analogue of ubiquinone, is used in combination with proguanil to treat and prevent *P. falciparum* malaria (Baggish and Hill, 2002; Nixon et al., 2013a; Osei-Akoto et al., 2005). It inhibits the *bc*₁ complex by competing with ubiquinol, disrupting the mitochondrial function (Biagini et al., 2006; Srivastava et al., 1997). However, the parasite has developed resistance, primarily due to point mutation at position 268 in cytochrome *b* (Y268S, Y268N and Y268C), along with other less common variants (Gil et al., 2003; Jacobsen et al., 2021; Kessler et al., 2007; Korsinczyk et al., 2000; Peters et al., 2002; Plucinski et al., 2014; Schwartz et al., 2003; Schwöbel et al., 2003). Additionally, atovaquone is expensive, suffers from poor solubility, high lipophilicity (LogP ~5) and low bioavailability, requiring administration with fatty meals to enhance absorption (Nixon et al., 2014). Given these limitations, it is critical to identify new drug candidates targeting the *cyt bc*₁ complex that can overcome resistance and offer improved pharmacokinetics.

Hybrid molecules have emerged as promising candidates, designed to target multiple pathways in *P. falciparum*, enhancing potency and reducing the likelihood of resistance. Hybrids combining naphthoquinones and 1,2,3-triazoles have demonstrated strong antimalarial activity. Naphthoquinones target the *bc*₁ complex, while triazoles have shown efficacy against various parasites, including *P. falciparum* (Faidallah et al., 2016; Graciano et al., 2022; Prasad Raiguru et al., 2023). Triazole-naphthoquinone conjugates have exhibited some potent *in vitro* activity against chloroquine-sensitive F32 Tanzania *P. falciparum* strain (IC₅₀: 0.8 µM - >20 µM) (Oramas-Royo et al., 2019), and lapachol-derived hybrids have shown promising docking interactions with PfDHODH and activity against chloroquine-resistant W2 *P. falciparum* strain (IC₅₀: 4.0 µM–19.4 µM) (Soares, 2018), suggesting their potential as next-generation antimalarials.

This study investigates the *in vitro* efficacy of a series of 1,4-naphthoquinone-1,2,3-triazole hybrids (Erasmus et al., 2021) against an atovaquone-resistant *P. falciparum* strain. Molecular modeling was used to explore the binding behavior within the Y268S mutant model, the most common resistance-conferring variant. *In silico* analyses were also conducted to assess pharmacokinetic properties and drug-likeness.

Due to the lack of a *P. falciparum bc*₁ crystal structure, *S. cerevisiae bc*₁ complex (PDB 3CX5) was used as a model, given its high sequence identity (about 68 %) with *Plasmodium* cytochrome *b* (Kessler et al., 2003, 2005) and similar sensitivity to atovaquone (IC₅₀: 3–4 nM) (Valli  res et al., 2013). This makes yeast a suitable model for studying drug interactions and resistance mechanism targeting the *bc*₁ complex.

While *in silico* modelling using different species with high sequence similarity can aid hypothesis generation, it has limitations. A 68 % sequence similarity does not guarantee functional equivalence; as critical differences may exist in binding or allosteric sites. Structural variations can influence ligand binding and enzyme activity, and species-specific metabolic pathways may alter the physiological response, requiring *in vitro* or *in vivo* validation.

Despite limitations, *in silico* approaches offer a cost-effective and rapid means to prioritize compounds for further testing. However, due to possible false positive or negatives, experimental validation remains essential.

This study adopted a dual approach, combining *in silico* and *in vitro* methods to evaluate the antimalarial potential of 1,4-naphthoquinone-1,2,3-triazole hybrids. The *in vitro* assays were conducted against the *P. falciparum* FCR3 strain, known for atovaquone-resistance (Carrasco et al., 2013; Rodrigues et al., 2009; Yeates et al., 2008). Molecular modeling used the yeast *bc*₁ complex (PDB 3CX5) to stimulate binding to

the Y279S mutant, analogous to the Y268S mutation in *P. falciparum*. Results from both approaches were compared to atovaquone to assess whether the hybrids demonstrated superior activity and pharmacokinetic properties, supporting their potential as next-generation antimalarial agents.

2. Materials and methods

2.1. Naphthoquinone-1,2,3-triazole hybrids

A series of ten 1,4-naphthoquinone-1,2,3-triazole hybrids previously synthesised and characterised by Erasmus et al. (2021) was investigated for antimalarial activity (Table 1).

2.2. *In vitro* anti-plasmodial activity

The FCR3 strain of *P. falciparum* was maintained continuously *in vitro* in supplemented RPMI-1640 culture media at 37 °C and gassed with a mixture of 2 % O₂, 5 % CO₂, and 93 % N₂ (Trager and Jensen, 1976). To evaluate the antimalarial activity of the hybrids, the 5 % D-sorbitol synchronized ring-stage parasites (Lambros and Vanderberg, 1979) were adjusted to a final parasitemia of 2 % and 2 % hematocrit, to which serial dilutions of the hybrids and positive control, atovaquone, were added followed by incubation for 72 h. Uninfected erythrocytes and drug-free parasitized erythrocytes were used as negative controls. Following 72 h of incubation period, the plates were frozen at –70 °C for 1 h and thawed after 1 h. The lysate (25 µl) was transferred to a second plate, to which 100 µl Malstat™ and 20 µl nitroblue tetrazolium-phenazine thiosulphate (1:1) mixture was added to each well and incubated for 40 min at 37 °C to determine the parasite lactate dehydrogenase (pLDH) activity (Makler et al., 1993). Thereafter, 5 % acetic acid (50 µl) was added to each well, and the absorbance of the formazan products recorded at 620 nm, as an indicator of parasite viability. The percentage of parasite growth, taking the appropriate controls into account, was calculated and used to determine the concentration required to inhibit parasite growth by 50 % (IC₅₀ value) from log sigmoid dose–response curves using the GraphPad Prism® 5.0.0 software (GraphPad Software, CA, USA). A four-parameter logistic regression model was applied to characterize the dose-response relationship, accommodating asymptotic behaviour at both low and high concentration extremes where parasite growth inhibition did not converge to absolute 0 % or 100 %. This modelling approach permits estimation of non-fixed lower and upper asymptotes, thereby capturing intrinsic biological variability and enhancing the robustness of curve fitting in biological assay conditions (Straetmans, 2012). Each experiment was repeated at least in triplicate, with the average and standard deviation (S.D) reported (van Zyl et al., 2002).

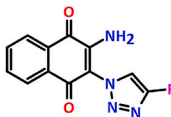
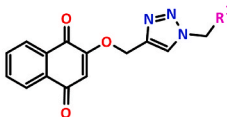
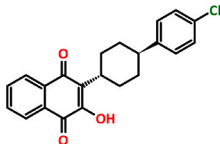
2.3. *In silico* studies

Due to the unknown allelic status of the PfFCR3 strain used in the *in vitro* study, the *in silico* model was constructed based on the most prevalent mutation (Y268S) reported in the resistant isolates (Akhoon et al., 2014; Fisher et al., 2012; Plucinski et al., 2014). While this approach provides a reasonable approximation, we acknowledge this as a limitation of the study, as the actual genotype may differ.

2.3.1. Molecular modelling

3D structures of ligands were prepared using LigPrep program of Schr  dinger Maestro software (Schr  dinger Release Notes - Release, 2023-2) and OPLS4 force field, with protonation states being predicted using Epik with a target pH of 7.0 ± 2.0 (Schr  dinger Release, 2023-2: Epik) (Shelley et al., 2007). The protein structure was prepared using the protein preparation wizard from Schr  dinger (Schr  dinger Release, 2023-2: Protein Preparation Workflow) (Solmaz and Hunte, 2008). All extra chains and ligands were deleted, keeping only the chains C and P

Table 1*In vitro* assay results and summary of docking and MM-GBSA scores for hybrids docked in 3CX5 (Y279S mutant model) and atovaquone.

Hybrid ID	Structures of hybrids	Antimalarial activity <i>Pf</i> FCR3 (IC ₅₀ ±S.D.; μM)	3CX5 mutant (Y279S) model		Key interactions
			Docking score	MM-GBSA (kcal/mol)	
	 D3, D7, D8  D12 – D18  Atovaquone				
D3	R = Phe	0.471 ± 0.128	–8.94	–38.76	Glu ²⁷² (water bridge, aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D7	R = 3,5-diCF ₃ Phe	0.596 ± 0.003	–8.27 (pose 1); –8.07 (pose 2)	–44.56 (pose 1); –45.13 (pose 2)	No interactions with His ¹⁸¹ , Glu ²⁷² and Ser ²⁷⁹ . Only intramolecular aromatic H-bond was observed.
D8	R = CH ₂ SPhe	0.651 ± 0.153	–9.62	–50.87	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge)
D12	R¹ = Phe	0.028 ± 0.006	–10.37 (pose 1); –10.31 (pose 2)	–40.02 (pose 1); –40.00 (pose 2)	Glu ²⁷² (water bridge and aromatic H-bond), Met ¹³⁹ (aromatic H-bond)/ His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge and aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D13	R¹ = 4-Me Phe	0.033 ± 0.004	–10.77	–56.54	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge)
D14	R ¹ = 4-tBu Phe	0.401 ± 0.077	–12.09	–47.86	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge and aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D15	R ¹ = 4-Br Phe	0.191 ± 0.049	–10.34 (pose 1); –10.12 (pose 2)	–44.49 (pose 1), –49.43 (pose 2)	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge, aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D16	R ¹ = 3-Br, 5-F Phe	0.074 ± 0.021	–10.65	–42.47	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge and aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D17	R ¹ = 4-NO ₂ Phe	0.259 ± 0.026	–9.96	–45.65	His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge, aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
D18	R ¹ = 4-CF ₃ Phe	0.110 ± 0.023	–10.91 (pose 1); –10.62 (pose 2)	–41.80 (pose 1); –49.32 (pose 2)	Glu ²⁷² (water bridge and aromatic H-bond), Met ¹³⁹ (aromatic H-bond)/ His ¹⁸¹ (H-bond), Glu ²⁷² (water bridge, aromatic H-bond), Met ¹³⁹ (aromatic H-bond)
Atova-quone	–	1.713 ± 0.014	–8.230	–21.66	No interactions with His ¹⁸¹ , Glu ²⁷² and Ser ²⁷⁹

involved in the binding pocket. To build the mutant model, tyrosine at position 279 (equivalent to position 268 of *Plasmodium* parasite) in the wild type (WT) model was replaced by serine to account for the Y279S mutation (equivalent to Y268S in *P. falciparum*) (Chebon-Bore et al., 2021; Jacobsen et al., 2021; Song et al., 2015). The protein was later pre-processed and minimised to relieve unfavourable constraints.

Molecular docking was performed using Glide extra precision (XP) scoring function, and ligand structures were treated as flexible, which allowed for sampling of ring conformations (including input ring conformation) and nitrogen inversion, to obtain five poses per ligand (Friesner et al., 2004, 2006). Prime MM-GBSA (Molecular Mechanics/Generalized Born Model and Solvent Accessibility) v3.000 program was used to estimate the binding free energy (or ΔG) for each docked pose complex with the VSGB solvation model and OPLS4 force field (Genheden and Ryde, 2015; Li et al., 2011; Mulakala and Viswanadhan, 2013).

The complexes with the best binding pose score (most negative ΔG value) were submitted for 100 ns molecular dynamic (MD) simulations using the Desmond program found within the Schrödinger software suite and the OPLS4 force field (Abdullah et al., 2023). Water molecules present in the binding pocket from the docking procedure were retained, and the system was solvated using the TIP4P water model. An orthorhombic simulation box was set up with boundaries 10 Å away from the protein, containing more than 130,000 atoms. The rest of the settings were kept on default and MD simulation was done for 100 ns with NPT conditions (300 K, 1.103 bar and 2000 bar Å). The MD simulations were analysed using the simulation interaction diagram tool within the Schrödinger Suite (Schrödinger Release, 2023-2: Desmond Molecular Dynamics System).

2.3.2. *In silico* ADMET and drug-likeness

ADMET properties, including the molecular properties that are incorporated in Lipinski's rule of five (Ro5), gastrointestinal (GI)

absorption, blood-brain barrier (BBB) permeability, along with number of rotatable bonds (RB), topological polar surface area (TPSA), and bioavailability were calculated using SwissADME program (Daina et al., 2017; SwissADME). ProTox-3.0 tool was used to predict the potential toxicity of chemical hybrids (Drwal et al., 2014; ProTox-3.0) with acute toxicity (rodent) predicted as LD₅₀ in mg/kg body weight, organ toxicity and toxicological endpoints.

3. Results and discussion

3.1. Antimalarial activity and SAR

All the test hybrids, along with atovaquone, evaluated for *in vitro* antimalarial activity against the FCR3 strain of *P. falciparum* displayed a dose dependent effect (Table 1).

All the hybrids showed promising antiplasmodial activities with IC₅₀ values < 1 μM against the FCR3 strain. Hybrids **D12** and **D13** exhibited the best activities with IC₅₀ values respectively, 61-fold and 52-fold lower than atovaquone (Table 1). Examination of the bioactivity data for the FCR3 plasmodial strain disclosed interesting SAR among this set of tested hybrids. It was observed that there were two series of hybrids – 2-amino-3-(1H-1,2,3-triazol-1-yl)-1,4-naphthoquinones (**D3**, **D7** and **D8**) and 2-[(1H-1,2,3-triazol-4-yl)methoxy]-1,4-naphthoquinones (**D12** to **D18**) (Table 1). According to the findings of the *in vitro* assay, hybrids **D12** to **D18** belonging to 2-[(1H-1,2,3-triazol-4-yl)methoxy]-1,4-naphthoquinones exhibited better antiplasmodial activities compared to hybrids **D3**, **D7** and **D8** (2-amino-3-(1H-1,2,3-triazol-1-yl)-1,4-naphthoquinones), suggesting that the presence of methoxy oxygen played a key role in the binding of hybrids at the binding pocket such that the hybrids could produce their inhibitory effects. Further analysis revealed that the anti-plasmodial activity depended on the electron-donating and electron-withdrawing nature of the substituent attached to the phenyl ring. Hybrid **D12**, with the best antiplasmodial activity,

had no substituent on the phenyl ring. Hybrid **D12** has also been reported to inhibit F32 Tanzania (chloroquine-sensitive) strain of *P. falciparum* with IC₅₀ value of $17.9 \pm 2.5 \mu\text{M}$ (Oramas-Royo et al., 2019). Hybrid **D13** with a 4-methyl phenyl group showed a slight increase in the IC₅₀ value (around 1.2 fold) compared to **D12** with unsubstituted phenyl. While hybrid **D14** with the *tert*-butyl group at the para position displayed a further increase in the IC₅₀ value, which could be accounted for by the bulky and electron-donating nature of the *tert*-butyl group. The presence of halogen groups (F or Br) improved the inhibitory activities of hybrids **D15**, **D16**, and **D18**. The presence of a halogen atom was also observed to enhance the antimalarial activities of quinolone-based compounds, as reported against the atovaquone-resistant *P. falciparum* TM90-C2B strain (Cross et al., 2010). Di-substitution improved the inhibitory efficiency of hybrid **D16** compared to mono-substituted **D15**. The presence of the nitro group at the para position in **D17** did not further improve the activity of the hybrid. Whilst, hybrid **D18**, with a para-trifluoromethyl substituent, displayed improved activity compared to **D14**, likely attributed to the presence of halogen atoms.

From hybrids **D3**, **D7** and **D8**, hybrid **D3** with an unsubstituted phenyl ring displayed improved antimalarial activity. On the other hand, hybrid **D7** with two trifluoromethyl groups might face binding hindrance at the active site due to the bulky nature of the compound (Báez-Grez and Pino-Rios, 2023; Huber et al., 2023; Iversen et al., 2001).

3.2. In silico studies

3.2.1. Molecular modelling

To verify that the hybrids targeted the cyt *bc*₁ complex, molecular docking studies were performed to investigate their binding modes within the yeast cyt *bc*₁ binding pocket (Valli  res et al., 2013) (Table 1). The docking scores for all test hybrids demonstrated higher ranked values (ranging from -8.07 to -12.09 kcal/mol) compared to atovaquone (-8.23 kcal/mol), suggesting stronger binding affinities towards the active site of cyt *bc*₁ complex (Table 1). The best binding affinity was displayed by hybrid **D14** with a docking score of -12.09 kcal/mol. However, MM-GBSA binding free energy calculations showed that hybrid **D13** (docking score -10.77 kcal/mol) displayed a more favourable ΔG_{bind} value of -56.54 kcal/mol compared to hybrid **D14** ($\Delta G_{\text{bind}} = -47.86$ kcal/mol). The higher negative docking score of hybrid **D14** suggests strong initial binding poses, likely due to favourable hydrogen bonds and hydrophobic interactions from the *tert*-butyl group. However, MM-GBSA binding energy, which incorporates solvation effects and molecular mechanics, revealed that the strong docking poses may not translate into energetically favourable binding complexes. Conversely, hybrids with poorer docking scores, like hybrid **D13**, might have achieved lower desolvation penalties and better overall stabilization, as reflected by its more favourable MM-GBSA ΔG_{bind} value.

On analysing the interactions with the key residues His¹⁸¹ (chain P) and Glu²⁷² (chain C), it was observed that hybrid **D7** displayed no interactions with either His¹⁸¹ or Glu²⁷² and was observed to be binding at a site of the pocket away from these key residues. This seemed to be energetically more favourable for hybrid **D7**, as suggested by its MM-GBSA score ($\Delta G_{\text{bind}} = -45.13$ kcal/mol).

All the other test hybrids interacted with His¹⁸¹ and Glu²⁷² via hydrogen bond and water bridge formation, respectively, supporting that these two key interactions are important for keeping the ligand stable within the binding pocket of cyt *bc*₁ and impart their inhibitory activities (Fig. 1).

Compared to hybrid **D12**, hybrids **D15**, **D16** and **D18** displayed slightly improved docking and MM-GBSA scores with similar initial interactions with the binding pocket residues His¹⁸¹, Glu²⁷², and Met¹³⁹ (chain C). However, as suggested by the *in vitro* results, hybrid **D12** outperformed the rest in the antiplasmodial assay. This discrepancy highlights the inherent limitations of computational scoring functions,

which primarily account for static binding interactions within the binding pocket and often overlook critical biological factors that may account for favourable pharmacokinetic properties. Further analysis of the molecular properties presented in Table 2 revealed that hybrids **D15** (LogP 3.00), **D16** (LogP 3.29) and **D18** (LogP 3.45) possess higher lipophilicity compared to **D12** (LogP 2.45) which might have negatively impacted the bioavailability of these compounds. Notably, the LogP values of these hybrids are better compared to atovaquone (LogP 4.64), suggesting a potentially improved bioavailability and pharmacokinetic profile. This observation highlights the importance of integrating computational predictions with experimental validation, as molecular docking scores alone may not fully predict biological potency of compounds.

To support the study findings, atovaquone was analysed in two separate models, one for wild type (WT) and another accounting for Y279S mutations, to assess and observe the differences in its binding modes in the two models using docking and MD simulations; and further using those results to compare with the results of the test hybrids. It was observed that atovaquone interacted with His¹⁸¹ (via H-bond) and Glu²⁷² (via water bridge) when docked in the WT model (docking score -9.98), whereas in the resistant model, it lost these key interactions and moved away from the binding pocket to an energetically more favourable site as supported by its MM-GBSA score in resistant model ($\Delta G_{\text{bind}} = -21.66$ kcal/mol) vs MM-GBSA score in the WT model ($\Delta G_{\text{bind}} = -12.63$ kcal/mol) (Fig. 1). These observations were in agreement with the literature (Akhoo et al., 2014; Birth et al., 2014; Fisher et al., 2012; Kessl et al., 2005), and were also supported by the *in vitro* antiplasmodial assay performed to assess atovaquone activity against the sensitive PfNF54 strain (IC₅₀ value: $0.021 \pm 0.011 \mu\text{M}$), further supporting that the PfFCR3 strain is resistant to atovaquone.

For further assessment, test compounds were subjected to 100 ns MD simulations. The results for the two most active hybrids, **D12** and **D13**, are presented to understand their trajectory and key ligand-protein interactions in the Y279S mutant model where Tyr²⁷⁹ (chain C) was replaced by Ser²⁷⁹ (chain C).

3.2.1.1. Protein-ligand Root Mean Square Deviation (RMSD). RMSD determination helped examine the target protein's conformational changes when combined with ligand molecules for a 100 ns MD simulation. At the beginning of the simulation, there was a jump in the RMSD as the system equilibrated and reached a stationary state. This jump was higher for hybrid **D13** compared to hybrid **D12**, but subsequently the RMSD remained relatively stable until the end of the simulation (Fig. 2). The core structure of the protein remained stable, as indicated by localised analysis of key domains in the protein root mean square fluctuation (RMSF) values (supplementary information, Figure S.3). The RMSD value for both ligands changed within a narrow range and remained lower than the RMSD of the protein. This indicated that both ligands remained securely within the binding pocket and did not diffuse away, aligning with effective binding interactions typically observed in stable protein-ligand complexes (Gohlke and Klebe, 2002).

3.2.1.2. Ligand protein contacts. The ligand-protein interactions between cyt *bc*₁ and hybrids **D12** and **D13** (Fig. 3), indicated that the residues with the highest interaction fractions was His¹⁸¹ (chain P) for both hybrids **D12** and **D13**. In addition to His¹⁸¹, hybrid **D12** also displayed major interactions with Ser²⁷⁹ (chain C) and Phe¹²⁹ (chain C), whereas hybrid **D13** exhibited additional interactions with Phe¹²⁹ (chain C) and Glu²⁷² (chain C).

