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The evaluation of gold-based compounds as potential inhibitors of HIV-1 replication.

by

Morore Katlego Mphahlele

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine

October 2011

Declaration

I, Morore Katlego Mphahlele declare that the work presented in this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Medicine in the University of Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination at this or any other University.



.....

10th day of October, 2011

Dedication

I dedicate this dissertation to my wonderful family:

My wife, Mokgadi Mphahlele

My lovely mother, Mokgohloe Mphahlele

My sons, Mokgobi Neo Mphahlele and Mafadi Mphela Mphahlele

My sister, Rebotiloe Mogoba

My niece, Kekweditše Mogoba

All my brothers, uncles, aunts and cousins

Thank you very much.

Publications and conference proceedings

Publications

Hewer, R., Coyanis, C., Traut, T., Mphahlele, M., Coates, J., van der Lingen, E. (2009). Investigating the biomedical applications of gold. *The Southern African Institute of Mining and Metallurgy*, 253 – 257

Morore Mphahlele, Maria Papathanasopoulous, Maria Cinellu, Mabel Coyanis, Telisha Traut, Judy Coates, Raymond Hewer. The inhibition of HIV-1 reverse transcriptase activity by gold(III) complexes. *Bioorganic and Medicinal Chemistry* (**in preparation**).

Conference Oral Presentations

Hewer, R., Coyanis, C., Traut, T., Papathanasopoulos, M.A., **Mphahlele, M.,** Coates, J., van der Lingen, E. Investigating the biomedical applications of gold. Presented at the World Gold Conference 2009, 26 – 30 October 2009, Johannesburg, South Africa.

Conference Poster Presentations

Coyanis, E.M., Cinellu, M.A., Papathanasopoulos M.A., Hewer, R., **Mphahlele,** M. Binding studies of gold compounds to human serum albumin utilising NMR. Presented at *Gold 2009*, 26 – 29 July 2009, Heidelberg, Germany.

Mphahlele, M. Cinellu, M.A., Papathanasopoulos M.A., Coates, J., Hewer, R. The pharmalogical properties and anti-HIV activity of organomettalic gold complexes. Presented at *Gold 2009*. 26 – 29 July 2009, Heidelberg, Germany.

Mphahlele, M., Cinellu, M.A., Papathanasopoulos M.A., Coates, J., Hewer, R. Inhibition of HIV-1 reverse transcriptase by gold-based compounds with anti-cancer activity. Presented at *COST D39*, 24 – 25 September 2009, Debrecen, Hungary.

M.K Mphahlele, M.A. Papathanasopoulos, M.A. Cinellu, M. Coyanis, J. Coates, R. Hewer. Inhibition of HIV-1 reverse transcriptase (RT) activity by gold-based compounds. Presented at *HIV DART 2010*, 7-10 December 2010, Los Cabos, Mexico.

Abstract

Highly active antiretroviral therapy has successfully limited HIV-1 disease progression to AIDS, but is consistently compromised by the emergence and transmission of HIV-1 drug resistant strains. As a result, a continued search for novel anti-HIV-1 agents with improved pharmacological profiles has become fundamental. Chrysotherapy has been in use since the early 1920s in treatment of rheumatoid arthritis, and has since been investigated for various ailments including HIV/AIDS. This study evaluated 45 synthetic gold compounds for drug like properties using theoretical and experimental techniques with the aim of generating sufficient data to considerably aid the rational design of new anti-HIV agents. Theoretical techniques applied included the Osiris Property Explorer and the Lipinski's Rule of Five which assessed drug-likeness and bioavailability respectively. *In vitro* studies included aqueous solubility assays, cytotoxicity (PM1 cell lines and PBMCs) assays, antiviral assays in PBMCs, direct enzyme (RT and IN) inhibition, and the effect of serum protein binding and biological stability on antiviral efficacy. An overall low drug-likeness score and an intermediate bioavailability were predicted by the Osiris Molecular Property Explorer. Low drug-likeness was suggested to be due to a high frequency of foreign fragments in the synthetic gold compounds, while their high molecular weight reduced bioavailability. In general gold compounds exhibited cytotoxicity properties and moderate aqueous solubility *in vitro*. Overall, the 45 synthetic gold compounds did not show activity against HIV-1 replication *in vitro*. Seven compounds (AB05-AB11) exhibited direct HIV-1 RT inhibition, and compounds AB39 and AB04 demonstrated moderate direct HIV-1 IN inhibition, but this activity was abrogated in PBMC inhibition assays. Serum binding, compound stability and cytotoxicity were all implicated in the lack of HIV-1 inhibition in PBMCs. To this end, data obtained was sufficient to aid in the future rational design of second generation HIV RT and IN inhibitors with acceptable pharmacological properties.