The ligand-protein contacts analysis indicated that hybrids **D12** and **D13** interacted with His¹⁸¹ (chain P, via H-bonds) for 78 % and 70 % of the simulation time, respectively (Fig. 3). In addition, hybrid **D12** also interacted with Ser²⁷⁹ for 49 % of the simulation time via a H-bond and with Phe¹²⁹ for 34 % of the simulation time via π - π stacking. On the other hand, hybrid **D13** had additional interactions with Glu²⁷² via

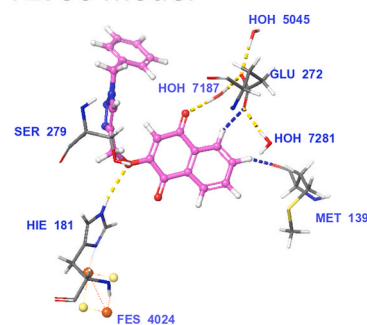
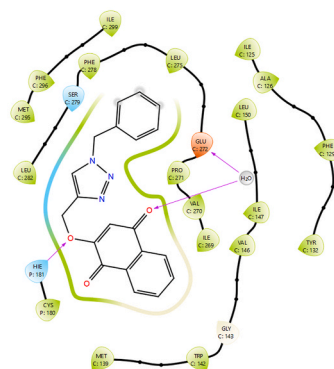
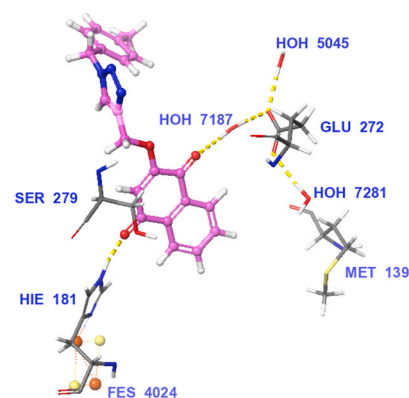
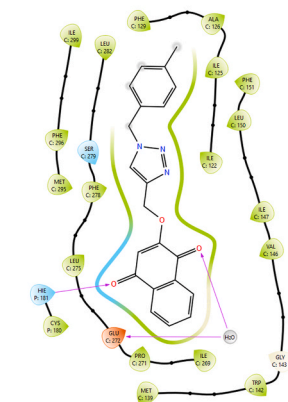
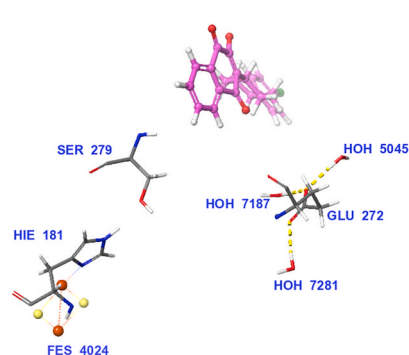
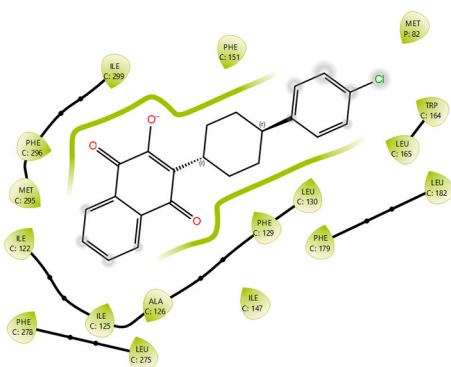
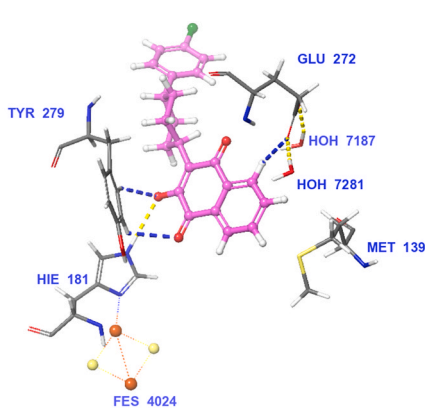
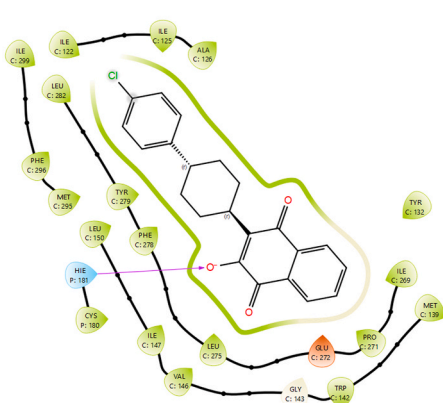
D12 in Y279S model**D13 in Y279S model****Atovaquone in Y279S model****Atovaquone in WT model**

Fig. 1. Ligand interaction diagram (LID) for hybrids **D12**, **D13** and **atovaquone** in **3CX5 (Y279S) mutant model** and their comparison with **atovaquone** in **3CX5 (WT) model** with 2-dimensional (2D) LIDs on the left-hand side and 3-dimensional (3D) LIDs on the right-hand side.

Table 2

Summary of physicochemical, drug-likeness and pharmacokinetic properties of hybrids compared to atovaquone.

Ligand ID	PHYSICOCHEMICAL PROPERTIES						DRUG-LIKENESS		PHARMACOKINETIC PROPERTIES	
	MW	LogP	#HBA	#HBD	#RB	TPSA	Lipinski #violations	Bioavailability Score	GI absorption	BBB permeant
	Criteria	≤500 g/mol (Lipinski)	≤5 (Lipinski)	≤10 (Lipinski)	≤5 (Lipinski)	≤10 (Veber)	≤140 Å ² (Veber)	≤1 violation	≥0.55	High is favourable
D3	316.31	1.93	4	1	2	90.87	0	0.55	High	No
D7	452.31	4	10	1	4	90.87	0	0.55	Low	No
D8	362.41	2.34	4	1	4	116.17	0	0.55	High	No
D12	345.35	2.42	5	0	5	74.08	0	0.85	High	Yes
D13	359.38	2.74	5	0	5	74.08	0	0.85	High	Yes
D14	401.46	3.62	5	0	6	74.08	0	0.85	High	Yes
D15	424.25	3	5	0	5	74.08	0	0.85	High	Yes
D16	442.24	3.29	6	0	5	74.08	0	0.85	High	Yes
D17	390.35	1.82	7	0	6	119.90	0	0.56	High	No
D18	413.35	3.45	8	0	6	74.08	0	0.85	High	No
Atovaquone	366.84	4.64	3	1	2	54.37	0	0.85	High	Yes

MW = molecular weight; LogP = log of octanol/water partition coefficient; #HBA = number of hydrogen bond acceptor(s); #HBD = number of hydrogen bond donor(s); #RB = number of rotatable bond(s); TPSA = topological polar surface area; Lipinski #violations = number of violations of Lipinski's rule; GI = gastrointestinal; BBB = blood-brain barrier.

water bridge for 40 % of the simulation time and with Phe¹²⁹ for 10 % to 28 % of the time via π - π stacking. Both hybrids showed comparable interactions with the binding pocket residues, with hybrid **D12** showing slightly better interactions in terms of time duration and with Ser²⁷⁹ which was present in the mutant model. These *in silico* results support the marginally better IC₅₀ value of hybrid **D12** compared to hybrid **D13** in the antiparasmodial assay.

3.3. Drug-likeness

All test hybrids, including atovaquone, complied with Lipinski's rule of five (Ro5) with no violations, indicating favourable drug-likeness (Table 2) (Lipinski, 2002). A bioavailability score of ≥0.55 further supported their potential as drug candidates. Though all these hybrids displayed satisfactory molecular properties and Lipinski's parameters within acceptable limits, hybrid **D7** exhibited #HBAs reaching the highest permitted value of 10, along with the highest MW (452.31 g/mol) and the highest LogP value of 4, suggesting that it might face challenges with absorption. Hybrids **D8** and **D17** displayed the highest topological polar surface area (TPSA) of 116.17 Å² and 119.90 Å², respectively, of all the test hybrids, suggesting that these two hybrids were more polar compared to other test hybrids and hence would be less extensively absorbed with low bioavailability. This observation was supported by their low predicted bioavailability score of 0.55 and 0.56, respectively.

In comparing the number of rotatable bonds (#RB), hybrid **D3** had the least #RB = 2, which was the same as that of the reference drug, atovaquone. Since the #RB helps to determine the conformational flexibility of the molecule, hybrid **D3** was the most rigid whereas hybrids **D14**, **D17** and **D18** were the most flexible with #RB = 6. Hybrids **D12**, **D13**, **D15** and **D16** had a balanced #RB = 5. A molecule with excessive rotatable bonds may be too flexible to achieve a stable binding with the target protein, while one with too few rotatable bonds may be overly rigid, limiting its ability to fit optimally into the binding site (Veber et al., 2002).

All hybrids were predicted to have high GI absorption except hybrid **D7** as supported by its high MW and high LogP values. Hybrids **D12**, **D13**, **D14**, **D15**, and **D16** were predicted to cross the BBB, making them suitable for the central nervous system (CNS) targeted therapies like cerebral malaria (Bensale and Gallego-Delgado, 2024; Postels and Birbeck, 2013). Hybrids **D3**, **D7**, **D8**, **D17** and **D18** were predicted to not cross the BBB, limiting their CNS-related applications.

3.3.1. BOILED egg (or Brain Or Intestinal EstimateD) plot

Like atovaquone, hybrids **D12**, **D13**, **D14**, **D15**, and **D16** were plotted in the yellow core suggesting their lipophilic nature and low polarity (TPSA <90 Å²), facilitating their ability to penetrate the BBB, and making them suitable as CNS drug candidates (Fig. 4). Hybrids **D3**, **D8**, **D17** and **D18** were plotted in the white region suggesting their good oral bioavailability and exhibiting a balance between lipophilicity and polarity required for absorption across the intestinal barrier. Hybrid **D7** was plotted outside the egg, in the grey region, exhibiting poor pharmacokinetics for oral administration or BBB permeability. It seemed to be very lipophilic hybrids (high WLogP) and might face issues like low solubility. None of the test hybrids falling in the egg regions were marked as a P-gp substrate, suggesting that their absorption or BBB permeability was unaffected by P-glycoprotein's active efflux.

3.3.2. Toxicity

Hybrids were evaluated for their safety profiles, LD₅₀ values, toxicity class, organ toxicity, and toxicity endpoints. Hybrids **D12**, **D13** and **D14** were the safest of all with LD₅₀ = 2000 mg/kg and toxicity class 4 which posed them to be harmful if swallowed (Table 3). Like atovaquone, hybrids **D12** and **D13** were predicted to be non-carcinogenic, non-immunotoxic and non-cytotoxic, with potential of respiratory and nephrotoxicity. Unlike atovaquone, these hybrids were flagged as mutagenic and neurotoxic raising concerns for their prolonged use.

The cytotoxicity of these hybrids was previously evaluated against HEK293 human embryonic kidney cell line, where hybrids **D13** and **D14**, along with atovaquone exhibited low toxicity with IC₅₀ values of 32.4 µM, 36.4 µM and 55.8 µM, respectively (Table 3) (Erasmus et al., 2021). All other tested hybrids displayed IC₅₀ values exceeding 100 µM, indicating minimal cytotoxic effects against the human HEK293 cells.

Additionally, observation of the supernatant while performing the antimalarial assay did not present any signs of blood lysis, indicating that the hybrids displayed inhibitory activity by targeting the parasite and not by causing haemolysis.

4. Conclusions

This study investigated a series of 1,4-naphthoquinone-1,2,3-triazole hybrids using a combination of *in vitro* and *in silico* approaches to identify potential antimalarial candidates. All hybrids demonstrated potent antiparasmodial activity against the *P. falciparum* FCR3 strain, with IC₅₀ values ranging from 0.028 µM to 0.651 µM, markedly superior to the reference drug atovaquone (IC₅₀ 1.713 µM). Among these, hybrids

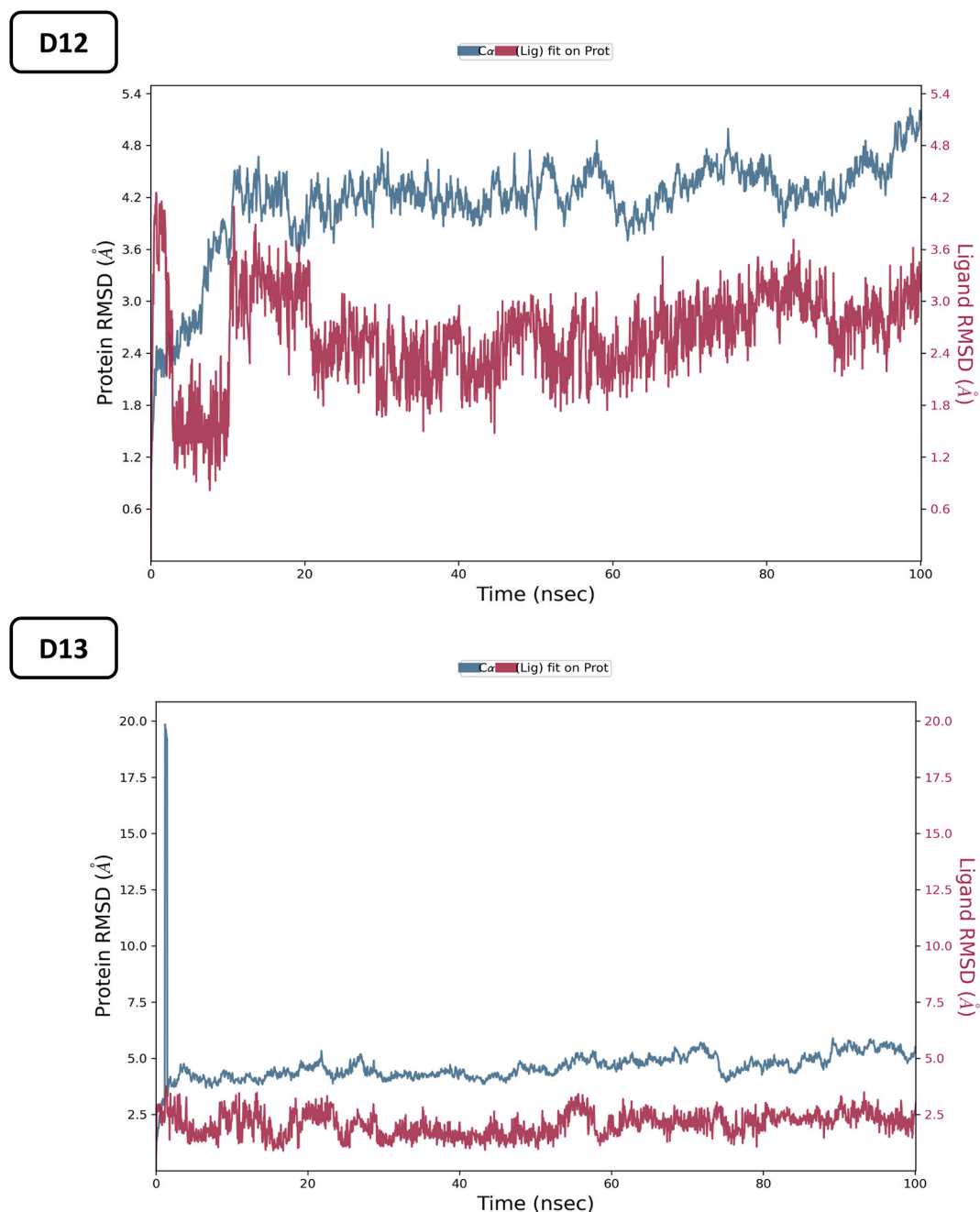


Fig. 2. Protein-Ligand (PL) Root Mean Square Deviation (RMSD) plots for hybrids **D12** and **D13** in complex with 3CX5 (Y279S) mutant-model over 100 ns of MD simulation obtained using Schrödinger Desmond. The blue curve represents the RMSD of the protein Ca atoms, providing insights into the backbone stability and overall conformational changes during the simulation. The red curve corresponds to the ligand RMSD, showing its movement relative to the protein binding site. A steady RMSD after 20 ns suggests system equilibration, indicating that both the protein and ligands had stabilized in their respective conformations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

D12 and **D13** emerged as the most promising hybrids with respective activities around 61- and 52-fold more than atovaquone, showing strong potential for further development against atovaquone-resistant strains. Molecular docking further supported these findings, showing that hybrids **D12** and **D13** established stable interactions with key residues His¹⁸¹ (chain P) and Glu²⁷² (chain C) within the binding pocket of the mutant model. In contrast, these interactions were lost when atovaquone was docked with the Y279S resistant model, indicating that stable binding to His¹⁸¹ (chain P) and Glu²⁷² (chain C) may be critical for a ligand's effectiveness against the resistant strain.

Cytotoxicity data against human HEK-293 cells revealed that all hybrids, except hybrids **D13** and **D14**, exhibited improved safety profile

compared to atovaquone, particularly highlighting hybrid **D12** as a favourable candidate based on the *in vitro* and *in silico* results. Additionally, predicted LogP values of the hybrids suggested improved bioavailability compared to atovaquone. Furthermore, *in silico* assessments of drug-likeness and toxicity profiles were consistent with the experimental results, underscoring the utility of computational tools in early-stage drug discovery.

Overall, these findings offer a strong foundation for future structural optimisation and preclinical development. Future studies should aim to enhance the pharmacokinetic profiles of these hybrid compounds and validate their efficacy *in vivo* to accelerate the development of effective antimalarial therapies against emerging drug resistance.

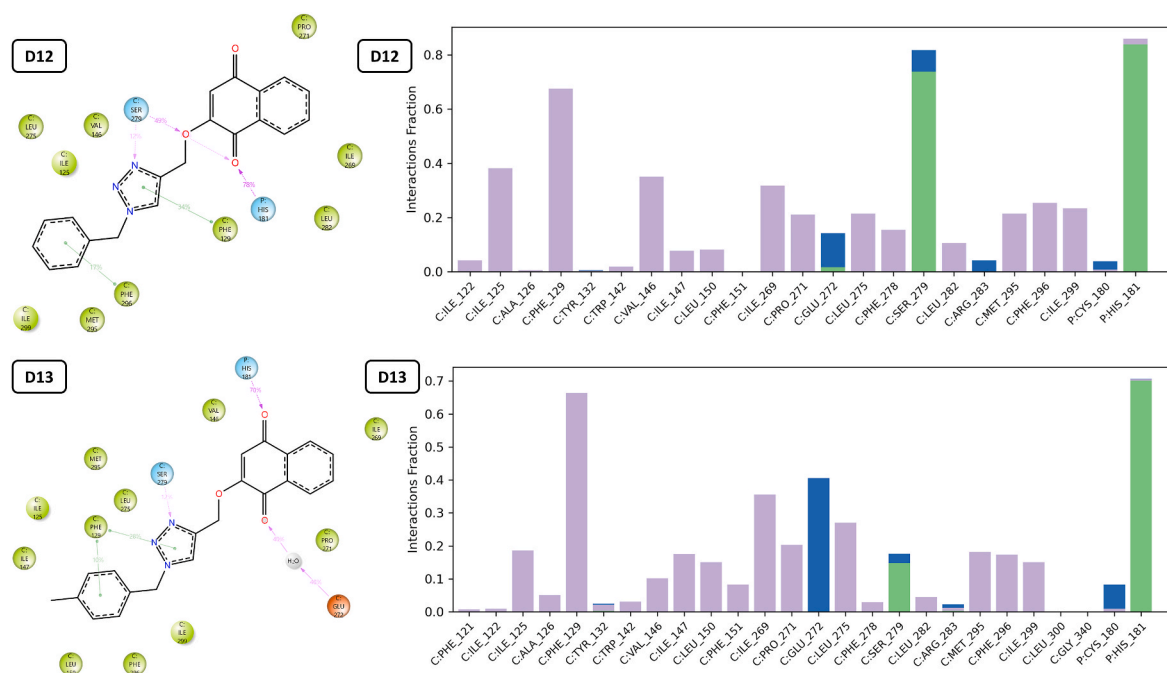


Fig. 3. Ligand-protein (LP) contact analysis from MD simulation for hybrids **D12** and **D13**, represented by LP contacts 2D summary diagrams (left) and LP contacts histograms (right) in 3CX5 (Y279S) mutant model. The 2D schematic interaction diagram visualising key interactions between the ligand and protein residues happening more than 10 % of the simulation time are shown. The bar chart represents the frequency of the protein-ligand interactions categorised into four types: hydrogen bonds (green), hydrophobic bonds (purple), ionic bonds (red) and water bridges (blue). Residues with higher interaction frequencies indicate key binding site contacts contributing to ligand stability. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

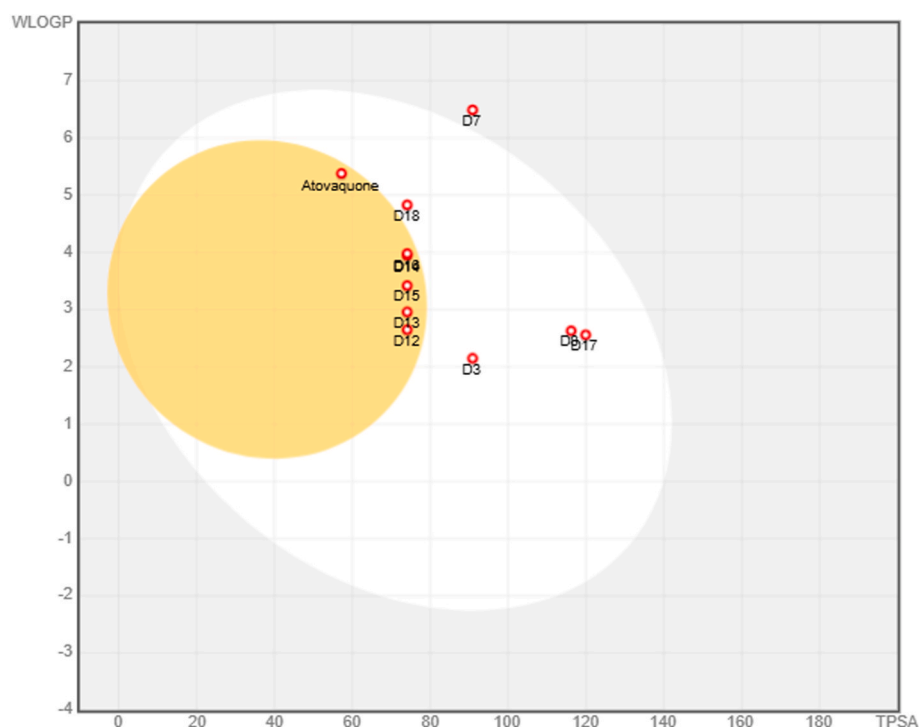


Fig. 4. BOILED Egg (or Brain Or Intestinal EstimateD) Plot of WLOGP vs TPSA for atovaquone and the test hybrids. Yellow indicates BBB-permeant, white indicates human intestinal permeant (HIA), i.e. gastrointestinal permeant; blue indicates P-glycoprotein positive, and red indicates P-glycoprotein negative. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CRediT authorship contribution statement

Priyanka Agarwal: Writing – review & editing, Writing – original

draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **David D. N'Da:** Writing – review & editing. **C. Johan van der Westhuizen:** Writing –

Table 3

Summary of the toxicity profiles for hybrids in comparison to atovaquone, based on ProTox 3.0 predictions.

Hybrid ID	HEK-293 (IC ₅₀ ; μ M) (Erasmus et al., 2021)	LD ₅₀ (mg/kg)	Toxicity Class	Organ Toxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
Atovaquone	55.85	2000	Class 4	Nephrotoxic Respiratory toxic	Non-carcinogenic	Non-immunotoxic	Non-mutagenic	Non-cytotoxic
D3	>100	400	Class 4	Hepatotoxic, Neurotoxic, Respiratory toxic	Carcinogenic	Non-immunotoxic	Mutagenic	Non-cytotoxic
D7	>100	1260	Class 4	Hepatotoxic, Neurotoxic	Carcinogenic	Non-immunotoxic	Mutagenic	Non-cytotoxic
D8	>100	1260	Class 4	Neurotoxic, Respiratory toxic	Carcinogenic	Non-immunotoxic	Mutagenic	Non-cytotoxic
D12	>100	2000	Class 4	Neurotoxic, Nephrotoxic, Respiratory toxic	Non-carcinogenic	Non-immunotoxic	Mutagenic	Non-cytotoxic
D13	32.4	2000	Class 4	Neurotoxic, Nephrotoxic, Respiratory toxic	Non-carcinogenic	Non-immunotoxic	Mutagenic	Non-cytotoxic
D14	36.42	2000	Class 4	Neurotoxic, Respiratory toxic	Non-carcinogenic	Immunotoxic	Non-mutagenic	Non-cytotoxic
D15	>100	2000	Class 4	Neurotoxic, Nephrotoxic, Respiratory toxic	Non-carcinogenic	Immunotoxic	Mutagenic	Non-cytotoxic
D16	>100	2000	Class 4	Hepatotoxic, Neurotoxic, Nephrotoxic, Respiratory toxic	Non-carcinogenic	Immunotoxic	Mutagenic	Non-cytotoxic
D17	>100	2000	Class 4	Respiratory toxic	Carcinogenic	Immunotoxic	Mutagenic	Non-cytotoxic
D18	>100	2000	Class 4	Hepatotoxic, Neurotoxic, Respiratory toxic	Non-carcinogenic	Immunotoxic	Mutagenic	Non-cytotoxic

review & editing. **Robyn L. van Zyl:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Conceptualization.

Ethics approval

This study used *Plasmodium falciparum* and human blood for culturing and performing *in vitro* assay with ethical clearance granted by the WITS Human Research Ethics Committee (Clearance Certificate No. M210121) and WITS Institutional Biosafety Committee (Clearance Certificate No. 20190404). Funding was obtained from the Faculty of Health Sciences Research Office.

Declaration of competing interest

Robyn van Zyl reports equipment, drugs, or supplies was provided by University of the Witwatersrand Johannesburg. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exppara.2025.108991>.

Data availability

Permission to share data to be obtained prior following request

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Simulation Interactions Diagram Report

Simulation Details

Jobname: merged
Entry title: 3CX5_truncated - prepared D12 + DPPC

CPU #	Job Type	Ensemble	Temp. [K]	Sim. Time [ns]	# Atoms	# Waters	Charge
Unknown*	Unknown*	Unknown*	300.0	100.146	131153	28651	0

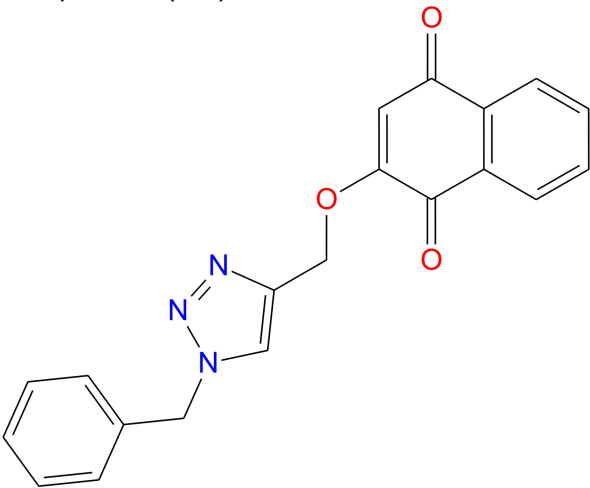
* The configuration file (-out.cfg) was not found. Keep it in same directory as .aef file.

Protein Information

	Tot. Residues	Prot. Chain(s)	Res. in Chain(s)	# Atoms	# Heavy Atoms	Charge
	577	'C', 'D', 'L', 'P'	values([388, 1, 1, 187]	9318	4633	-3
C	1	MAFRKSNVYLSLVNSYIIDSPQPSINYNWNNMGSLLGLCLVIQIVTGIFMAMHYSSNIELAFSSVEHIMR	5 10 15 20 25 30 35 40 45 50 55 60 65	70		
SSA						
P	31	KSTYRTPNPFDDVLKENNDADKGRSYAYFMVGAMGLSSAGAKSTVETFISSMTATADVLAMAKVEVNLA	35 40 45 50 55 60 65 70 75 80 85 90 95	100		
SSA						
C	71	DVHNGYILRYLHANGASFFFMVFMHMAKGLYGSYRSPRVTLWNVGVIIFILTITATAFLGYCCVYGQMS	75 80 85 90 95 100 105 110 115 120 125 130 135	140		
SSA						
P	101	IPLGKNVVVKWQGPVFIHRTPEHIEQANSVDMSEALKDPQTDADRVKDPQWLIMLGICTHLGCVPIGEA	105 110 115 120 125 130 135 140 145 150 155 160 165	170		
SSA						
C	141	HWGATVITNLFSAIPFVGNDIVSWLWGGFSVSNPTIQRFFALHYLVPFIIAAMVIMHLMALHIHGSSNPL	145 150 155 160 165 170 175 180 185 190 195 200 205	210		
SSA						
P	171	GDFGGWFCPCGSHYDISGRIRKGPAPLNLEIPAYEFDGDKVIVGX	175 180 185 190 195 200 205 210 215	215A		
SSA						
C	211	GITGNLDRIPMHSYFIFKDLVTVFLFMLILALFVFYSPNTLGHDPDNYIPGNPLVTPASIVPEWYLLPFSA	215 220 225 230 235 240 245 250 255 260 265 270 275	280		
SSA						
C	281	ILRSIPDKLLGVITMFAAILVLLVLPFTDRSVVRGNTFKVLSKFFFFIFVFNFVLLGQIGACHVEVPYVL	285 290 295 300 305 310 315 320 325 330 335 340 345	350		
SSA						
C	351	MGQIATFIYFAYFLIIVPVISTIENVLFYIGRVNKKX	355 360 365 370 375 380	4033		
SSA						

Ligand Information

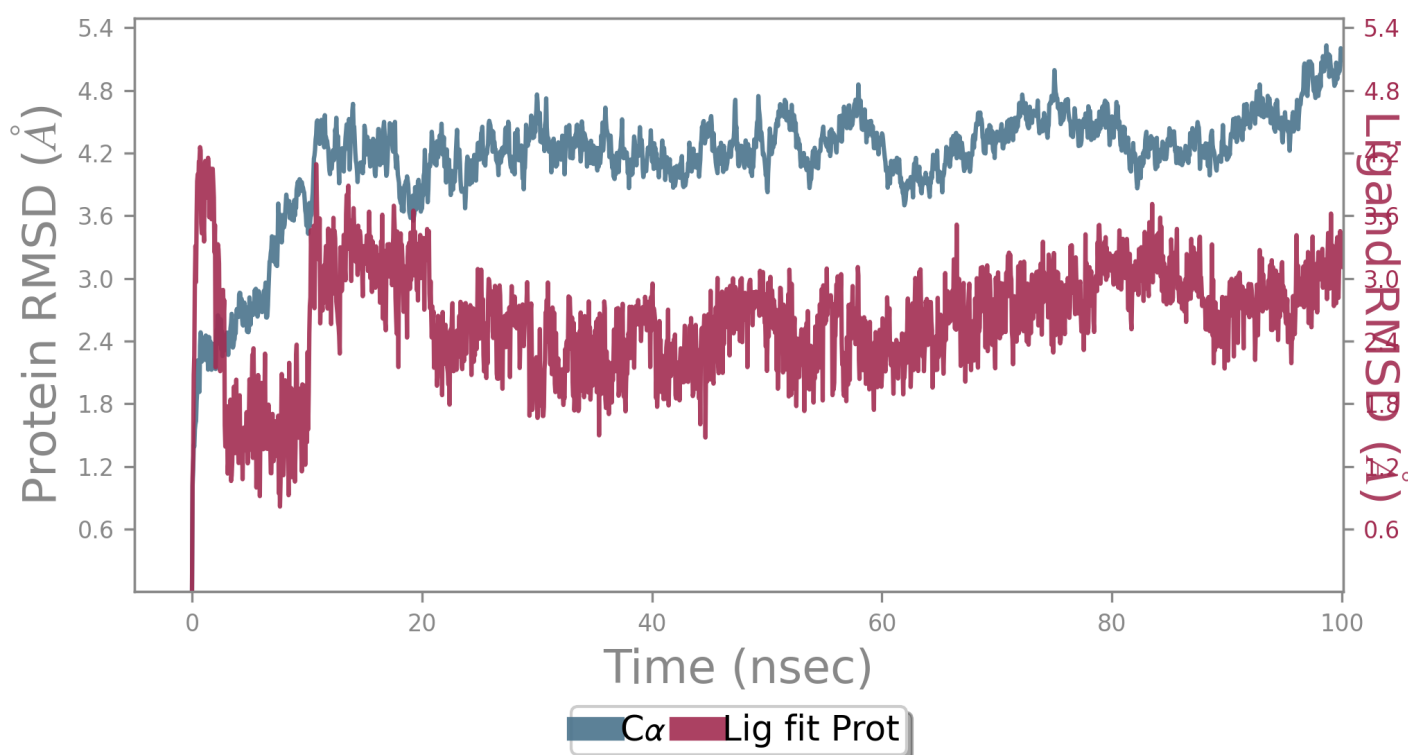
SMILES	c1cccc(c12)C(=O)C=C(C2=O)OCc3cn(nn3)Cc4ccccc4
PDB Name	'UNK'
Num. of Atoms	41 (total) 26 (heavy)
Atomic Mass	345.361 au
Charge	0
Mol. Formula	C20H15N3O3
Num. of Fragments	4
Num. of Rot. Bonds	5



Counter Ion/Salt Information

Type	Num.	Concentration [mM]	Total Charge
Na	88	55.844	+88
Cl	79	50.133	-79

Protein-Ligand RMSD



The Root Mean Square Deviation (RMSD) is used to measure the average change in displacement of a selection of atoms for a particular frame with respect to a reference frame. It is calculated for all frames in the trajectory. The RMSD for frame x is:

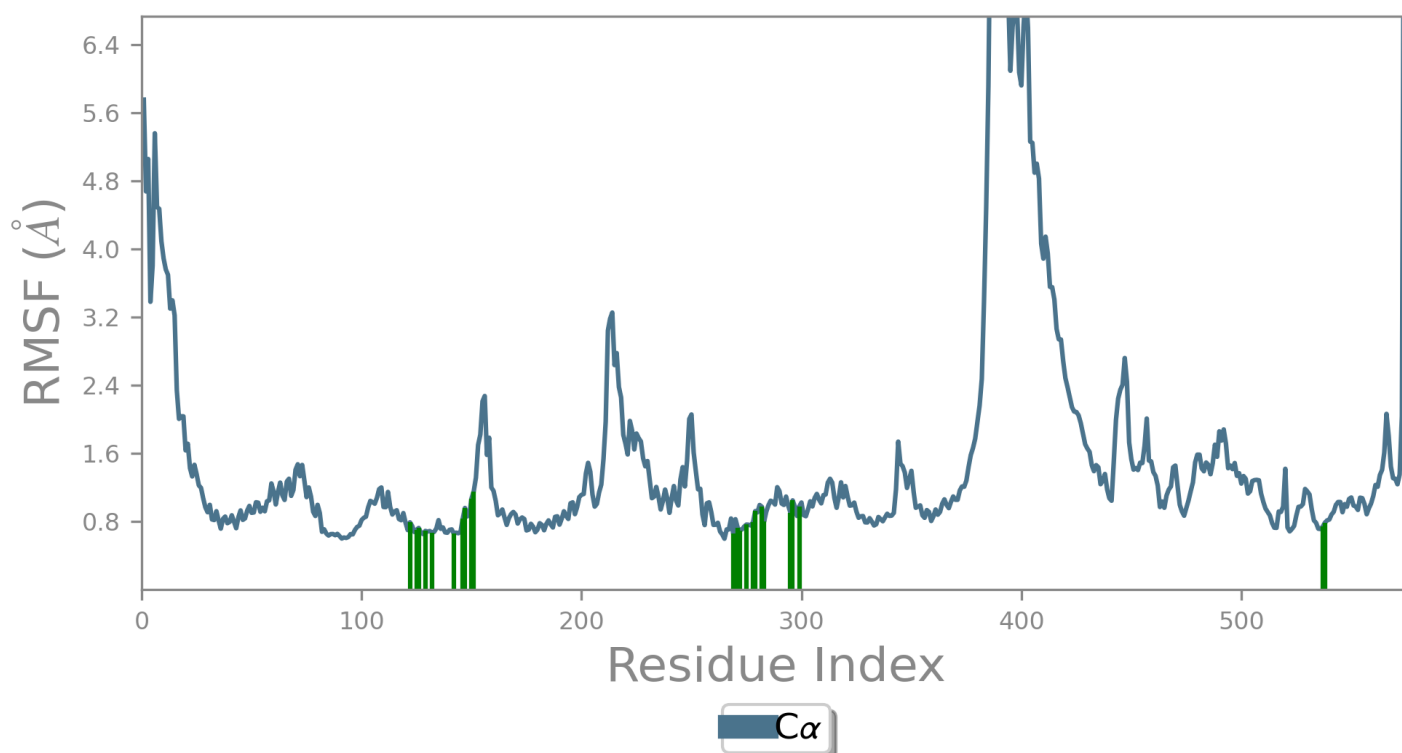
$$RMSD_x = \sqrt{\frac{1}{N} \sum_{i=1}^N (r'_i(t_x) - r_i(t_{ref}))^2}$$

where N is the number of atoms in the atom selection; t_{ref} is the reference time, (typically the first frame is used as the reference and it is regarded as time $t=0$); and r' is the position of the selected atoms in frame x after superimposing on the reference frame, where frame x is recorded at time t_x . The procedure is repeated for every frame in the simulation trajectory.

Protein RMSD: The above plot shows the RMSD evolution of a protein (left Y-axis). All protein frames are first aligned on the reference frame backbone, and then the RMSD is calculated based on the atom selection. Monitoring the RMSD of the protein can give insights into its structural conformation throughout the simulation. RMSD analysis can indicate if the simulation has equilibrated — its fluctuations towards the end of the simulation are around some thermal average structure. Changes of the order of 1-3 Å are perfectly acceptable for small, globular proteins. Changes much larger than that, however, indicate that the protein is undergoing a large conformational change during the simulation. It is also important that your simulation converges — the RMSD values stabilize around a fixed value. If the RMSD of the protein is still increasing or decreasing on average at the end of the simulation, then your system has not equilibrated, and your simulation may not be long enough for rigorous analysis.

Ligand RMSD: Ligand RMSD (right Y-axis) indicates how stable the ligand is with respect to the protein and its binding pocket. In the above plot, 'Lig fit Prot' shows the RMSD of a ligand when the protein-ligand complex is first aligned on the protein backbone of the reference and then the RMSD of the ligand heavy atoms is measured. If the values observed are significantly larger than the RMSD of the protein, then it is likely that the ligand has diffused away from its initial binding site.

Protein RMSF



The Root Mean Square Fluctuation (RMSF) is useful for characterizing local changes along the protein chain. The RMSF for residue i is:

$$RMSF_i = \sqrt{\frac{1}{T} \sum_{t=1}^T \langle (r'_i(t)) - r_i(t_{ref})^2 \rangle}$$

where T is the trajectory time over which the RMSF is calculated, t_{ref} is the reference time, r_i is the position of residue i ; r' is the position of atoms in residue i after superposition on the reference, and the angle brackets indicate that the average of the square distance is taken over the selection of atoms in the residue.

On this plot, peaks indicate areas of the protein that fluctuate the most during the simulation. Typically you will observe that the tails (N - and C -terminal) fluctuate more than any other part of the protein. Secondary structure elements like alpha helices and beta strands are usually more rigid than the unstructured part of the protein, and thus fluctuate less than the loop regions.

Ligand Contacts: Protein residues that interact with the ligand are marked with green-colored vertical bars.