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List of Abbreviations

Units of measurement

%	Percent
Å	Angstrom
°C	degree Celsius
Ci	Curies
cpm	counts per minute
g	gram
K	kelvin
kDa	kilo Daltons
M	molar
mg	milligram
MHz	megahertz
ml	millilitre
mM	millimolar
mm^3	cubic millimetre
mol	mole
mU	milli-units
mU/ml	milliunits per milliliter
ng	nanogram
nm	nanometer/nanomoles
nM	nanomoles
ppm	parts per million
rpm	revolutions per minute
U	Units
µl	microlitre
µM	micromolar

Amino acids

Cys	Cysteine
Glu	Glutamine
His	Histidine
Lys	Lysine
Ser	Serine
Thr	Threonine

Microorganisms

A. niger	*Aspergillus niger*
C. albicans	*Candida albicans*
E. coli	*Escherichia coli*
P. aeruginosa	*Pseudomonas aeruginosa*
P. berghei	*Plasmodium berghei*
P. falciparum	*Plasmodium falciparum*
P. vaginalis	*Trichomonas vaginalis*
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
T. cruzi	*Trypanosoma cruzi*

Countries and agencies

BMS	Bristol Myers Squibb
DRC	Democratic Republic of Congo
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
RTECS	Registry of Toxic Effects of Chemical Substances
USA	United States of America
USAID	United States Agency for International Development
US FDA	United States Food and Drug Administration

Abbreviations used in the text

3TC	2', 3'-dideoxy-3'-thiacytidine
AIDS	acquired immunodeficiency syndrome
ABTS	2, 2’-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)

ACN	acetonitrile
ADMET	adsorption, distribution, metabolism, excretion, toxicity
Anti-DIG-POD	anti-digoxigenin-peroxidase
ARV	antiretroviral
AZT	azidothymidine
CC_{50}	Cytotoxic concentration
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CHR	C-terminal heptad repeat
CI	confidence interval
CRF	circulating recombinant forms
CRA	co-receptor antagonist
CXCR4	CXC chemokine receptor type 4
dH_20	distilled water
DMSO	dimethyl sulfoxide
DNA/ cDNA	deoxyribonucleic acid/ complementary deoxyribonucleic acid
DTT	dithiothreitol
D_2O	deuterated oxide
ED_{50}	effective dose required to produce 50% inhibition
ELISA	enzyme-linked immunosorbent assay
$FCIC_{50}$	fold change in IC_{50}
FCS	foetal calf serum
FI	fusion inhibitor
FP	fusion peptide
gp	glycoprotein
HAART	highly active antiretroviral therapy
HBV	hepatitis B virus
HIV	human immunodeficiency virus
^1H NMR	proton nuclear magnetic resonance
HSA	human serum albumin
HTLV	human T-lymphotropic virus
IC_{50}	concentration required to induce 50% inhibition
IL	interleukin

INI	integrase inhibitor
Iκβ	inhibitor kappa beta
LAV	lymphadenopathy associated virus
LEDGF/p75	lens epithelium-derived growth factor/p75
Lipinski's RO5	Lipinski's rule of five
LTR	long terminal repeat
MA	matrix
MHC	major histocompatibility complex
MOI	multiplicity of infection
MP	micro-plate
NC	nucleocapsid
NF-κβ	nuclear factor kappa beta
NHR	N-terminal heptad repeat
NRTI	nucleoside reverse transcriptase inhibitor
NtRTI	nucleotide reverse transcriptase inhibitor
NNRTI	non-nucleoside reverse transcriptase inhibitor
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PHA	phytohaemaglutinin
PHI	pre-hairpin intermediate
PI	protease inhibitor
PIC	pre-integration complex
P-TEF-b	positive-transcriptional elongation factor-b
PTD	pretransmembrane domain
RA	rheumatoid arthritis
RTIs	RT inhibitors
RNA	ribonucleic acid
SAR	structure activity relationship
SHB	six helix bundle
SI	selective index
SISA	simple interactive statistical analysis
SIV	simian immunodeficiency virus
STD	saturation transfer difference
TAR	transactivation response region