Protein Secondary Structure

% Helix

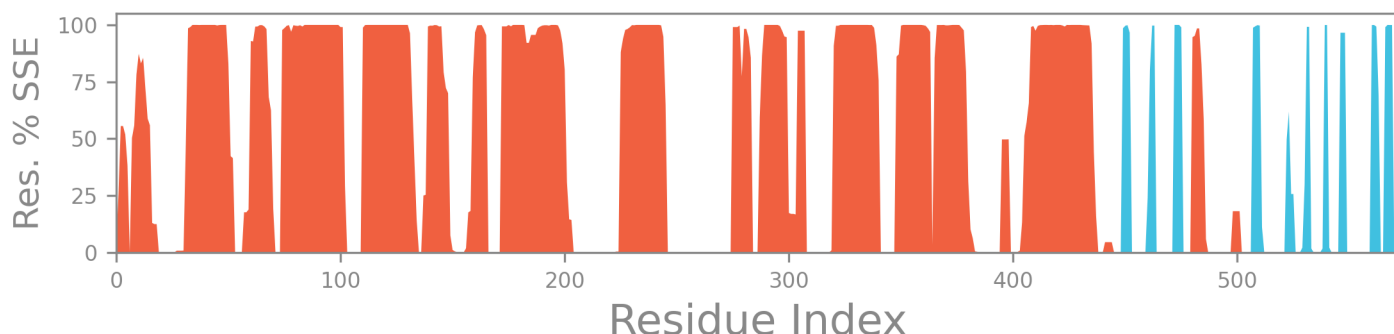
47.50

% Strand

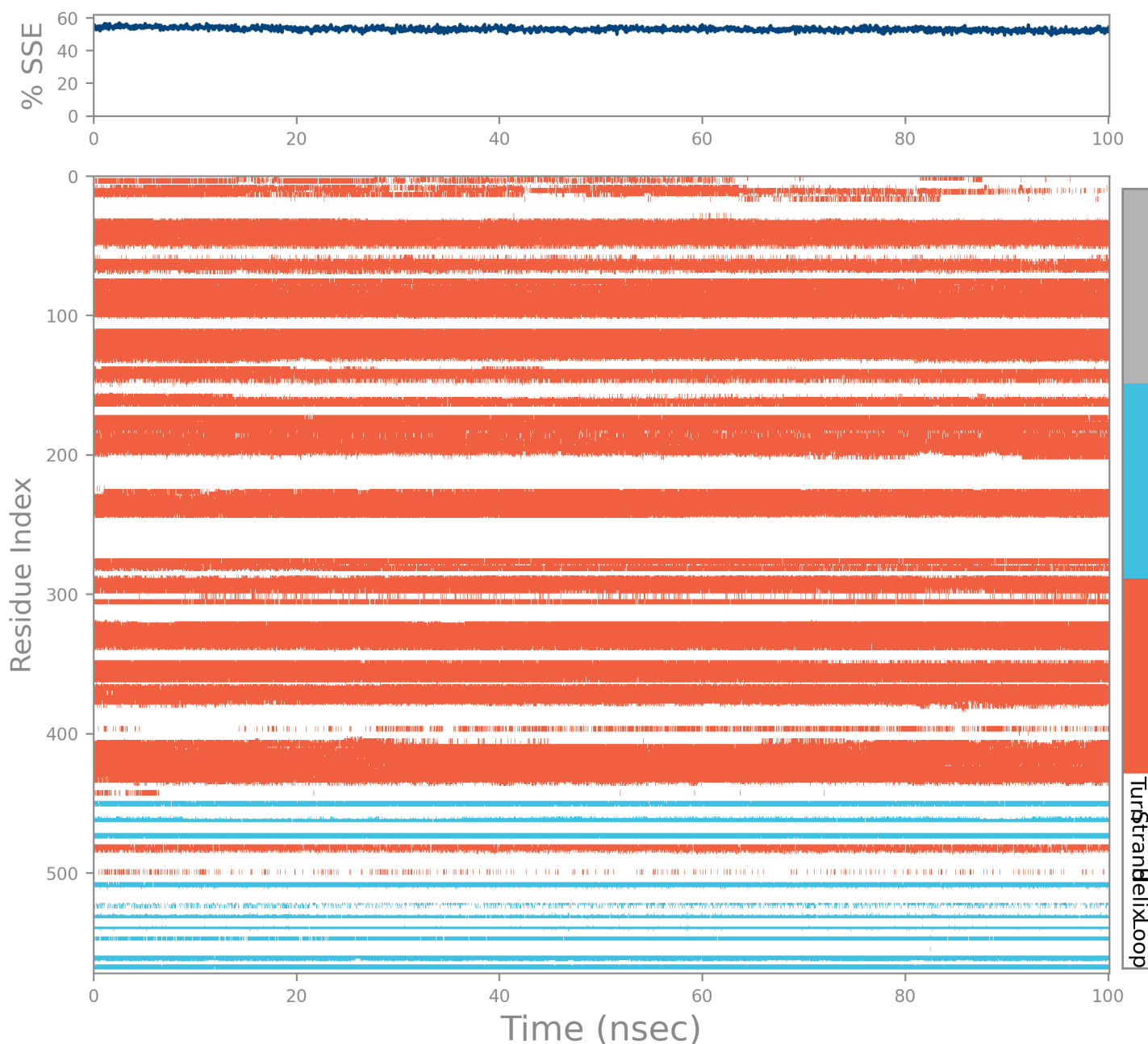
5.53

% Total SSE

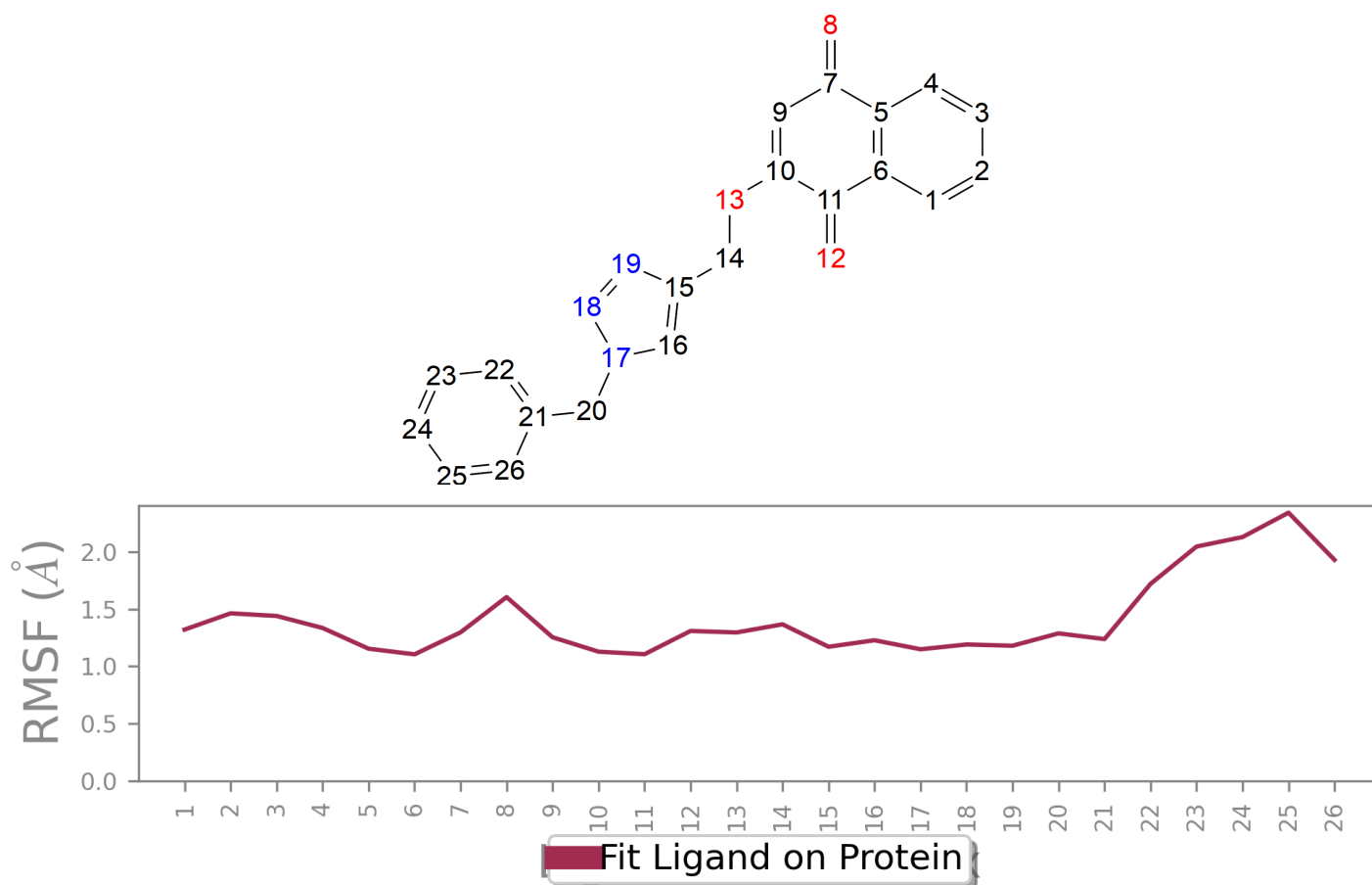
53.03



Protein secondary structure elements (SSE) like **alpha-helices** and **beta-strands** are monitored throughout the simulation. The plot above reports SSE distribution by residue index throughout the protein structure. The plot below summarizes the SSE composition for each trajectory frame over the course of the simulation, and the plot at the bottom monitors each residue and its SSE assignment over time.



Ligand RMSF



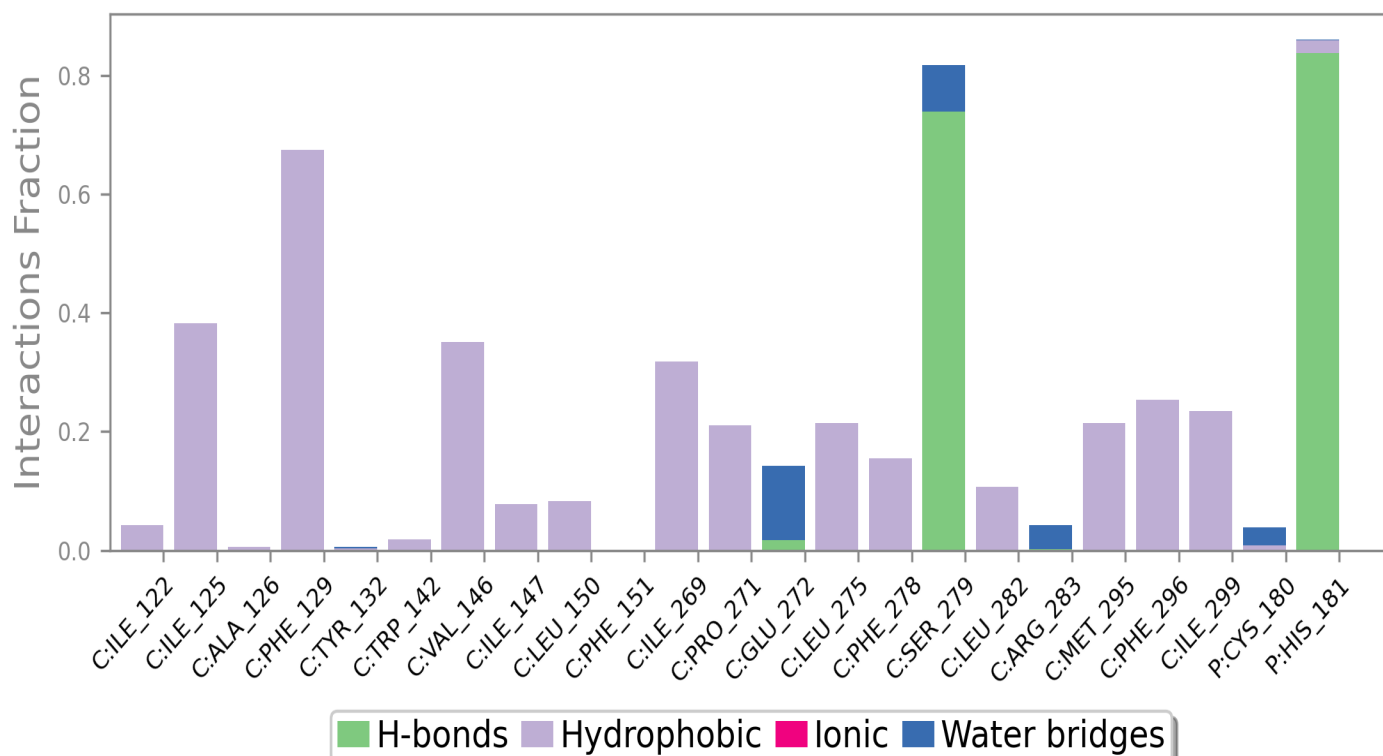
The Ligand Root Mean Square Fluctuation (L-RMSF) is useful for characterizing changes in the ligand atom positions. The RMSF for atom i is:

$$RMSF_i = \sqrt{\frac{1}{T} \sum_{t=1}^T (r'_i(t) - r_i(t_{ref}))^2}$$

where T is the trajectory time over which the RMSF is calculated, t_{ref} is the reference time (usually for the first frame, and is regarded as the zero of time); r is the position of atom i in the reference at time t_{ref} and r' is the position of atom i at time t after superposition on the reference frame.

Ligand RMSF shows the ligand's fluctuations broken down by atom, corresponding to the 2D structure in the top panel. The ligand RMSF may give you insights on how ligand fragments interact with the protein and their entropic role in the binding event. In the bottom panel, the 'Fit Ligand on Protein' line shows the ligand fluctuations, with respect to the protein. The protein-ligand complex is first aligned on the protein backbone and then the ligand RMSF is measured on the ligand heavy atoms.

Protein-Ligand Contacts



Protein interactions with the ligand can be monitored throughout the simulation. These interactions can be categorized by type and summarized, as shown in the plot above. Protein-ligand interactions (or 'contacts') are categorized into four types: Hydrogen Bonds, Hydrophobic, Ionic and Water Bridges. Each interaction type contains more specific subtypes, which can be explored through the 'Simulation Interactions Diagram' panel. The stacked bar charts are normalized over the course of the trajectory: for example, a value of 0.7 suggests that 70% of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts of same subtype with the ligand.

Hydrogen Bonds: (H-bonds) play a significant role in ligand binding. Consideration of hydrogen-bonding properties in drug design is important because of their strong influence on drug specificity, metabolism and adsorption. Hydrogen bonds between a protein and a ligand can be further broken down into four subtypes: backbone acceptor; backbone donor; side-chain acceptor; side-chain donor.

The current geometric criteria for protein-ligand H-bond is: distance of 2.5 Å between the donor and acceptor atoms (D—H...A); a donor angle of $\geq 120^\circ$ between the donor-hydrogen-acceptor atoms (D—H...A); and an acceptor angle of $\geq 90^\circ$ between the hydrogen-acceptor-bonded_atom atoms (H...A—X).

Hydrophobic contacts: fall into three subtypes: π -Cation; π - π ; and Other, non-specific interactions. Generally these type of interactions involve a hydrophobic amino acid and an aromatic or aliphatic group on the ligand, but we have extended this category to also include π -Cation interactions.

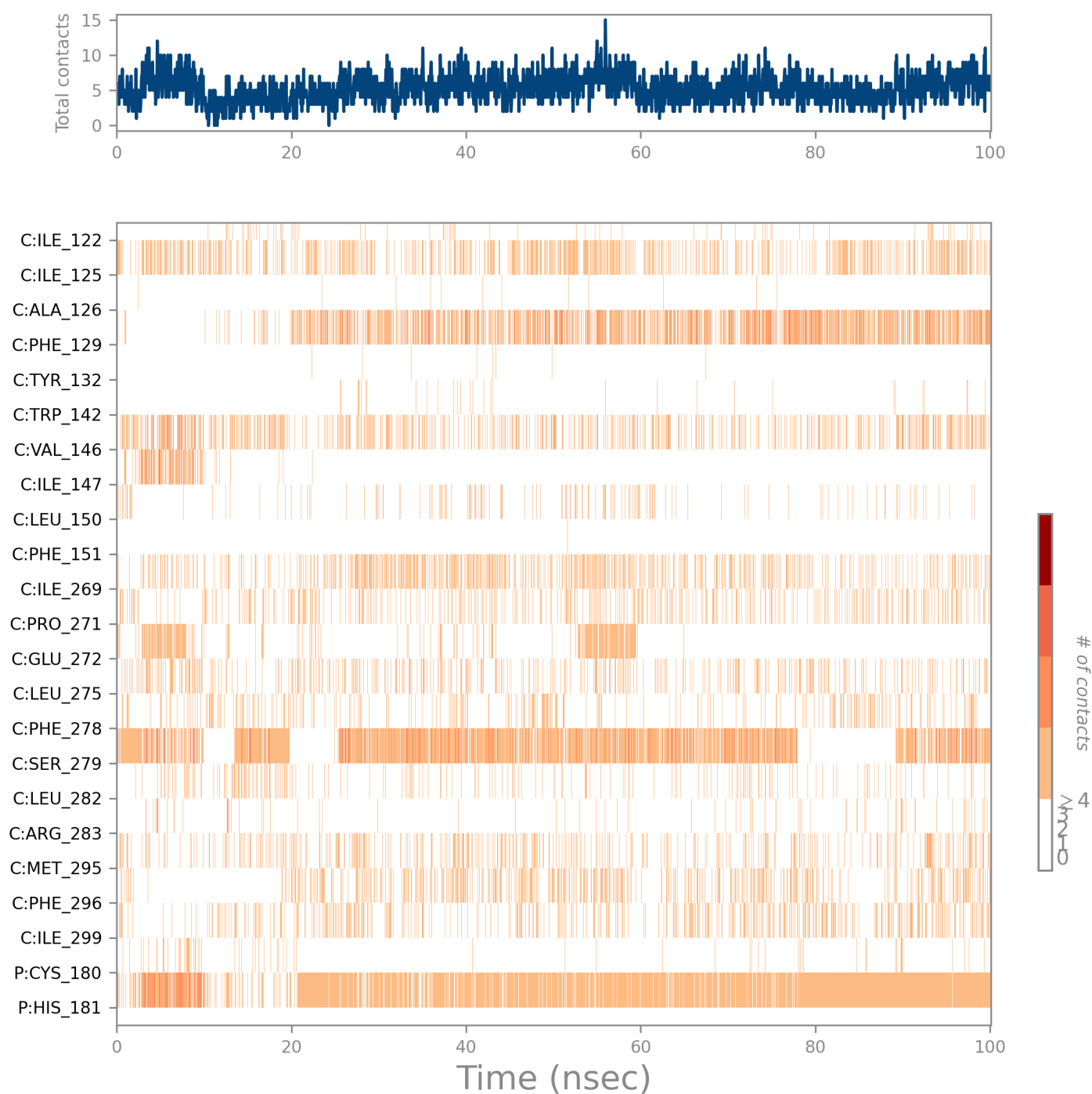
The current geometric criteria for hydrophobic interactions is as follows: π -Cation — Aromatic and charged groups within 4.5 Å; π - π — Two aromatic groups stacked face-to-face or face-to-edge; Other — A non-specific hydrophobic sidechain within 3.6 Å of a ligand's aromatic or aliphatic carbons.

Ionic interactions: or polar interactions, are between two oppositely charged atoms that are within 3.7 Å of each other and do not involve a hydrogen bond. We also monitor Protein-Metal-Ligand interactions, which are defined by a metal ion coordinated within 3.4 Å of protein's and ligand's heavy atoms (except carbon). All ionic interactions are broken down into two subtypes: those mediated by a protein backbone or side chains.

Water Bridges: are hydrogen-bonded protein-ligand interactions mediated by a water molecule. The hydrogen-bond geometry is slightly relaxed from the standard H-bond definition.

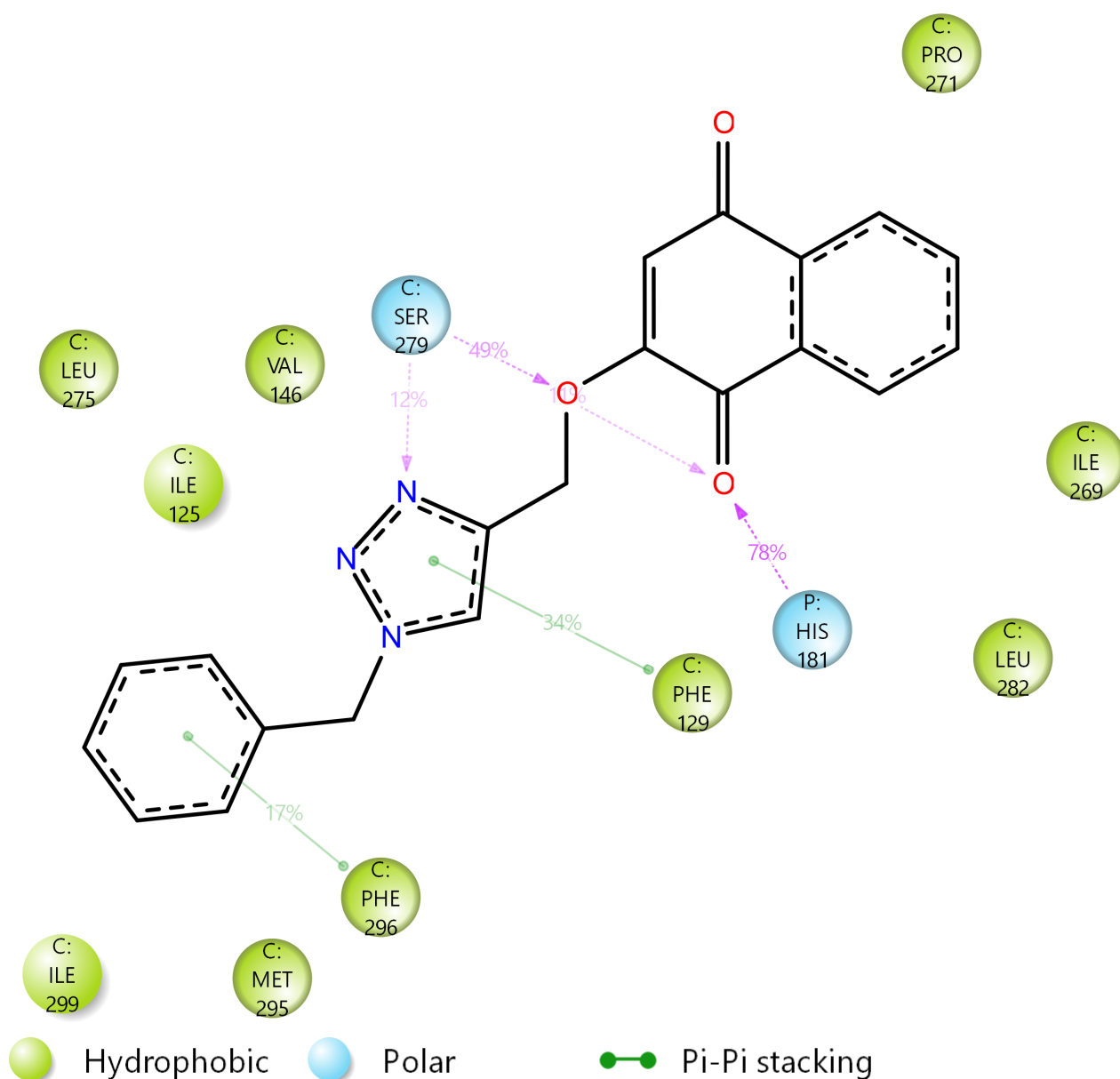
The current geometric criteria for a protein-water or water-ligand H-bond are: a distance of 2.8 Å between the donor and acceptor atoms (D—H...A); a donor angle of $\geq 110^\circ$ between the donor-hydrogen-acceptor atoms (D—H...A); and an acceptor angle of $\geq 90^\circ$ between the hydrogen-acceptor-bonded_atom atoms (H...A—X).

Protein-Ligand Contacts (cont.)



A timeline representation of the interactions and contacts (**H-bonds, Hydrophobic, Ionic, Water bridges**) summarized in the previous page. The top panel shows the total number of specific contacts the protein makes with the ligand over the course of the trajectory. The bottom panel shows which residues interact with the ligand in each trajectory frame. Some residues make more than one specific contact with the ligand, which is represented by a darker shade of orange, according to the scale to the right of the plot.

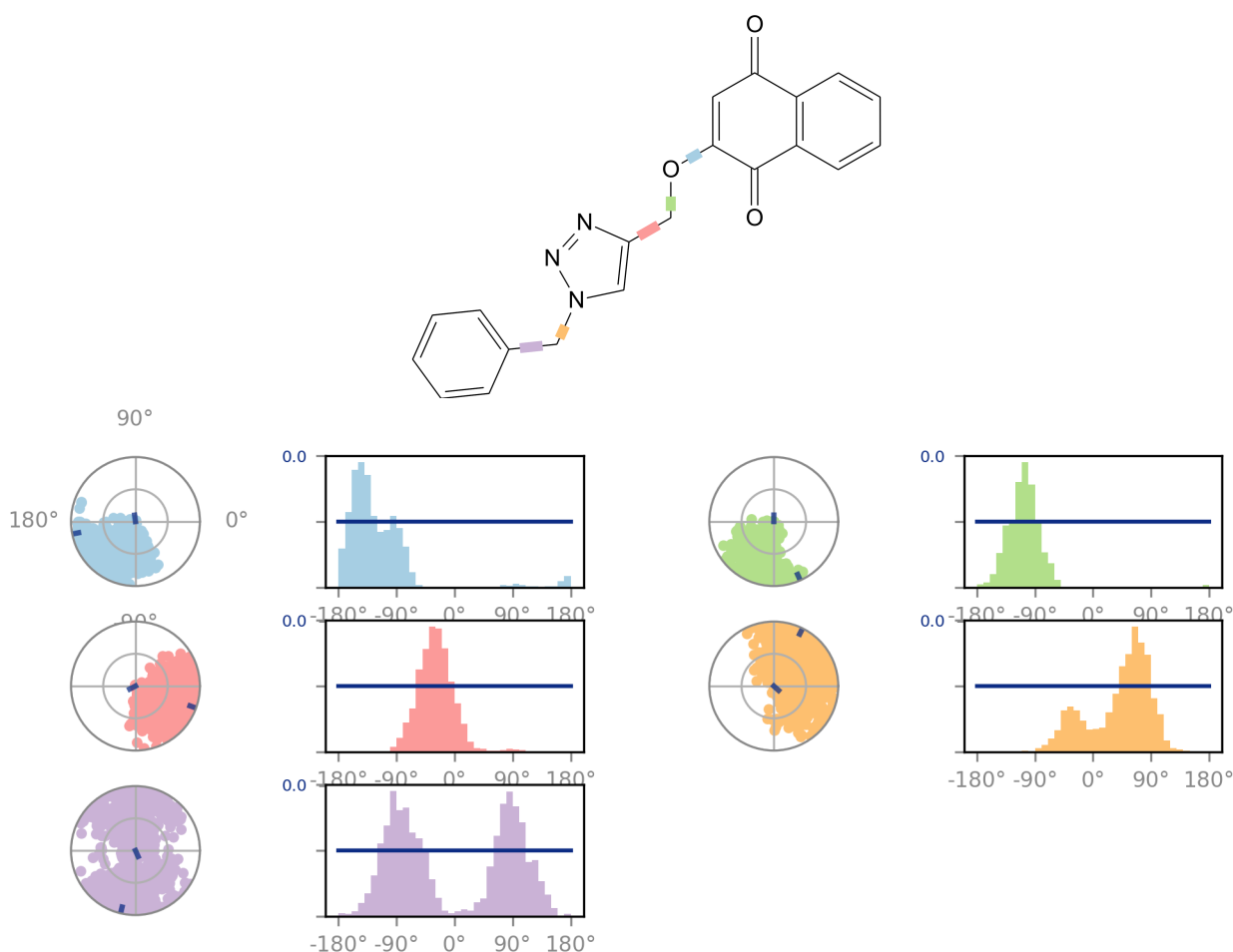
Ligand-Protein Contacts



A schematic of detailed ligand atom interactions with the protein residues. Interactions that occur more than **10.0%** of the simulation time in the selected trajectory (0.00 through 100.10 nsec), are shown.

Note: it is possible to have interactions with >100% as some residues may have multiple interactions of a single type with the same ligand atom. For example, the ARG side chain has four H-bond donors that can all hydrogen-bond to a single H-bond acceptor.

Ligand Torsion Profile

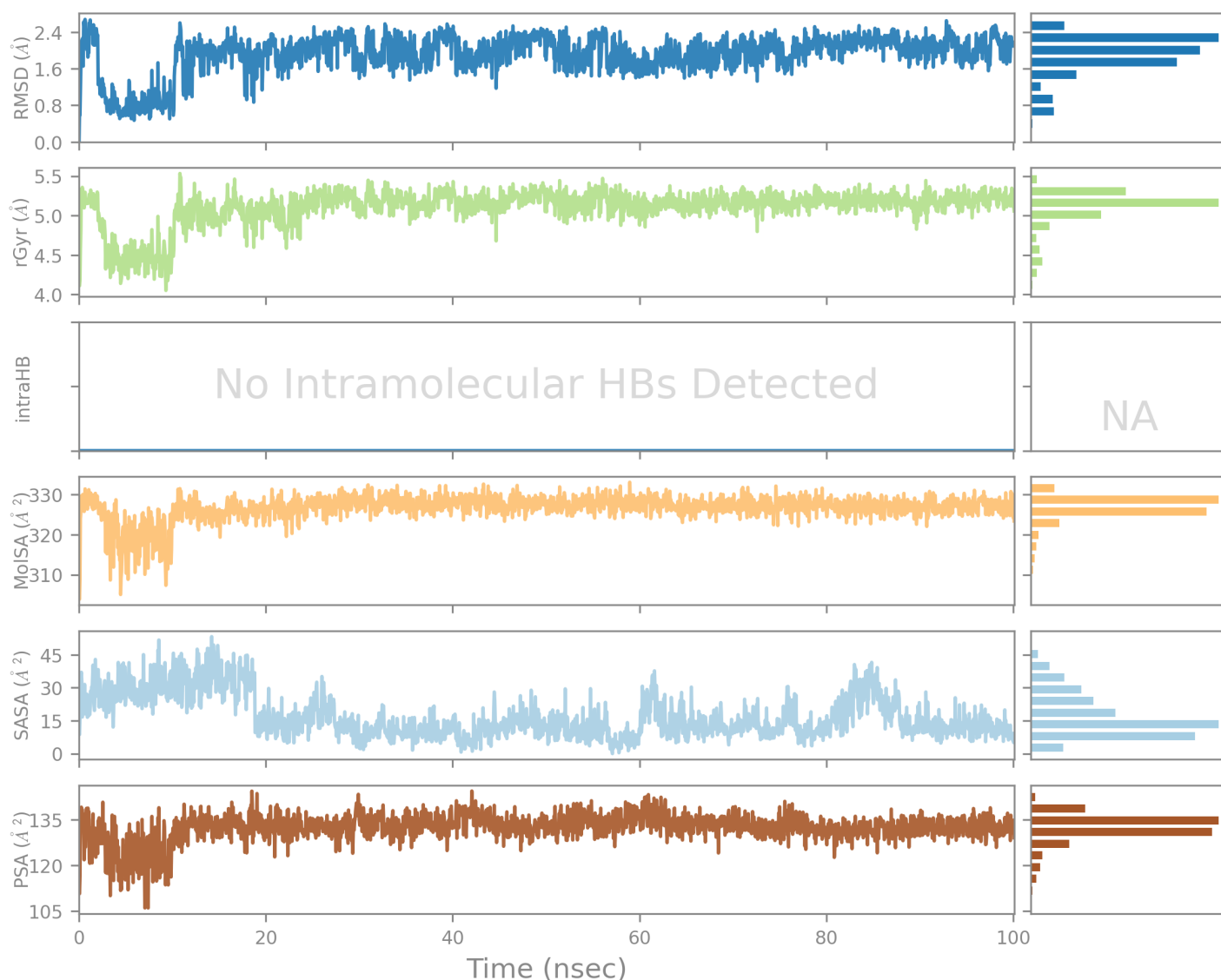


The ligand torsions plot summarizes the conformational evolution of every rotatable bond (RB) in the ligand throughout the simulation trajectory (0.00 through 100.10 nsec). The top panel shows the 2d schematic of a ligand with color-coded rotatable bonds. Each rotatable bond torsion is accompanied by a dial plot and bar plots of the same color.

Dial (or radial) plots describe the conformation of the torsion throughout the course of the simulation. The beginning of the simulation is in the center of the radial plot and the time evolution is plotted radially outwards.

The bar plots summarize the data on the dial plots, by showing the probability density of the torsion. If torsional potential information is available, the plot also shows the potential of the rotatable bond (by summing the potential of the related torsions). The values of the potential are on the left Y-axis of the chart, and are expressed in *kcal/mol*. Looking at the histogram and torsion potential relationships may give insights into the conformational strain the ligand undergoes to maintain a protein-bound conformation.

Ligand Properties



Ligand RMSD: Root mean square deviation of a ligand with respect to the reference conformation (typically the first frame is used as the reference and it is regarded as time $t=0$).

Radius of Gyration (rGyr): Measures the 'extendedness' of a ligand, and is equivalent to its principal moment of inertia.

Intramolecular Hydrogen Bonds (intraHB): Number of internal hydrogen bonds (HB) within a ligand molecule.

Molecular Surface Area (MolSA): Molecular surface calculation with 1.4 Å probe radius. This value is equivalent to a van der Waals surface area.

Solvent Accessible Surface Area (SASA): Surface area of a molecule accessible by a water molecule.

Polar Surface Area (PSA): Solvent accessible surface area in a molecule contributed only by oxygen and nitrogen atoms.

Simulation Interactions Diagram Report

Simulation Details

Jobname: merged
Entry title: 3CX5_truncated - prepared D13 + DPPC

CPU #	Job Type	Ensemble	Temp. [K]	Sim. Time [ns]	# Atoms	# Waters	Charge
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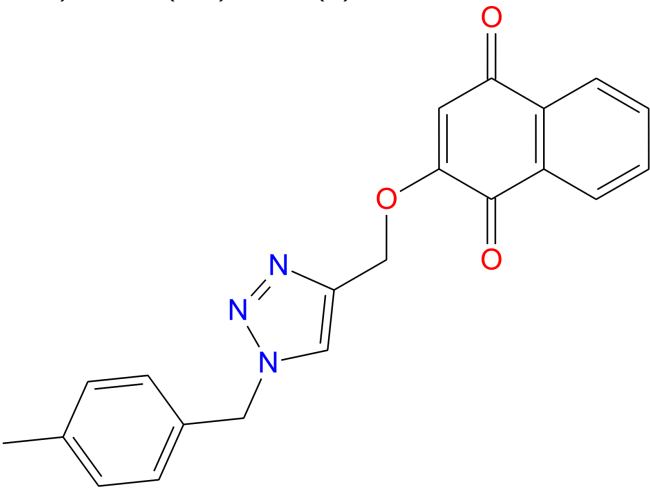
* The configuration file (-out.cfg) was not found. Keep it in same directory as .aef file.

Protein Information

	Tot. Residues	Prot. Chain(s)	Res. in Chain(s)	# Atoms	# Heavy Atoms	Charge
	577	'C', 'D', 'L', 'P'	values([388, 1, 1, 187]	9318	4633	-3
C	1	MAFRKSNVYLSLVNSYIIDSPQPSSINYYWNNMGSLLGLCLVIQIVTGIFMAMHYSSNIELAFSSVEHIMR	70			
SSA						
P	31	KSTYRTPNFDDVLKENNDADKGRSYAYFMVGAMGLSSAGAKSTVETFISSMTATADVLAMAKVEVNLA	100			
SSA						
C	71	DVHNGYILRYLHANGASFFFMVFMHMAKGLYGSYRSPRVTLWNVGVIIIFILTITATFLGYCCVYGQMS	140			
SSA						
P	101	IPLGKNVVVKWQGPVFIHRTPHEIQEANSVDMALKDPQTDADRVKDPQWLIMLGICTHLGCVPIGEA	170			
SSA						
C	141	HWGATVITNLFSAIPFVGNDIVSWLWGGFSVSNPTIQRFFALHYLVPFIIAAMVIMHLMALHIHGSSNPL	210			
SSA						
P	171	GDFGGWFCPCCHGSHYDISGRIRKGPAPLNLEIPAYEFDGDKVIVGX	215A			
SSA						
C	211	GITGNLDRIPMHSYFIFKDLVTVFLFMLILALFVFYSPNTLGHDPDNYIPGNPLVTPASIVPEWYLLPFSA	280			
SSA						
C	281	ILRSIPDKLLGVITMFAAILVLLVLPFTDRSVVRGNTFKVLISKFFFFIFVFNFVLLGQIGACHVEVPYVL	350			
SSA						
C	351	MGQIATFIYFAYFLIIVPVISTIENVLFYIGRVNKXX	4033			
SSA						

Ligand Information

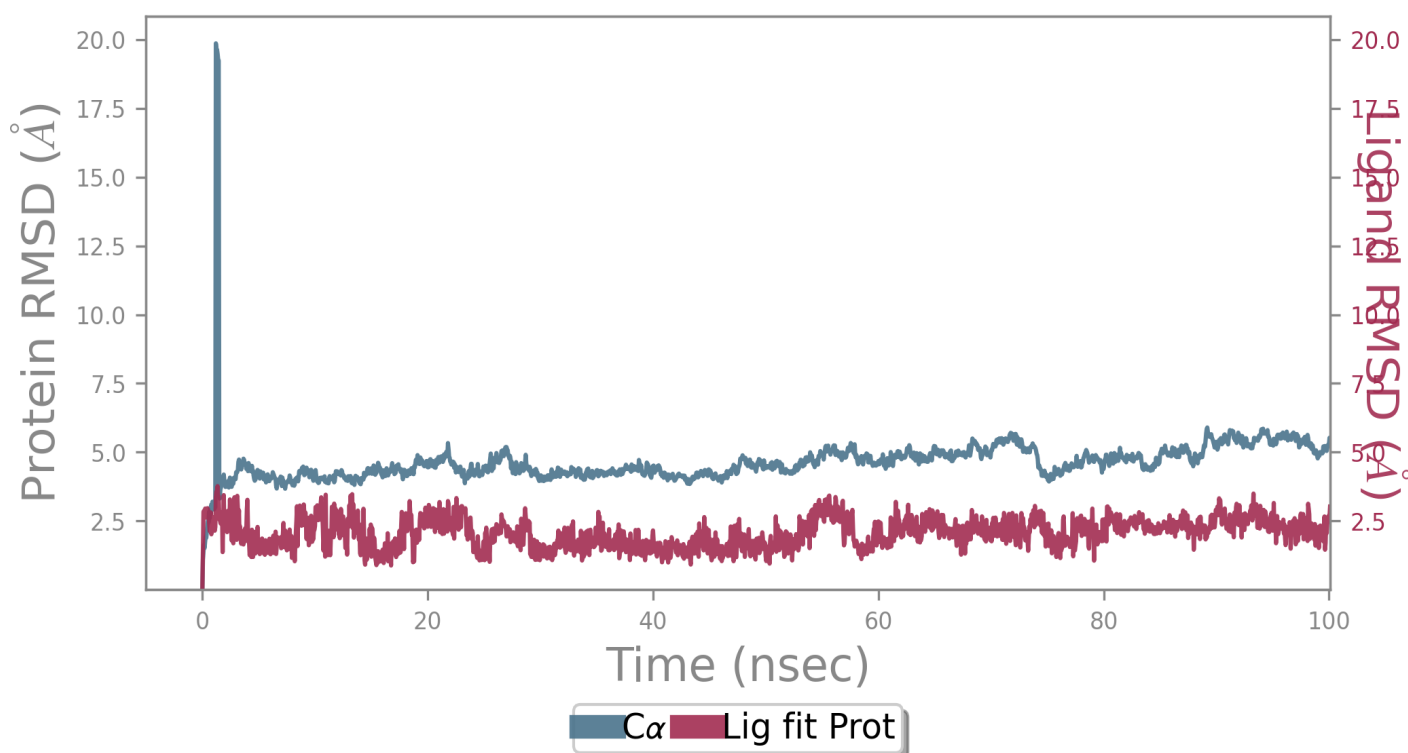
SMILES	c1cccc(c12)C(=O)C=C(C2=O)OCc3cn(nn3)Cc4ccc(C)cc4
PDB Name	'UNK'
Num. of Atoms	44 (total) 27 (heavy)
Atomic Mass	359.388 au
Charge	0
Mol. Formula	C21H17N3O3
Num. of Fragments	4
Num. of Rot. Bonds	5



Counter Ion/Salt Information

Type	Num.	Concentration [mM]	Total Charge
Na	88	55.893	+88
Cl	79	50.177	-79

Protein-Ligand RMSD



The Root Mean Square Deviation (RMSD) is used to measure the average change in displacement of a selection of atoms for a particular frame with respect to a reference frame. It is calculated for all frames in the trajectory. The RMSD for frame x is:

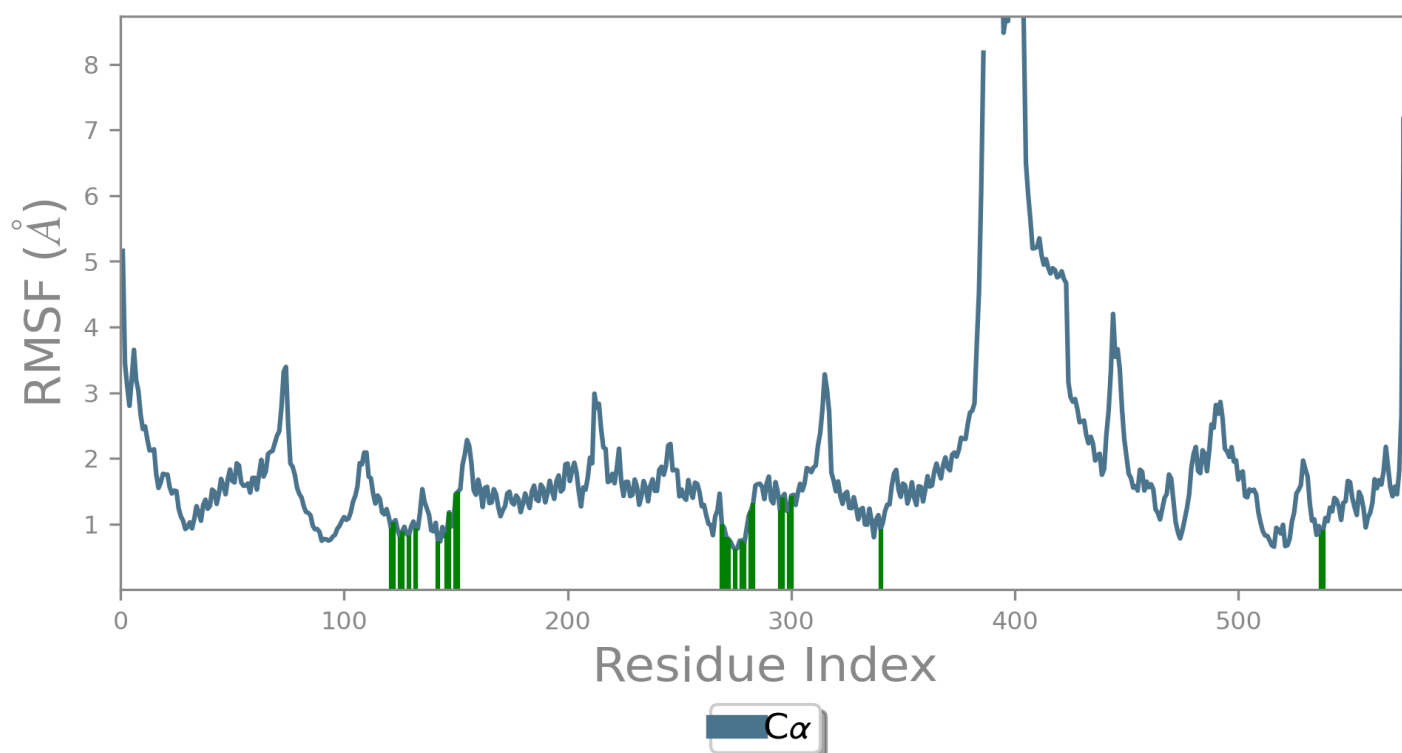
$$RMSD_x = \sqrt{\frac{1}{N} \sum_{i=1}^N (r'_i(t_x) - r_i(t_{ref}))^2}$$

where N is the number of atoms in the atom selection; t_{ref} is the reference time, (typically the first frame is used as the reference and it is regarded as time $t=0$); and r' is the position of the selected atoms in frame x after superimposing on the reference frame, where frame x is recorded at time t_x . The procedure is repeated for every frame in the simulation trajectory.

Protein RMSD: The above plot shows the RMSD evolution of a protein (left Y-axis). All protein frames are first aligned on the reference frame backbone, and then the RMSD is calculated based on the atom selection. Monitoring the RMSD of the protein can give insights into its structural conformation throughout the simulation. RMSD analysis can indicate if the simulation has equilibrated — its fluctuations towards the end of the simulation are around some thermal average structure. Changes of the order of 1-3 Å are perfectly acceptable for small, globular proteins. Changes much larger than that, however, indicate that the protein is undergoing a large conformational change during the simulation. It is also important that your simulation converges — the RMSD values stabilize around a fixed value. If the RMSD of the protein is still increasing or decreasing on average at the end of the simulation, then your system has not equilibrated, and your simulation may not be long enough for rigorous analysis.

Ligand RMSD: Ligand RMSD (right Y-axis) indicates how stable the ligand is with respect to the protein and its binding pocket. In the above plot, 'Lig fit Prot' shows the RMSD of a ligand when the protein-ligand complex is first aligned on the protein backbone of the reference and then the RMSD of the ligand heavy atoms is measured. If the values observed are significantly larger than the RMSD of the protein, then it is likely that the ligand has diffused away from its initial binding site.

Protein RMSF



The Root Mean Square Fluctuation (RMSF) is useful for characterizing local changes along the protein chain. The RMSF for residue i is:

$$RMSF_i = \sqrt{\frac{1}{T} \sum_{t=1}^T \langle (r'_i(t)) - r_i(t_{ref})^2 \rangle}$$

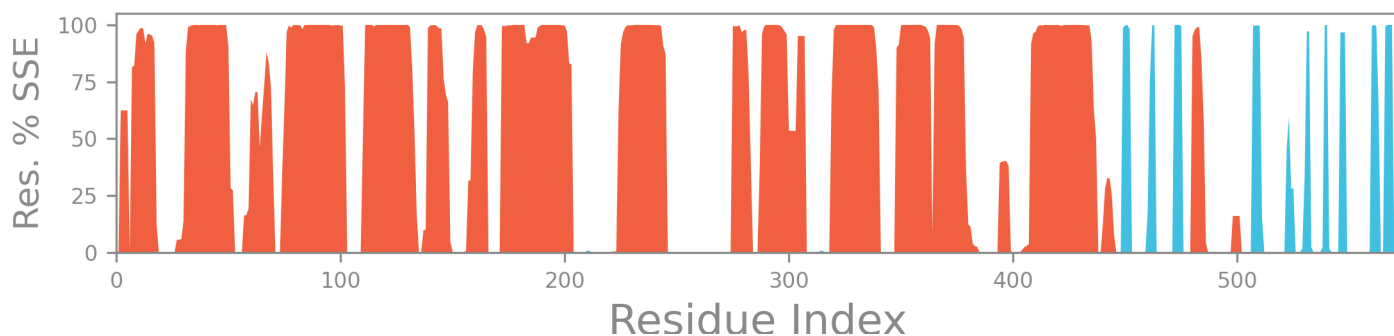
where T is the trajectory time over which the RMSF is calculated, t_{ref} is the reference time, r_i is the position of residue i ; r' is the position of atoms in residue i after superposition on the reference, and the angle brackets indicate that the average of the square distance is taken over the selection of atoms in the residue.

On this plot, peaks indicate areas of the protein that fluctuate the most during the simulation. Typically you will observe that the tails (N - and C -terminal) fluctuate more than any other part of the protein. Secondary structure elements like alpha helices and beta strands are usually more rigid than the unstructured part of the protein, and thus fluctuate less than the loop regions.