TNF-α	tumour necrosis factor alpha
tRNA	transfer RNA
TI	Therapeutic index
URF	unique recombinant forms

CHAPTER 1
INTRODUCTION

1.1. Introduction to HIV and AIDS

1.1.1.The history of HIV

Acquired Immunodeficiency Syndrome (AIDS) was first identified in homosexual patients and described as an immune deficiency state associated with multiple microbial infections (Gottlieb *et al*., 1981). The viral agent associated with AIDS was isolated in 1983 from a lymph node of a homosexual patient with multiple lymphadenopathies, and was named Lymphadenopathy Associated Virus (LAV; Barre-Sinoussi *et al*., 1983). The name LAV was later changed to Human T-Lymphotropic Virus Type (HTLV)-III due to the viral agent's genomic similarities with HTLV-I (Gallo *et al*., 1984). HTLV-III was subsequently reported to be the causative agent of AIDS (Gallo *et al*., 1984 and Popovic *et al*., 1984). In 1986 the causative agent of AIDS was renamed Human Immunodeficiency Virus type 1 (HIV-1) with relation to the immune depletion condition it causes (Coffin *et al*., 1986). In the same year, HIV-2 was isolated from West African commercial sex workers (Marx, 1986).

Until recently the only known historical HIV-1 sample was ZR59; a tissue sample obtained in 1959 from a Bantu male in Kinshasa, Democratic Republic of Congo (DRC) then Léopoldville (Zhu *et al*., 1998). The HIV-1 from ZR59 was later found to be closely related to a simian immunodeficiency virus (SIV) strain that originates in chimpanzees of the subspecies *Pan Troglodytes troglodytes* (Keele *et al*., 2006). Another HIV-1 sample (DR60) obtained from a female in Léopoldville was recently recovered. DR60 was a lymph node biopsy taken in 1960 and was since preserved in paraffin wax (Worobey *et al*., 2008). Genetic variations in the order of 12% introduced by mutations, between HIV-1 recovered from DRC60 and ZR59, suggested the presence of HIV-1 in humans for a significant period before 1960

(Worobey *et al*., 2008). An earlier report by Korber *et al*., (2000) compared the genetic make-up of modern HIV-1 to that from ZR59. It was estimated that HIV-1 was present in humans between 1915 and 1941, with an optimum estimate for a common ancestor of the main HIV-1 group to be 1931 (Korber *et al*., 2000).

1.1.2.The epidemiology of HIV

HIV-1/AIDS has since its discovery become a global public health predicament of pandemic magnitude (Fauci, 2006). Recent studies have estimated that by the end of 2009, approximately 33.3 million (range: 31.4 million - 35.3 million) people were living with HIV-1 worldwide (UNAIDS, 2010). Sub-Saharan Africa was reported as the region most affected by the HIV-1 pandemic (UNAIDS, 2010). The region accounts for approximately 67% of the total number of people living with HIV-1 and 75% of all AIDS-related mortalities (UNAIDS, 2010). In the same study South Africa was reported to be the country with the highest global estimates of people living with HIV-1 infection (UNAIDS, 2010). An estimated 5.7 million people are reported to be living with HIV-1 in South Africa with over 250,000 AIDS related mortalities in 2008 alone (Department of Health, 2010). The South African national HIV-1 survey estimates the national prevalence of HIV-1 at 10-11%. Approximately 10.9% of all South Africans over two years of age were reported to be living with HIV-1 in 2008, with adults having the highest rates of HIV-1 at 16.9% (Department of Health, 2010). Children between the ages 2-14 have an estimated HIV-1 prevalence of approximately 2.5%, and HIV-1 prevalence among the youth (ages 14-24) was estimated at approximately 8.7 % in South Africa (Department of Health, 2010). The HIV-1 prevalence is highest among the African population at 13.6%. The coloured, white and Indian population's HIV-1 prevalence rates are estimated at 1.7%, 0.3% and 0.3% respectively (Department of Health, 2010). The prevalence of HIV-1 infection among women attending antenatal clinics across South Africa has been estimated at approximately 28% (Department of Health, 2010); with Kwazulu-Natal, Mpumalanga and the Free State being provinces with the highest recorded HIV-1 prevalence rates at 37.4%, 32% and 33%, respectively (Department of Health, 2010). The Western Cape and the Northern Cape are provinces with the lowest HIV-1 prevalence rates of 6.1% and 12.6%, respectively, with heterosexual transmission being the main route of transmission (Department of Health, 2010). Other HIV-1

transmission modes include homosexual transmission, intravenous drug use and mother to child transmission (Global HIV Prevention Working Group, 2007).