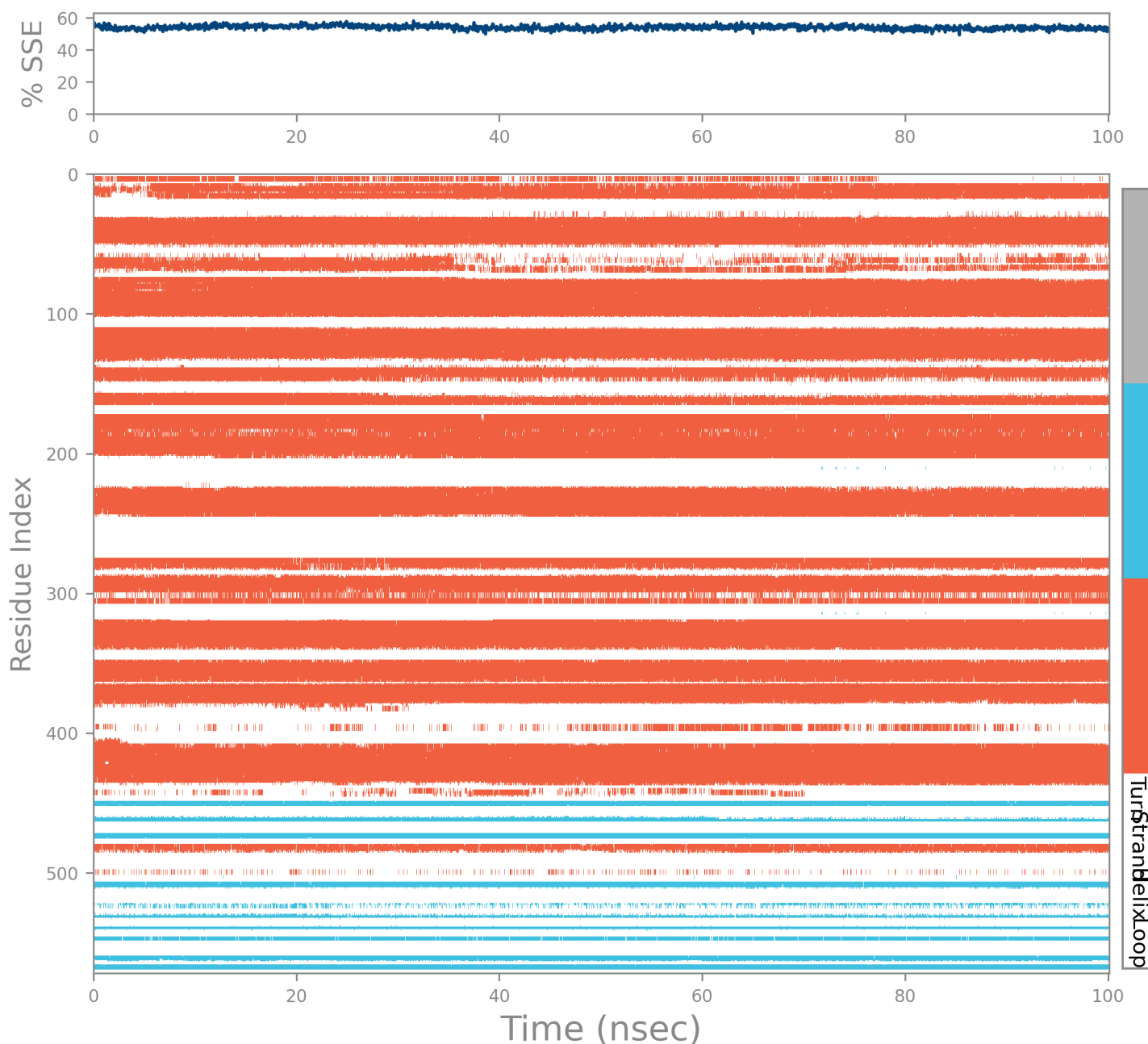
Ligand Contacts: Protein residues that interact with the ligand are marked with green-colored vertical bars.

Protein Secondary Structure

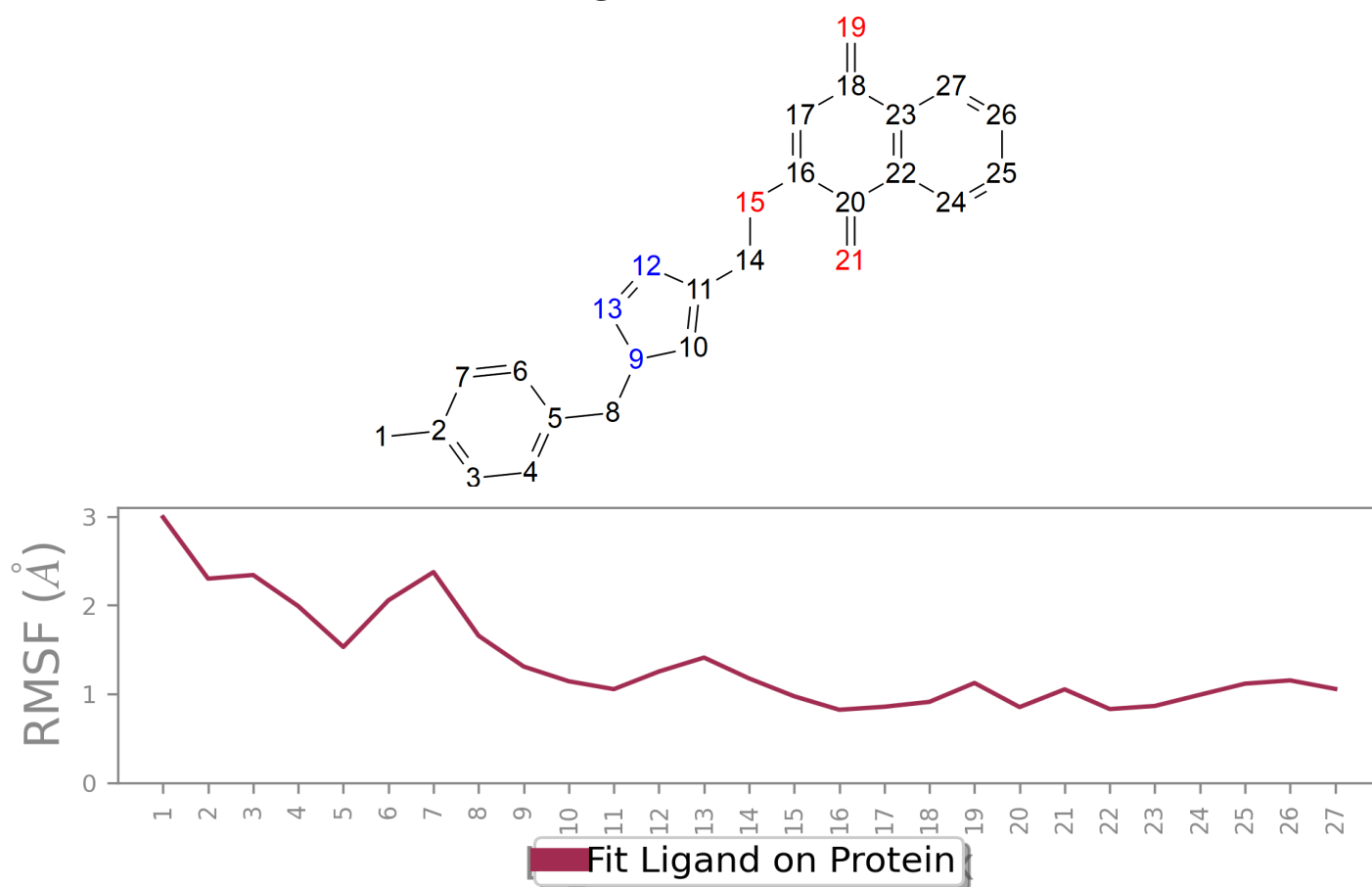
% Helix 48.48 % Strand 5.52 % Total SSE 54.00



Protein secondary structure elements (SSE) like **alpha-helices** and **beta-strands** are monitored throughout the simulation. The plot above reports SSE distribution by residue index throughout the protein structure. The plot below summarizes the SSE composition for each trajectory frame over the course of the simulation, and the plot at the bottom monitors each residue and its SSE assignment over time.



Ligand RMSF



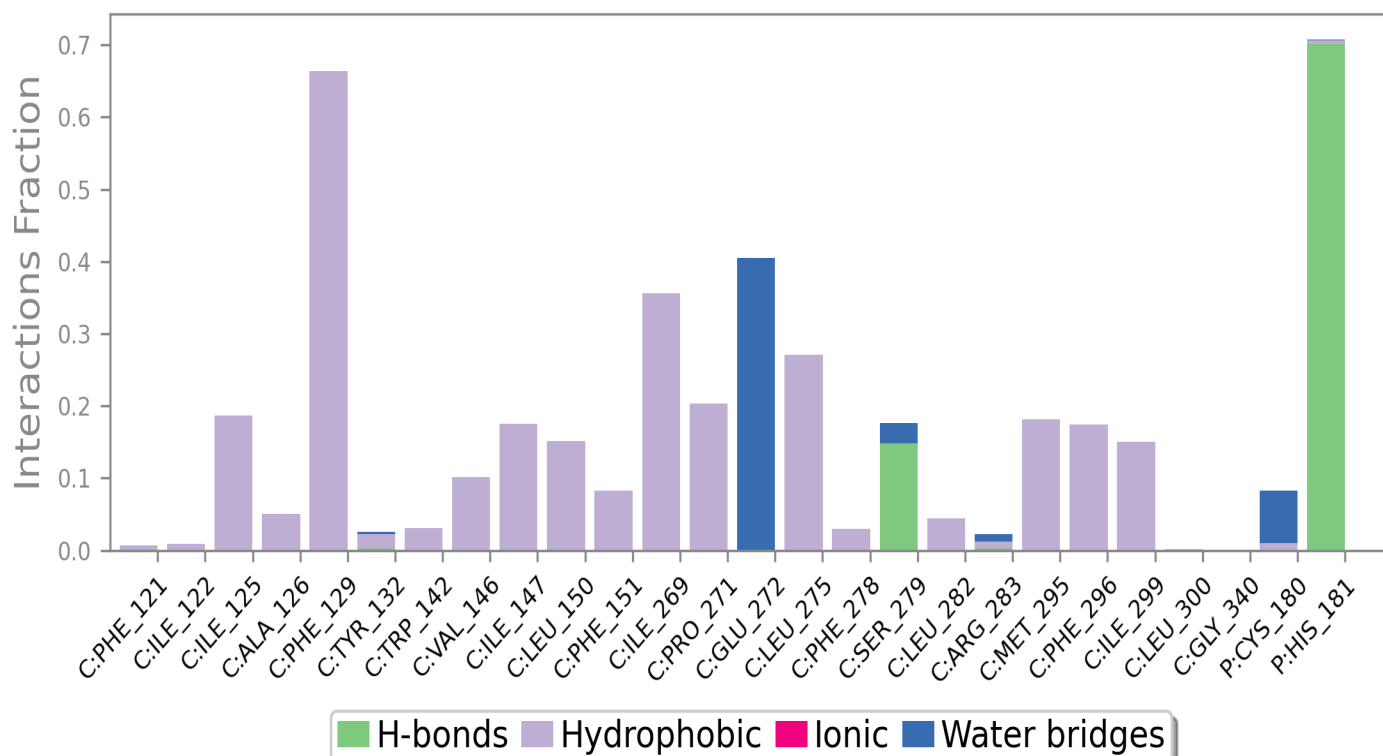
The Ligand Root Mean Square Fluctuation (L-RMSF) is useful for characterizing changes in the ligand atom positions. The RMSF for atom i is:

$$RMSF_i = \sqrt{\frac{1}{T} \sum_{t=1}^T (r'_i(t) - r_i(t_{ref}))^2}$$

where T is the trajectory time over which the RMSF is calculated, t_{ref} is the reference time (usually for the first frame, and is regarded as the zero of time); r is the position of atom i in the reference at time t_{ref} and r' is the position of atom i at time t after superposition on the reference frame.

Ligand RMSF shows the ligand's fluctuations broken down by atom, corresponding to the 2D structure in the top panel. The ligand RMSF may give you insights on how ligand fragments interact with the protein and their entropic role in the binding event. In the bottom panel, the 'Fit Ligand on Protein' line shows the ligand fluctuations, with respect to the protein. The protein-ligand complex is first aligned on the protein backbone and then the ligand RMSF is measured on the ligand heavy atoms.

Protein-Ligand Contacts



Protein interactions with the ligand can be monitored throughout the simulation. These interactions can be categorized by type and summarized, as shown in the plot above. Protein-ligand interactions (or 'contacts') are categorized into four types: Hydrogen Bonds, Hydrophobic, Ionic and Water Bridges. Each interaction type contains more specific subtypes, which can be explored through the 'Simulation Interactions Diagram' panel. The stacked bar charts are normalized over the course of the trajectory: for example, a value of 0.7 suggests that 70% of the simulation time the specific interaction is maintained. Values over 1.0 are possible as some protein residue may make multiple contacts of same subtype with the ligand.

Hydrogen Bonds: (H-bonds) play a significant role in ligand binding. Consideration of hydrogen-bonding properties in drug design is important because of their strong influence on drug specificity, metabolism and adsorption. Hydrogen bonds between a protein and a ligand can be further broken down into four subtypes: backbone acceptor; backbone donor; side-chain acceptor; side-chain donor.

The current geometric criteria for protein-ligand H-bond is: distance of 2.5 Å between the donor and acceptor atoms (D—H...A); a donor angle of $\geq 120^\circ$ between the donor-hydrogen-acceptor atoms (D—H...A); and an acceptor angle of $\geq 90^\circ$ between the hydrogen-acceptor-bonded_atom atoms (H...A—X).

Hydrophobic contacts: fall into three subtypes: π -Cation; π - π ; and Other, non-specific interactions. Generally these type of interactions involve a hydrophobic amino acid and an aromatic or aliphatic group on the ligand, but we have extended this category to also include π -Cation interactions.

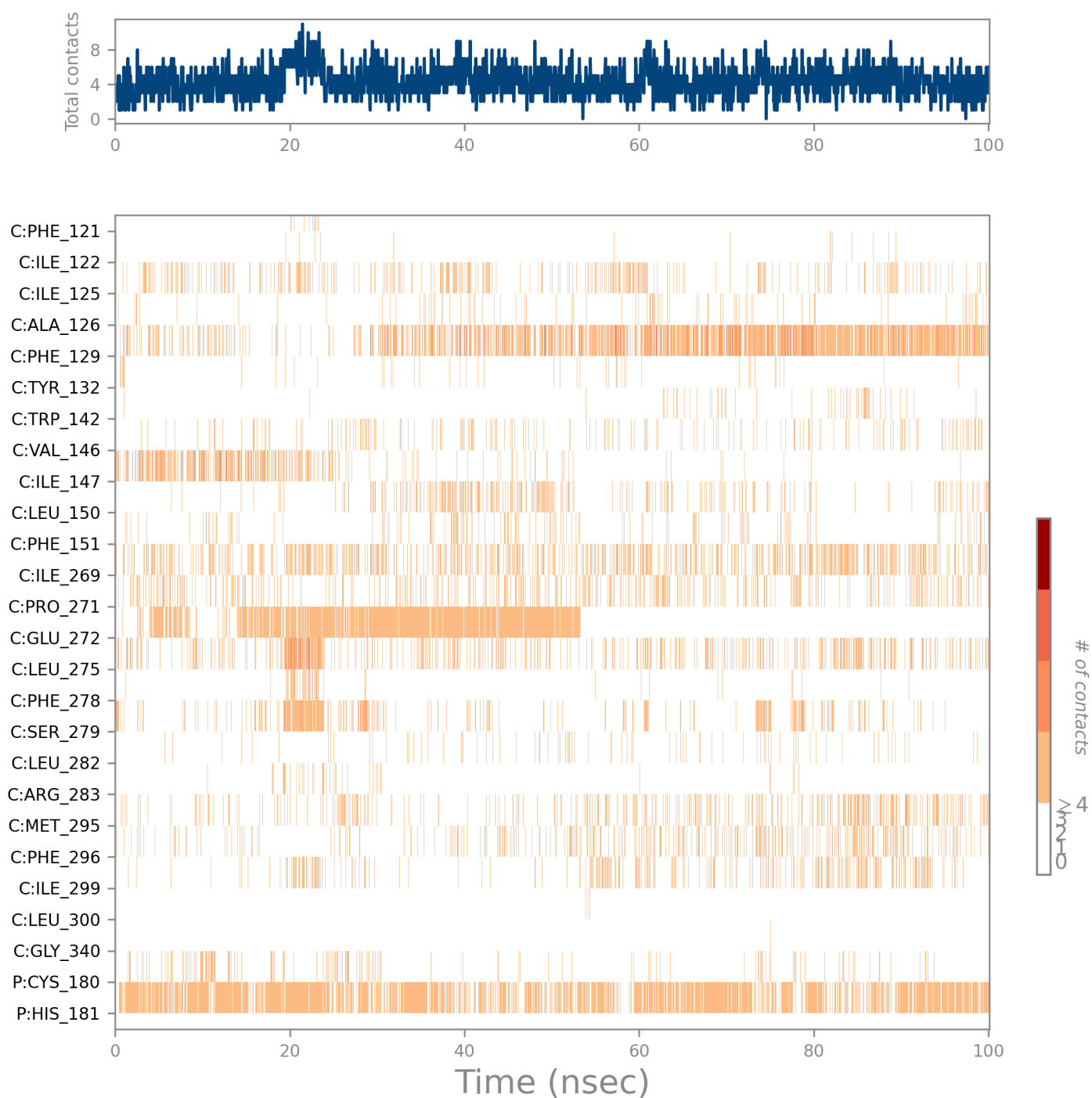
The current geometric criteria for hydrophobic interactions is as follows: π -Cation — Aromatic and charged groups within 4.5 Å; π - π — Two aromatic groups stacked face-to-face or face-to-edge; Other — A non-specific hydrophobic sidechain within 3.6 Å of a ligand's aromatic or aliphatic carbons.

Ionic interactions: or polar interactions, are between two oppositely charged atoms that are within 3.7 Å of each other and do not involve a hydrogen bond. We also monitor Protein-Metal-Ligand interactions, which are defined by a metal ion coordinated within 3.4 Å of protein's and ligand's heavy atoms (except carbon). All ionic interactions are broken down into two subtypes: those mediated by a protein backbone or side chains.

Water Bridges: are hydrogen-bonded protein-ligand interactions mediated by a water molecule. The hydrogen-bond geometry is slightly relaxed from the standard H-bond definition.

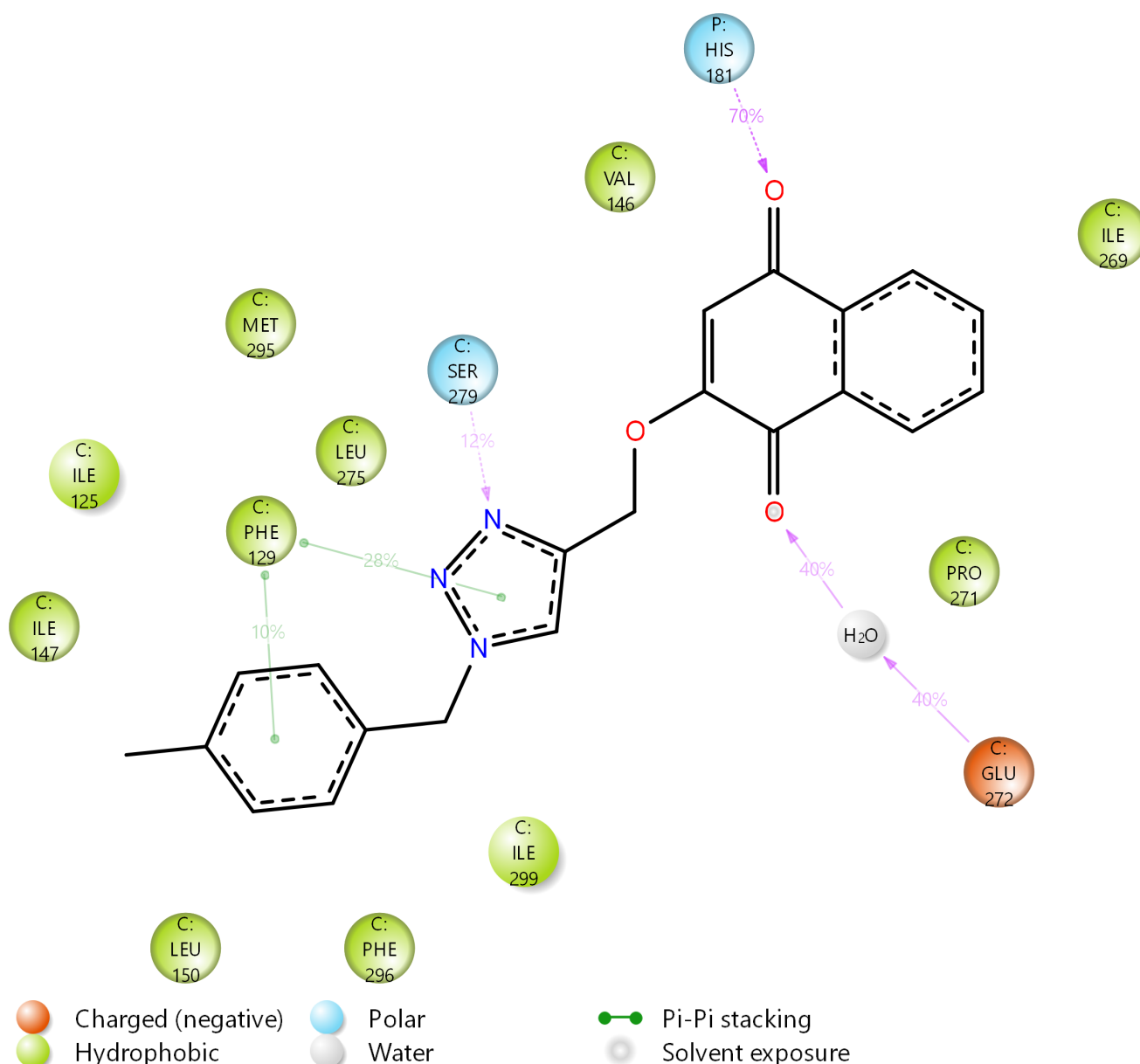
The current geometric criteria for a protein-water or water-ligand H-bond are: a distance of 2.8 Å between the donor and acceptor atoms (D—H...A); a donor angle of $\geq 110^\circ$ between the donor-hydrogen-acceptor atoms (D—H...A); and an acceptor angle of $\geq 90^\circ$ between the hydrogen-acceptor-bonded_atom atoms (H...A—X).

Protein-Ligand Contacts (cont.)



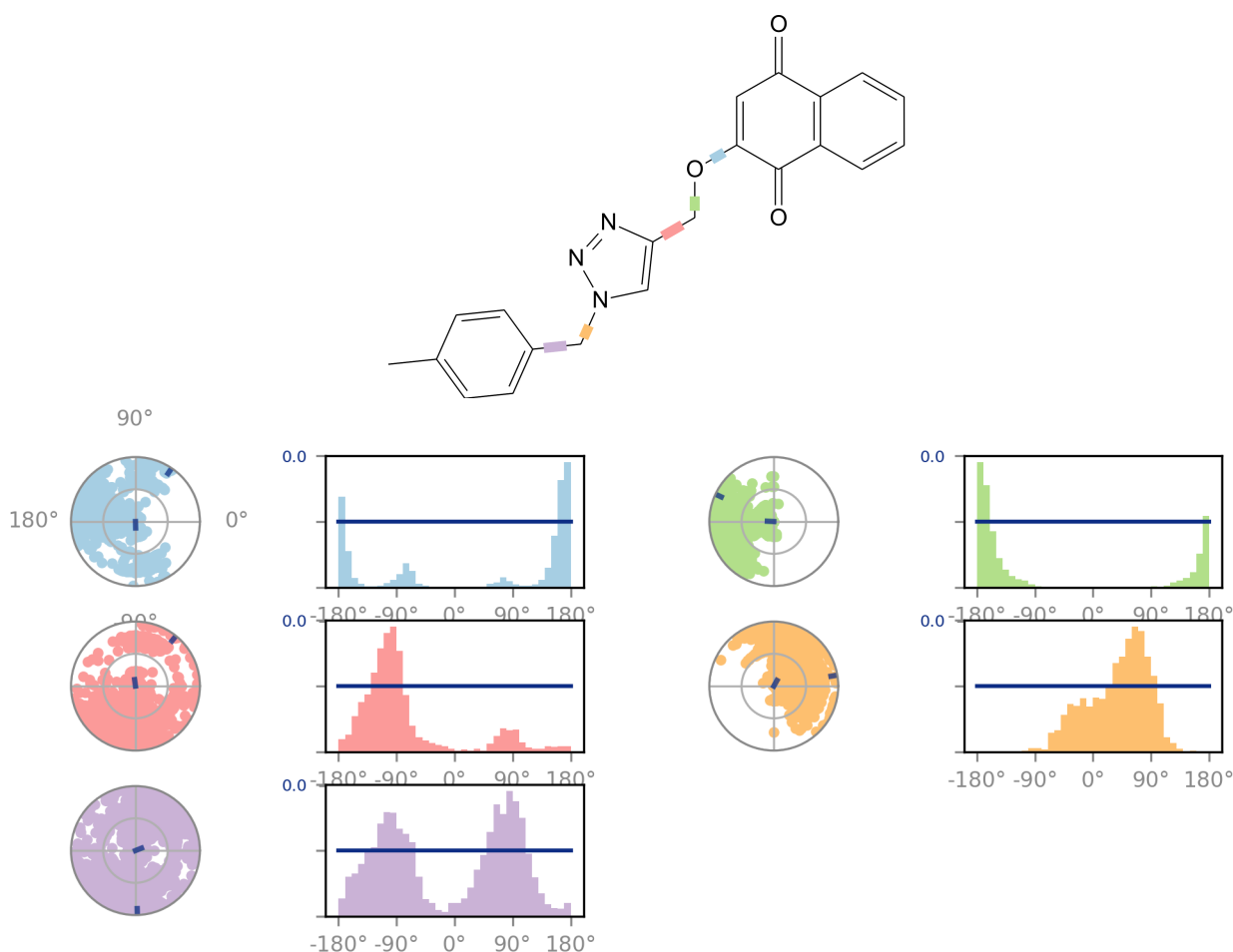
A timeline representation of the interactions and contacts (**H-bonds, Hydrophobic, Ionic, Water bridges**) summarized in the previous page. The top panel shows the total number of specific contacts the protein makes with the ligand over the course of the trajectory. The bottom panel shows which residues interact with the ligand in each trajectory frame. Some residues make more than one specific contact with the ligand, which is represented by a darker shade of orange, according to the scale to the right of the plot.

Ligand-Protein Contacts



A schematic of detailed ligand atom interactions with the protein residues. Interactions that occur more than **10.0%** of the simulation time in the selected trajectory (0.00 through 100.10 nsec), are shown. Note: it is possible to have interactions with >100% as some residues may have multiple interactions of a single type with the same ligand atom. For example, the ARG side chain has four H-bond donors that can all hydrogen-bond to a single H-bond acceptor.

Ligand Torsion Profile

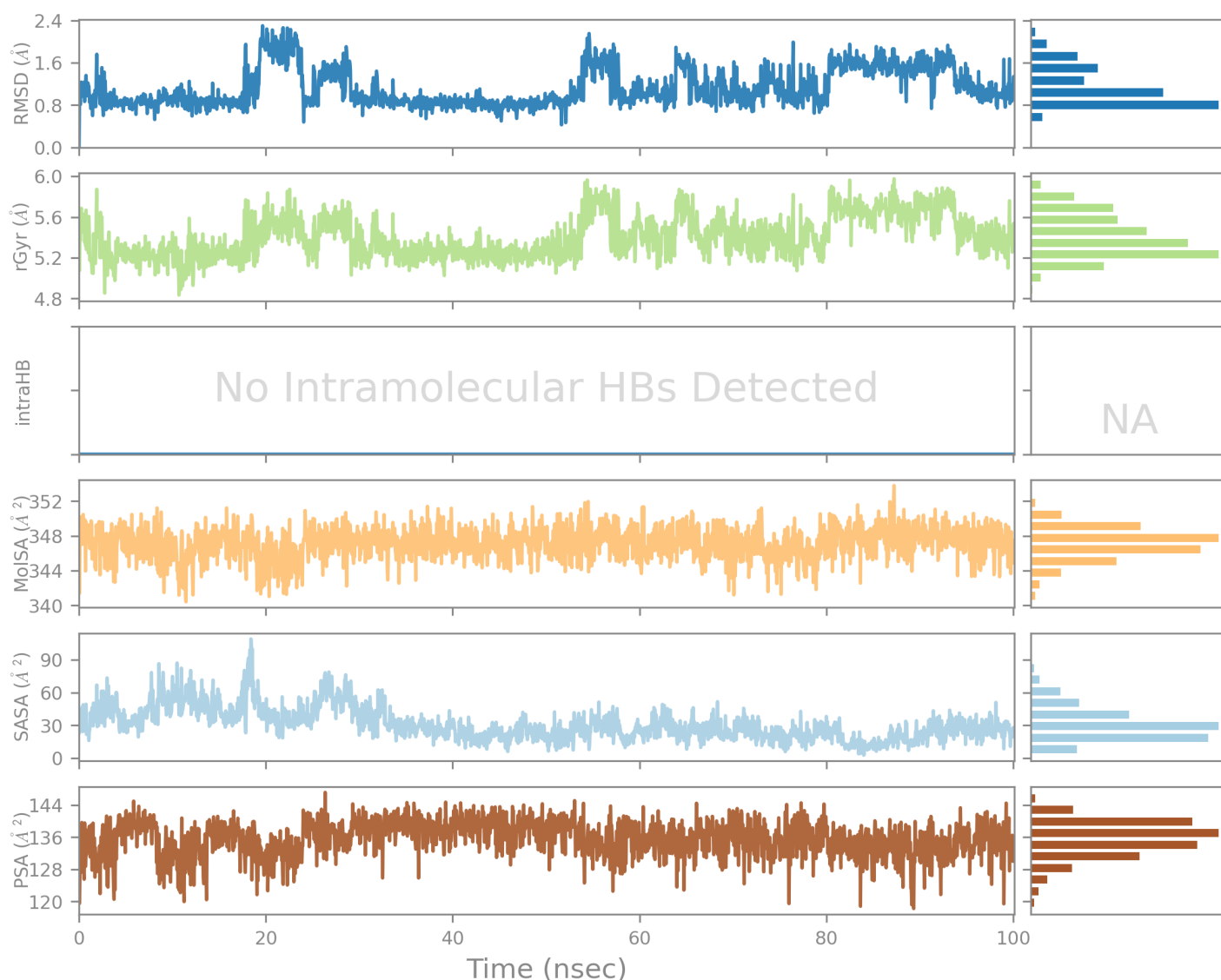


The ligand torsions plot summarizes the conformational evolution of every rotatable bond (RB) in the ligand throughout the simulation trajectory (0.00 through 100.10 nsec). The top panel shows the 2d schematic of a ligand with color-coded rotatable bonds. Each rotatable bond torsion is accompanied by a dial plot and bar plots of the same color.

Dial (or radial) plots describe the conformation of the torsion throughout the course of the simulation. The beginning of the simulation is in the center of the radial plot and the time evolution is plotted radially outwards.

The bar plots summarize the data on the dial plots, by showing the probability density of the torsion. If torsional potential information is available, the plot also shows the potential of the rotatable bond (by summing the potential of the related torsions). The values of the potential are on the left Y-axis of the chart, and are expressed in *kcal/mol*. Looking at the histogram and torsion potential relationships may give insights into the conformational strain the ligand undergoes to maintain a protein-bound conformation.

Ligand Properties



Ligand RMSD: Root mean square deviation of a ligand with respect to the reference conformation (typically the first frame is used as the reference and it is regarded as time $t=0$).

Radius of Gyration (rGyr): Measures the 'extendedness' of a ligand, and is equivalent to its principal moment of inertia.

Intramolecular Hydrogen Bonds (intraHB): Number of internal hydrogen bonds (HB) within a ligand molecule.

Molecular Surface Area (MolSA): Molecular surface calculation with 1.4 Å probe radius. This value is equivalent to a van der Waals surface area.

Solvent Accessible Surface Area (SASA): Surface area of a molecule accessible by a water molecule.

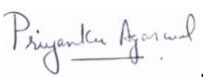
Polar Surface Area (PSA): Solvent accessible surface area in a molecule contributed only by oxygen and nitrogen atoms.

Appendix H.4

Chapter 5: Targeting Atovaquone-Resistant Malaria: *In vitro* and *In silico* Insights into 1,4-naphthoquinone-1,2,3-triazole Hybrid Molecules

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, **Priyanka Agarwal**, student number **2286575**, declare that this Thesis/~~Dissertation/Research Report~~ is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis/~~Dissertation/Research Report~~.

Signature of Student  Date...05-March-2025.....

Name of Primary Supervisor...Prof Robyn L. van Zyl....

Signature of Primary Supervisor Date.....

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/~~Dissertation/Research Report~~. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

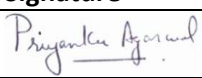
Article 1:

Title: Targeting Atovaquone-Resistant Malaria: *In vitro* and *In silico* Insights into 1,4-Naphthoquinone Hybrid Molecules

Changed to

A Dual *In vitro* and *In silico* Approach to Evaluate 1,4-Naphthoquinone-1,2,3-triazole Hybrids Against Atovaquone-Resistant Malaria

Journal name, year, volume and page numbers: ...Experimental Parasitology.....

Authors	Name	Signature	Date
1 st author	Priyanka Agarwal		05-March-2025
2 nd author	David D. N'Da		07-March-2025
3 rd author	C. Johan van der Westhuizen		07-March-2025
4 th author	Robyn L. van Zyl		
5 th author			
6 th author			

Comments by primary supervisor:

.....
.....

Appendix I

Chapter 6: Evaluation of naphthyl bearing 1,3,4-thiadiazoleacetamide hybrids as *Plasmodium* cytochrome *bc*₁ complex inhibitors

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, **Priyanka Agarwal**, student number **2286575**, declare that this Thesis/~~Dissertation/Research Report~~ is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis/~~Dissertation/Research Report~~.

Signature of Student  Date...31-March-2025.....

Name of Primary Supervisor...Prof Robyn L. van Zyl....

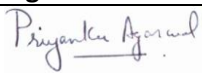
Signature of Primary Supervisor Date.....

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/~~Dissertation/Research Report~~. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

Article 2:

Title: Naphthyl bearing 1,3,4-thiadiazoleacetamide targeting the *Plasmodium falciparum* cytochrome *bc*₁ complex

Journal name, year, volume and page numbers: ... Experimental Parasitology.....

Authors	Name	Signature	Date
1 st author	Priyanka Agarwal		31-March-2025
2 nd author	C. Johan van der Westhuizen		31-March-2025
3 rd author	Robyn L. van Zyl		31-March-2025
4 th author			
5 th author			
6 th author			

Comments by primary supervisor:

Student with her own knowledge of the possible cyt bc1 inhibitors identified that this set under investigation could potentially inhibit this site - undertook *in vitro* antimalarial, toxicity as well as *in silico* studies. Drafted manuscript.

Appendix J

Chapter 7: Designing novel *Plasmodium falciparum* cytochrome *bc*₁ inhibitors using computer-aided drug discovery (CADD)

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, **Priyanka Agarwal**, student number **2286575**, declare that this Thesis/~~Dissertation/Research Report~~ is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis/~~Dissertation/Research Report~~.

Signature of Student  Date...31-March-2025.....

Name of Primary Supervisor...Prof Robyn L. van Zyl....

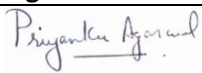
Signature of Primary Supervisor Date.....

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/~~Dissertation/Research Report~~. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

Article 3:

Title: Computational design, molecular modelling, and pharmacokinetic profiling of chalcones as novel inhibitors of *Plasmodium falciparum* cytochrome *bc*₁

Journal name, year, volume and page numbers: ...Journal of Molecular Graphics and Modelling.....

Authors	Name	Signature	Date
1 st author	Priyanka Agarwal		31-March-2025
2 nd author	C. Johan van der Westhuizen		31-March-2025
3 rd author	Robyn L. van Zyl		31-March-2025
4 th author			
5 th author			
6 th author			

Comments by primary supervisor:

Based on initial collated literature, the student identified the chalcones as an under explored group of compounds to investigate. Undertook *in silico* analysis, drug design to write and edit this draft paper.

Simulation Interactions Diagram Report

Simulation Details

Jobname: merged

Entry title: 3CX5_Apo-protein-1 + DPPC

CPU #	Job Type	Ensemble	Temp. [K]	Sim. Time [ns]	# Atoms	# Waters	Charge
Unknown*	Unknown*	Unknown*	300.0	100.599	130861	28564	0

* The configuration file (-out.cfg) was not found. Keep it in same directory as .aef file.

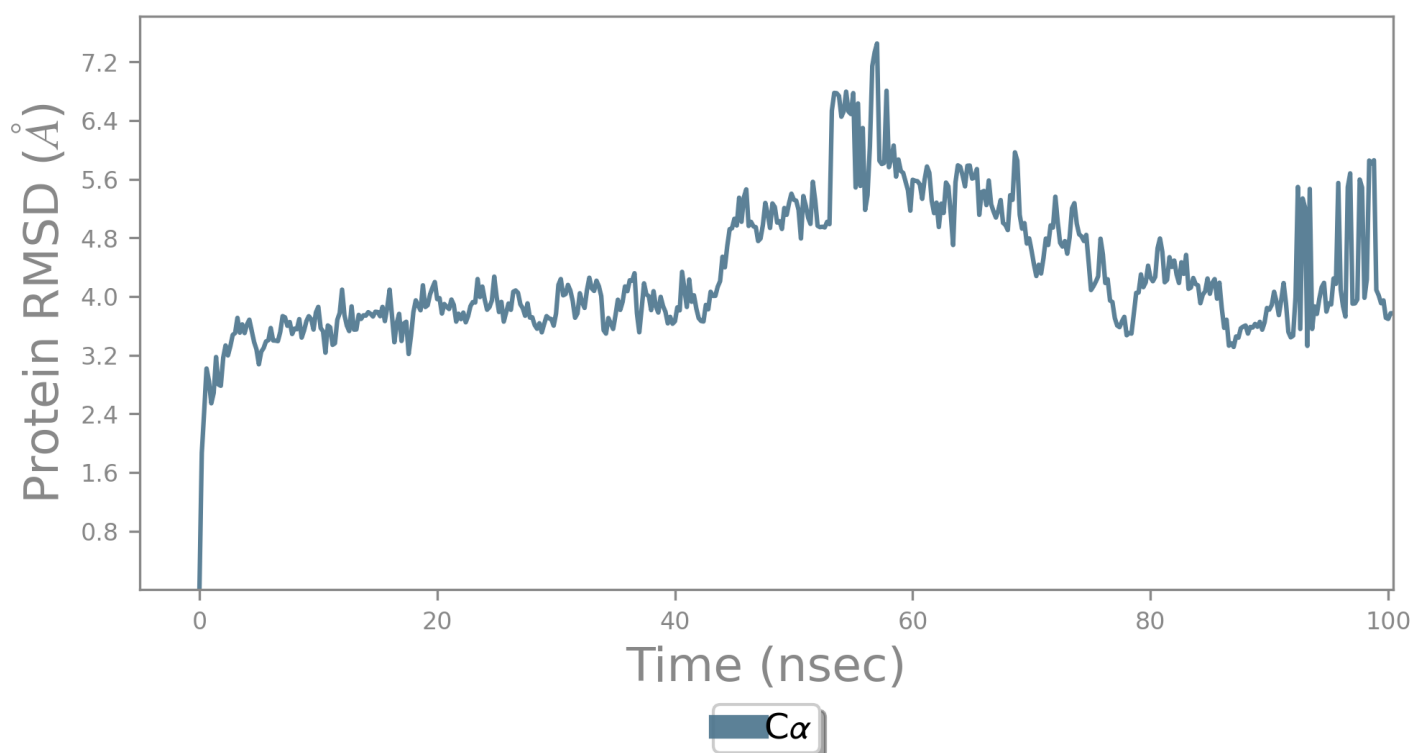
Protein Information

	Tot. Residues	Prot. Chain(s)	Res. in Chain(s)	# Atoms	# Heavy Atoms	Charge										
	577	'C', 'D', 'L', 'P'	Res. values([388, 1, 1, 187]	9328	4639	-3										
C	1	MAFRKSNVYLSLVNSYIIDS	35	30	35	40	45	50	55	60	65	70				
SSA																
P	31	KSTYRTPNFDDVLKENNDADKGRSYAYFMVGAMGLSSAGAKSTVETFISSMTATADVLAMAKVEVNLA	35	40	45	50	55	60	65	70	75	80	85	90	95	100
SSA																
C	71	DVHNGYILRYLHANGASFFFMVFMHMAKGLYGSYRSPRVTLWNVGVIIIFILTIATAFLGYCCVYGQMS	75	80	85	90	95	100	105	110	115	120	125	130	135	140
SSA																
P	101	IPLGKNVVVKWQGPVFIHRTPHEIQEANSVDMSALKDPQTDADRVKDPQWLIMLGICTHLGCVPIGEA	105	110	115	120	125	130	135	140	145	150	155	160	165	170
SSA																
C	141	HWGATVITNLFSAIPFVGNDIVSWLWGGFVSNSPTIQRFFALHYLVFPFIIAAMVIMHLMALHIHGSSNPL	145	150	155	160	165	170	175	180	185	190	195	200	205	210
SSA																
P	171	GDFGGWFPCCHGSHYDISGRIRKGPAPLNLEIPAYEFDGDKVIVGX	175	180	185	190	195	200	205	210	215	215A				
SSA																
C	211	GITGNLDRIPMHSYFIFKDLVTVFLFMLILALFVFYSPNTLGHDPDNYIPGNPLVTPASIVPEWYLLPFYA	215	220	225	230	235	240	245	250	255	260	265	270	275	280
SSA																
C	281	ILRSIPDKLLGVITMFAAILVLLVLPFTDRSVVRGNTFKVLSKFFFFIFVFNFVLLGQIGACHVEVPYVL	285	290	295	300	305	310	315	320	325	330	335	340	345	350
SSA																
C	351	MGQIATFIYFAYFLIIVPVISTIENVLFYIGRVNKKX	355	360	365	370	375	380	4033							
SSA																

Counter Ion/Salt Information

Type	Num.	Concentration [mM]	Total Charge
Na	88	56.015	+88
Cl	79	50.286	-79

Protein-Ligand RMSD



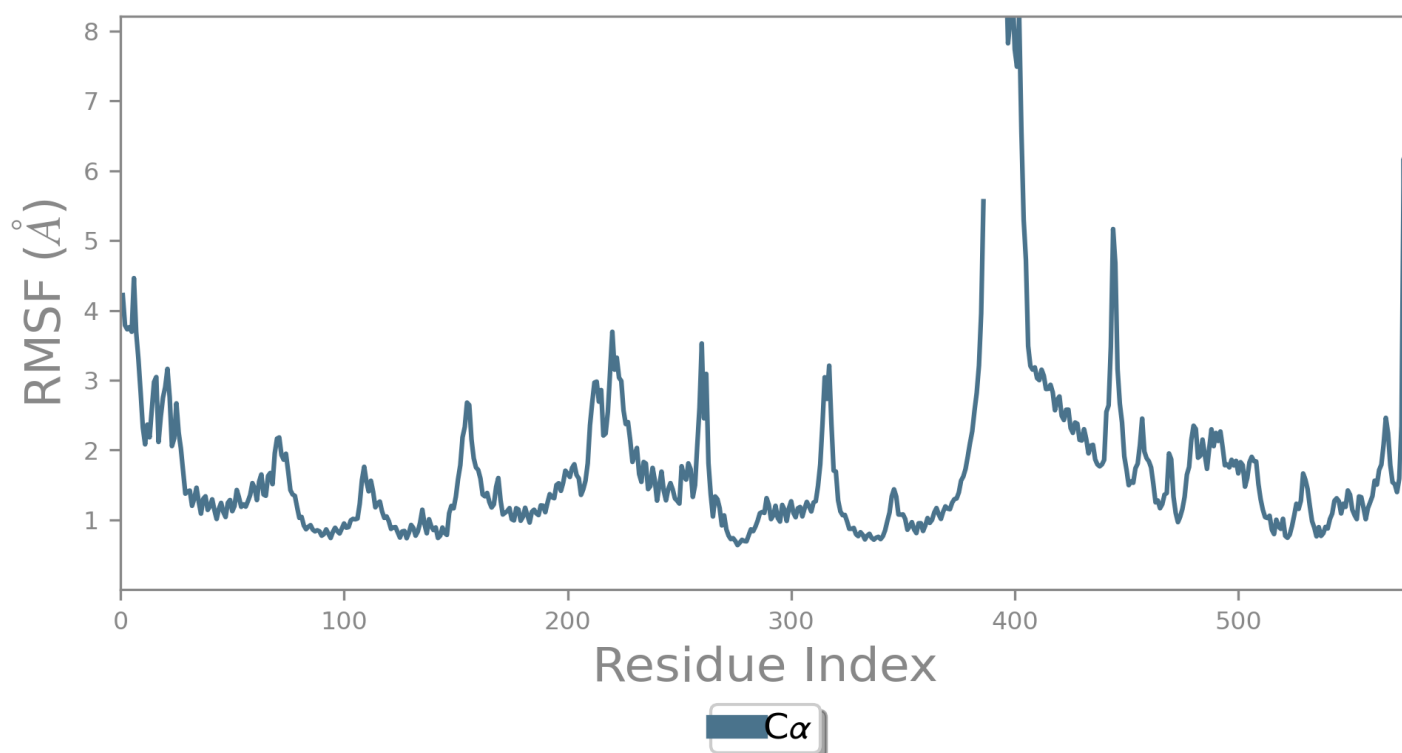
The Root Mean Square Deviation (RMSD) is used to measure the average change in displacement of a selection of atoms for a particular frame with respect to a reference frame. It is calculated for all frames in the trajectory. The RMSD for frame x is:

$$RMSD_x = \sqrt{\frac{1}{N} \sum_{i=1}^N (r'_i(t_x) - r_i(t_{ref}))^2}$$

where N is the number of atoms in the atom selection; t_{ref} is the reference time, (typically the first frame is used as the reference and it is regarded as time $t=0$); and r' is the position of the selected atoms in frame x after superimposing on the reference frame, where frame x is recorded at time t_x . The procedure is repeated for every frame in the simulation trajectory.