1.1.3. Nomenclature and phylogeny of HIV

The HI virus is classified as a member of the family Retroviridae, subfamily Orthoretrovirinae and genus lentivirus (*ICTVdB* – The Universal virus database, version 4. http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/ accessed 08-09-2010). There are 2 distinct types of human HIV, namely HIV-1 and HIV-2. These viruses are distinguished on the basis of their genome organizations and evolutionary relations to primate lentiviruses as illustrated in Figure 1.1 Three transmissions of SIVcpz from the central African chimpanzee subspecies (*Pantroglodytes troglodytes* and *schweinfurthii*) gave rise to HIV-1 groups M, N and O and the other SIVsmm gave rise to HIV-2 groups A and H (Chen *et al*., 1997; Gao *et al*., 1999; Santiago *et al*., 2002, Worobey *et al*., 2007).

HIV-2 consists of 5 distinct phylogenetic lineages designated as groups A, B, C, F and G, and one circulating recombinant form (CRF); (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html accessed 19-07-2010). HIV-1 was classified into 3 groups M, N and O with each lineage representing a separate transmission event in chimpanzees in central Africa (McCutchan, 2006). In 2009, a new HIV-1 sequence was isolated from a Cameroonian woman who was residing in France; the virus was designated HIV-1 group P and exhibited close relations to SIV discovered in gorillas (Plantier *et al.,* 2009). The HIV-1 group M which is responsible for most of the global HIV-1 epidemic consists of nine subtypes designated A-D, F-H, J, and K, 48 CRFs, and numerous unique recombinant forms (URFs) (Robertson *et al*., 2000; Hemelaar *et al*., 2006; McCutchan, 2006 and http://www.hiv.lanl.gov accessed 19-07-2010). Formation of new lineages occurs as a result of the HIV-1's high mutation rate. The sequence mutations are introduced during reverse transcription since the viral reverse trancriptase (RT) enzyme lacks proof reading ability and as a result of viral recombination among quasispecies (Coffin, 1986; Hahn *et al*., 1986; Richetti *et al*., 1990; Zhang *et al*., 1994; Robertson *et al*., 1995; Ho, 1997 and Levy *et al*., 2004). These evolutionary processes had produced the well-Characterised CRFs such as CRF02_AG, CRF08_BC,

CRF15_01B, CRF09_cpx, CRF01_AE and CRF04_cpx within HIV-1 group M (McCutchan, 2006). CRFs are numbered sequentially as they become known and designated according to HIV-1 subtypes recombined. For instance, CRF02_AG represents a subtype A and a subtype G recombinant (Howard and Rasheed, 2006). In a situation where recombination has occurred between more than two subtypes the CRF is labelled with a designation cpx (complex). For instance, CRF04_cpx represents a recombinant form comprised of four subtypes, namely A, G, H, K and some unclassified regions (Paraskevis *et al.*, 2001). To date, 48 HIV-1 and 1 HIV-2 CRF has been described (http://www.hiv.lanl.gov accessed 19-07-2010). Numerous URFs have been identified, and represent a large and heterogeneous group of HIV-1 strains (McCutchan, 2006). They are a reflection of a mixture of subtypes in a population where they are found and some may be generated within an individual with dual infection of HIV-1 (infection by two or more distinct viruses; Gerhardt *et al.*, 2005; McCutchan *et al.*, 2005).

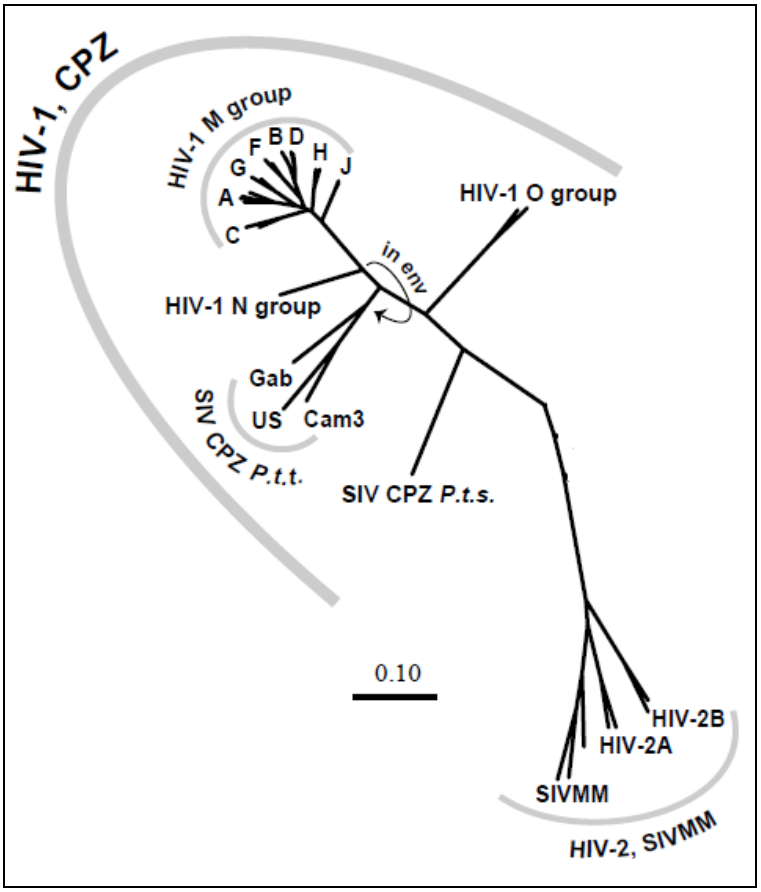


Figure 1.1: Lentivirus phylogenetic tree illustrating maximum likelihood relations between HIV-1, HIV-2 and SIV partial sequences. This Figure was obtained from Worobey, 2007.

1.1.4. Geographical distribution of HIV

HIV lineages are to a large degree associated with geographical distribution. HIV-2 infections are prevalent in West Africa. HIV-2 has a prevalence of more than 1% in West African nations such as Cape Verde, Ivory Coast, Gambia, Guinea-Bissau, Mali, Mauritania, Nigeria and Sierra Leone (Centres for Disease Control and Prevention: http://www.cdc.gov/hiv/resources/factsheets/hiv2.htm accessed 26-08-2010). Other West African countries that report an incidence of HIV-2 infection are Benin, Burkina Faso, Ghana, Guinea, Liberia, Niger, São Tomé, Senegal and Togo. Southern African states such as Angola and Mozambique have also reported an over 1% prevalence rate of HIV-2 infection (Centers for Disease Control and Prevention, Last Reviewed: August 11, 2010). HIV-2 is therefore considered to be less transmissible and less pathogenic than HIV-1 (McCutchan, 2006). HIV-1 group O is most prevalent in Cameroon and neighbouring West African countries, where it accounts for approximately 1 to 5% of HIV-1 positive samples (Peeters *et al*., 1997). Similarly, HIV-1 group N viruses have been identified in a few individuals from Cameroon (Simon *et al*., 1998; Ayouba *et al*., 2000 and Yamaguchi *et al*., 2006). HIV-1 group M viruses are responsible for the main pandemic worldwide (Human Retroviruses and AIDS 1999**:** A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences). Only six of all HIV-1 group M strains dominate the HIV-1 pandemic, namely; subtypes A, B, C, D, CRF01_AE and CRF02_AG (McCutchan, 2006). HIV-1 subtype K has been reported in the Democratic Republic of Congo and Cameroon (Hemelaar *et al*., 2006). HIV-1 subtypes F, G, H and J have been reported in South America and Western Europe; Africa and Central Europe; Central Africa; and the Caribbean, North, Central and West Africa (http://www.avert.org/hiv-types.htm accessed 20-49-2010). The HIV-1 group M subtypes also vary in geographical distribution. For instance, subtype A is prevalent in West Africa and the Republics of the Soviet Union (Robertson *et al*., 1995 and McCutchan, 2006). Subtype B is prevalent globally but dominates the epidemics in South and North America, Western Europe and Australia (Bobkov *et al*., 2004). Subtype C accounts for more than 50% of all infections worldwide but is predominant in Southern Africa, East Africa and India (Buonaguro *et al*., 2007). Subtype D predominantly circulates in East Africa (McCutchan, 2006). Similarly, CRF01_AE is the major strain throughout Southeast Asia and CRF02_AG dominates in West Africa (McCutchan, 2006). Related CRFs

such as CRF07_BC and CRF08_BC have been identified in China (McCutchan *et al*., 2002). CRF02_AG and the CRF09_cpx are prevalent in West Africa (McCutchan *et al*., 2004). Interestingly, approximately 30% of strains in East Africa are URF's (Dowling *et al*., 2002; Harris *et al*., 2002; Arroyo *et al*., 2004 and Arroyo *et al*., 2005); more URF's are also prevalent in the epidemics of West Central Africa and parts of South America (Carr *et al*., 2001a; Carr *et al*., 2001b; Thompson *et al*., 2004b and Sierra *et al*., 2005).