Protein RMSD: The above plot shows the RMSD evolution of a protein (left Y-axis). All protein frames are first aligned on the reference frame backbone, and then the RMSD is calculated based on the atom selection. Monitoring the RMSD of the protein can give insights into its structural conformation throughout the simulation. RMSD analysis can indicate if the simulation has equilibrated — its fluctuations towards the end of the simulation are around some thermal average structure. Changes of the order of 1-3 Å are perfectly acceptable for small, globular proteins. Changes much larger than that, however, indicate that the protein is undergoing a large conformational change during the simulation. It is also important that your simulation converges — the RMSD values stabilize around a fixed value. If the RMSD of the protein is still increasing or decreasing on average at the end of the simulation, then your system has not equilibrated, and your simulation may not be long enough for rigorous analysis.

Protein RMSF



The Root Mean Square Fluctuation (RMSF) is useful for characterizing local changes along the protein chain. The RMSF for residue i is:

$$RMSF_i = \sqrt{\frac{1}{T} \sum_{t=1}^T \langle (r'_i(t)) - r_i(t_{ref})^2 \rangle}$$

where T is the trajectory time over which the RMSF is calculated, t_{ref} is the reference time, r_i is the position of residue i ; r' is the position of atoms in residue i after superposition on the reference, and the angle brackets indicate that the average of the square distance is taken over the selection of atoms in the residue.

On this plot, peaks indicate areas of the protein that fluctuate the most during the simulation. Typically you will observe that the tails (N - and C -terminal) fluctuate more than any other part of the protein. Secondary structure elements like alpha helices and beta strands are usually more rigid than the unstructured part of the protein, and thus fluctuate less than the loop regions.

Protein Secondary Structure

% Helix

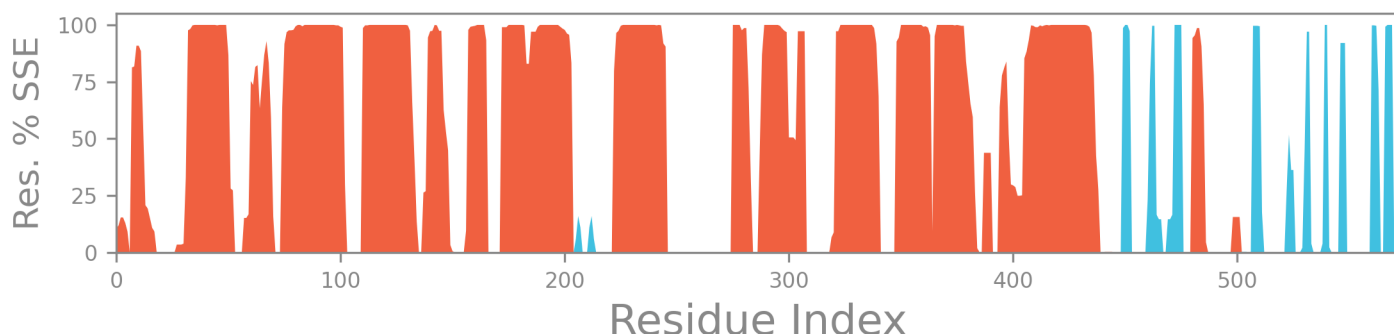
49.32

% Strand

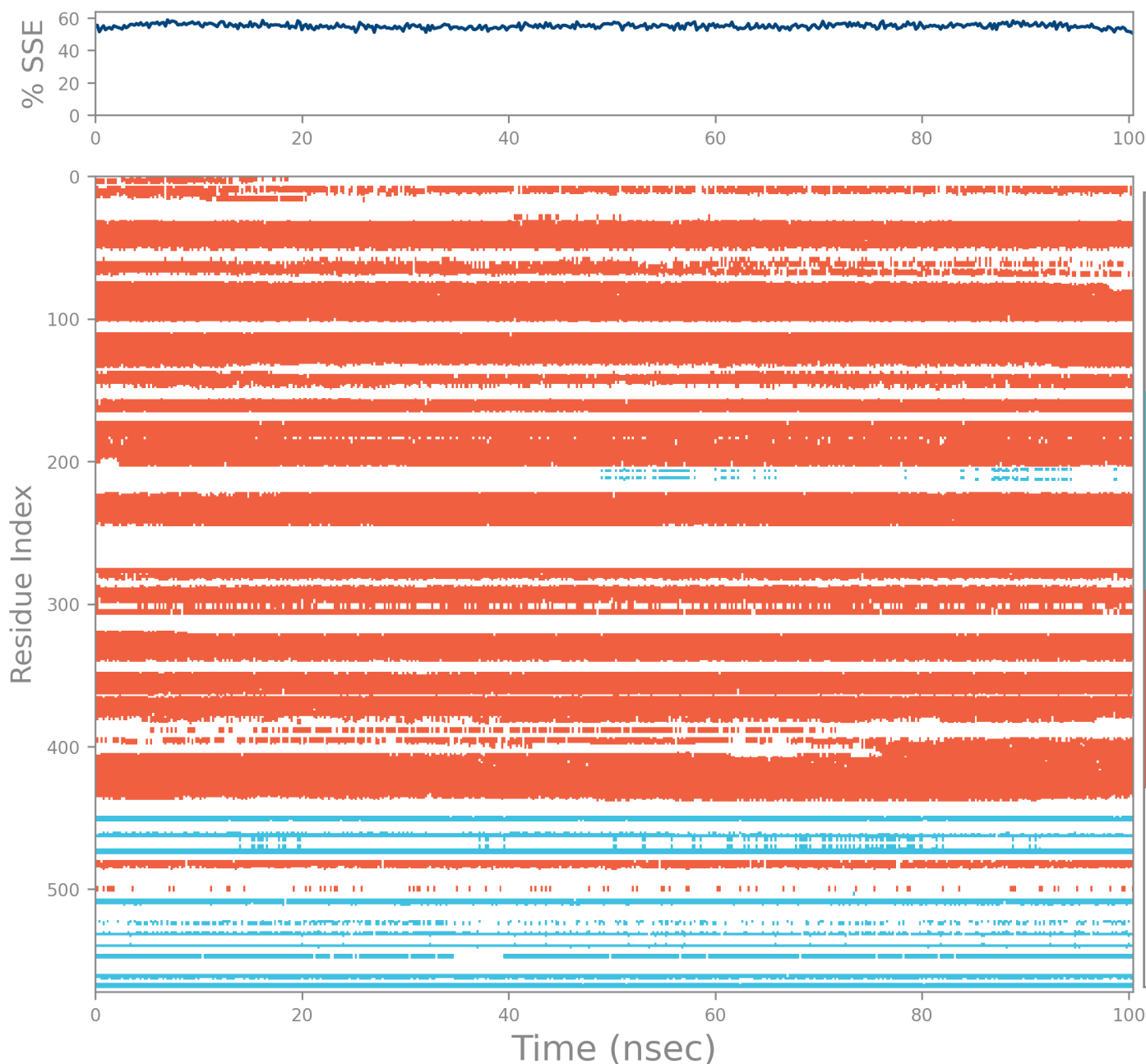
5.79

% Total SSE

55.12



Protein secondary structure elements (SSE) like **alpha-helices** and **beta-strands** are monitored throughout the simulation. The plot above reports SSE distribution by residue index throughout the protein structure. The plot below summarizes the SSE composition for each trajectory frame over the course of the simulation, and the plot at the bottom monitors each residue and its SSE assignment over time.



Simulation Interactions Diagram Report

Simulation Details

Jobname: merged

Entry title: 3CX5_truncated - prepared + DPPC

CPU #	Job Type	Ensemble	Temp. [K]	Sim. Time [ns]	# Atoms	# Waters	Charge
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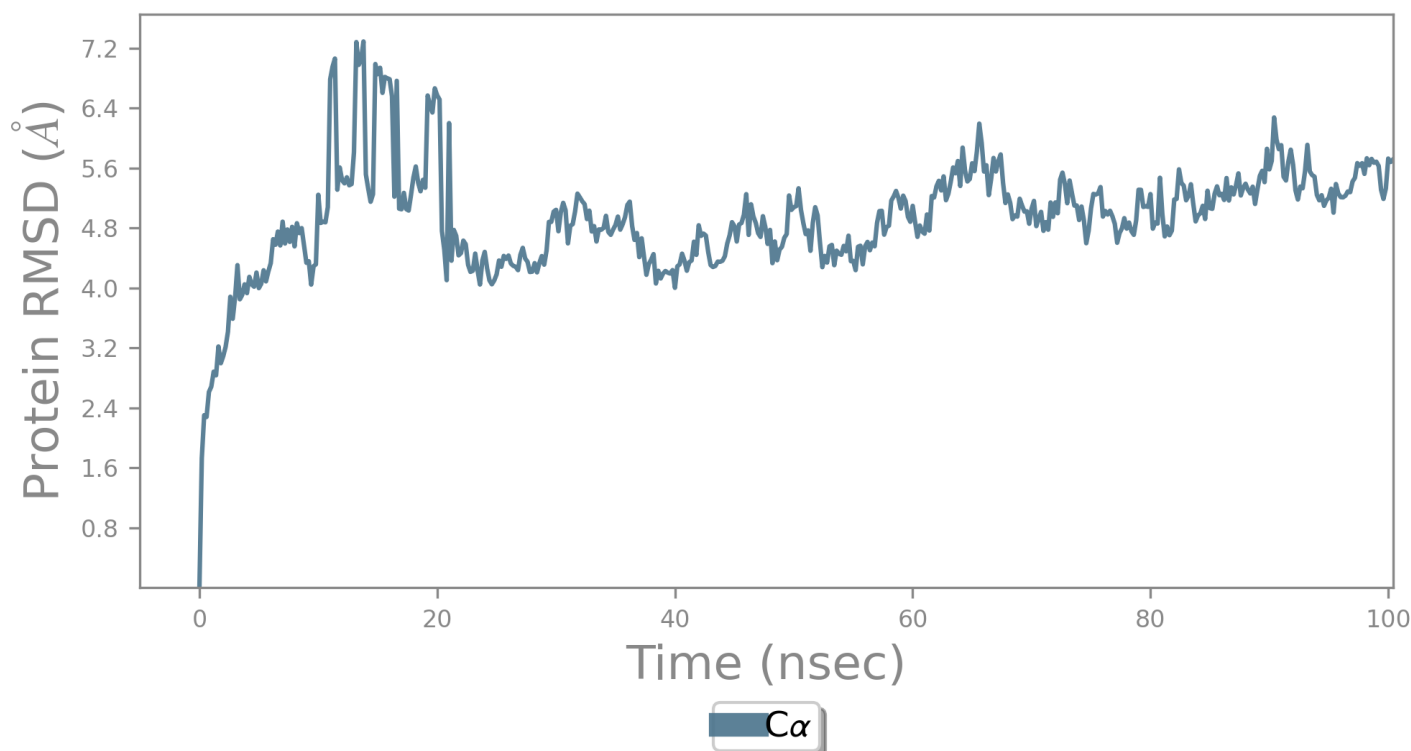
Protein Information

	Tot. Residues	Prot. Chain(s)	Res. in Chain(s)	# Atoms	# Heavy Atoms	Charge										
	577	'C', 'D', 'L', 'P'	Res. values([388, 1, 1, 187]	9318	4633	-3										
C	1	MAFRKSNVYLSLVNSYIIDS	35	30	45	55	60	65	70							
SSA																
P	31	KSTYRTPNFDDVLKENNDADKGRSYAYFMVGAMGLSSAGAKSTVETFISSMTATADVLAMAKVEVNLA	35	40	45	50	55	60	65	70	75	80	85	90	95	100
SSA																
C	71	DVHNGYILRYLHANGASFFFMVFMHMAKGLYGSYRSPRVTLWNVGVIIIFILTITATFLGYCCVYGQMS	75	80	85	90	95	100	105	110	115	120	125	130	135	140
SSA																
P	101	IPLGKNVVVKWQGPVFIHRTPEHIEANSVDMSALKDPQTDADRVDKPQWLIMLGICTHLGCVPIGEA	105	110	115	120	125	130	135	140	145	150	155	160	165	170
SSA																
C	141	HWGATVITNLFSAPFVGNDIVSWLWGGFSVSNPTIQRFFALHYLVFPFIIAAMVIMHLMALHIHGSSNPL	145	150	155	160	165	170	175	180	185	190	195	200	205	210
SSA																
P	171	GDFGGWFPCPCGSHYDISGRIRKGPAPLNLEIPAYEFDGDKVIVGX	175	180	185	190	195	200	205	210	215	215A				
SSA																
C	211	GITGNLDRIPMHSYFIFKDLVTVFLFMLILALFVFYSPNTLGHDPDNYIPGNPLVTPASIVPEWYLLPFSA	215	220	225	230	235	240	245	250	255	260	265	270	275	280
SSA																
C	281	ILRSIPDKLLGVITMFAAILVLLVLPFTDRSVVRGNTFKVLSKFFFFIFVFNFVLLGQIGACHVEVPYVL	285	290	295	300	305	310	315	320	325	330	335	340	345	350
SSA																
C	351	MGQIATFIYFAYFLIIVPVISTIENVLFYIGRVNKKX	355	360	365	370	375	380	4033							
SSA																

Counter Ion/Salt Information

Type	Num.	Concentration [mM]	Total Charge
Na	88	55.811	+88
Cl	79	50.103	-79

Protein-Ligand RMSD



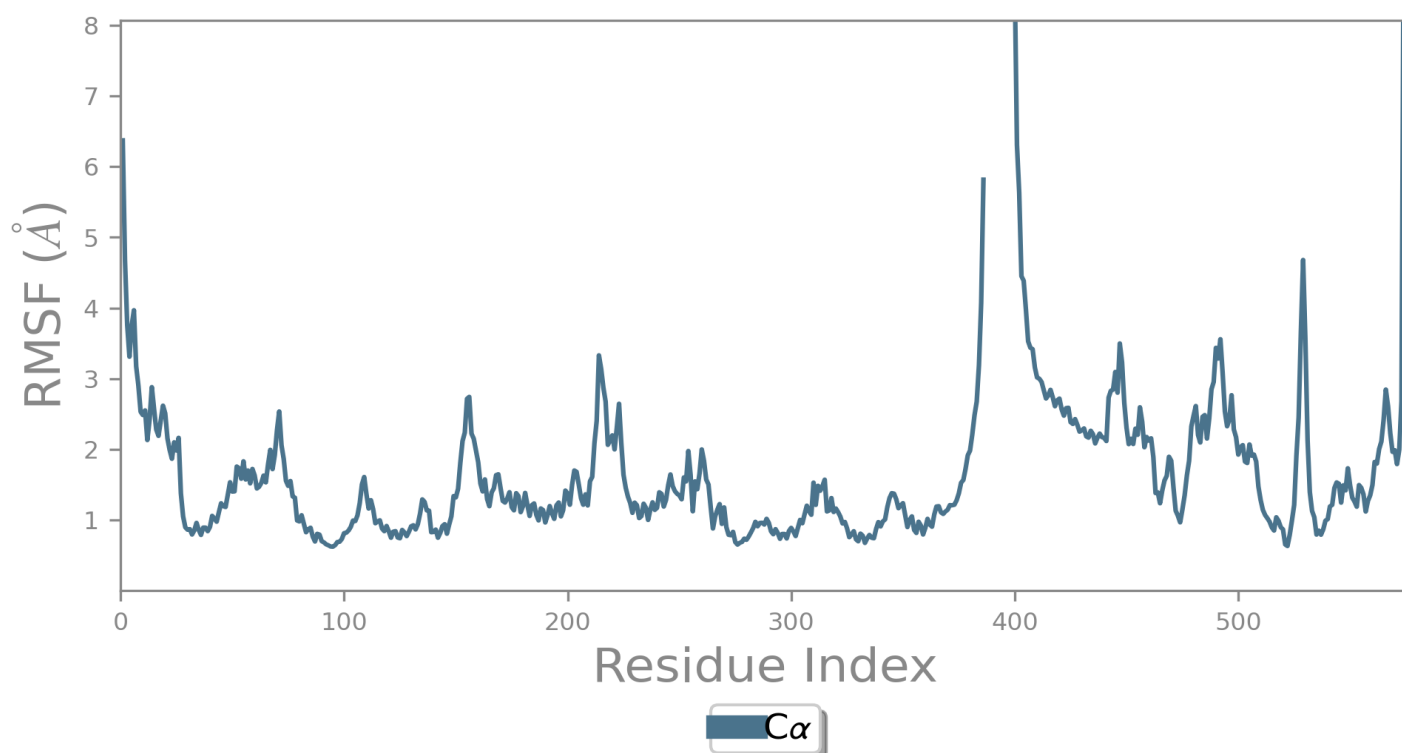
The Root Mean Square Deviation (RMSD) is used to measure the average change in displacement of a selection of atoms for a particular frame with respect to a reference frame. It is calculated for all frames in the trajectory. The RMSD for frame x is:

$$RMSD_x = \sqrt{\frac{1}{N} \sum_{i=1}^N (r'_i(t_x) - r_i(t_{ref}))^2}$$

where N is the number of atoms in the atom selection; t_{ref} is the reference time, (typically the first frame is used as the reference and it is regarded as time $t=0$); and r' is the position of the selected atoms in frame x after superimposing on the reference frame, where frame x is recorded at time t_x . The procedure is repeated for every frame in the simulation trajectory.

Protein RMSD: The above plot shows the RMSD evolution of a protein (left Y-axis). All protein frames are first aligned on the reference frame backbone, and then the RMSD is calculated based on the atom selection. Monitoring the RMSD of the protein can give insights into its structural conformation throughout the simulation. RMSD analysis can indicate if the simulation has equilibrated — its fluctuations towards the end of the simulation are around some thermal average structure. Changes of the order of 1-3 Å are perfectly acceptable for small, globular proteins. Changes much larger than that, however, indicate that the protein is undergoing a large conformational change during the simulation. It is also important that your simulation converges — the RMSD values stabilize around a fixed value. If the RMSD of the protein is still increasing or decreasing on average at the end of the simulation, then your system has not equilibrated, and your simulation may not be long enough for rigorous analysis.

Protein RMSF



The Root Mean Square Fluctuation (RMSF) is useful for characterizing local changes along the protein chain. The RMSF for residue i is:

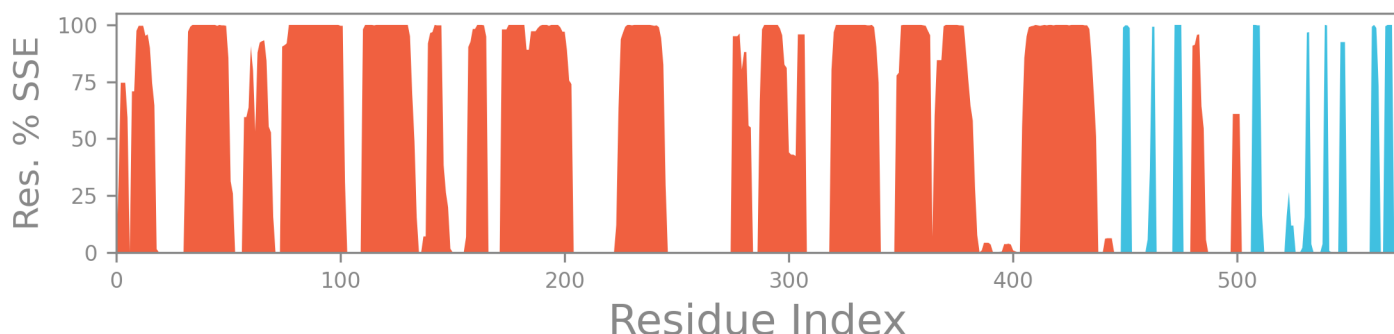
$$RMSF_i = \sqrt{\frac{1}{T} \sum_{t=1}^T \langle (r'_i(t)) - r_i(t_{ref})^2 \rangle}$$

where T is the trajectory time over which the RMSF is calculated, t_{ref} is the reference time, r_i is the position of residue i ; r' is the position of atoms in residue i after superposition on the reference, and the angle brackets indicate that the average of the square distance is taken over the selection of atoms in the residue.

On this plot, peaks indicate areas of the protein that fluctuate the most during the simulation. Typically you will observe that the tails (N - and C -terminal) fluctuate more than any other part of the protein. Secondary structure elements like alpha helices and beta strands are usually more rigid than the unstructured part of the protein, and thus fluctuate less than the loop regions.

Protein Secondary Structure

% Helix 49.10 % Strand 5.22 % Total SSE 54.33



Protein secondary structure elements (SSE) like **alpha-helices** and **beta-strands** are monitored throughout the simulation. The plot above reports SSE distribution by residue index throughout the protein structure. The plot below summarizes the SSE composition for each trajectory frame over the course of the simulation, and the plot at the bottom monitors each residue and its SSE assignment over time.