1.1.5. The HIV virion

A mature HIV virion is spherical to pleomorphic in shape with a diameter of 90-130nm (Gallo *et al*., 1984; Gonda *et al*., 1985 and Lytle *et al*., 1992). HIV is an enveloped virus as shown in Figure 1.2. The envelope of the virion is a lipid bilayer derived from the host cell plasma membrane (Arthur *et al*., 1992), within which the viral envelope glycoproteins are embedded. The envelope is comprised of 72 envelope glycoprotein projections which are 9-10nm in length and 14-15nm in diameter formed from a precursor molecule gp160 (Gelderblom, 1991 and Hallenberger *et al*., 1997). Each spike consists of three (trimeric) surface gp120 glycoproteins that are noncovalently bound to three transmembrane gp41 molecules (Earl *et al*., 1990 and Weiss *et al*., 1990).

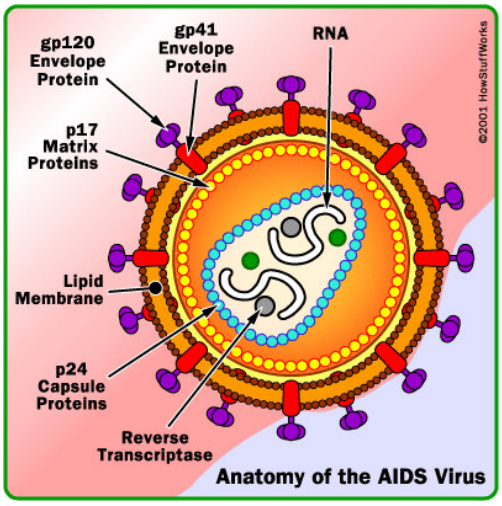


Figure 1.2: Schematic diagram of a mature virion. This Figure was taken from: http://health.howstuffworks.com/diseases-conditions/infectious/aids2.htm accessed 07-09-2011

HIV is a positive sense single stranded RNA virus with a genome representing approximately 2% of the overall viral mass. The genome is a monomer of approximately 9200 nucleotides that form three main genes in the order: 5'-*gag-pol-env*-3' as illustrated in Figure 1.3. The *gag* and the *env* genes encode structural proteins; the *pol* gene encodes the enzymes RT, RNaseH, integrase and protease (Gelderblom, 1991). HIV-1 consists of other genes whose functions are regulatory (*tat* and *rev*) and accessory (*vpr, vif, vpu* and *nef*) to the replication process (reviewed in Table 1.1). The HIV-2 genome is similar, but contains *vpx* instead of *vpu*. When integrated, the viral genome is flanked by two identical long terminal repeats (LTRs).

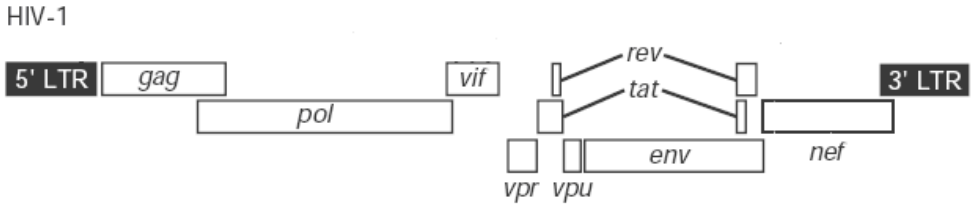


Figure 1.3: The complete HIV-1 genome. This Figure was taken from Jacque *et al*., 2002 and depicts the three main genes *gag*, *pol* and *env* and regulatory and accessory genes essential for HIV-1 replication.

Table 1.1: The size and functions of HIV-1 regulatory, accessory and structural proteins.

Gene Products	Size (kDa)	Function
Regulatory Gene Products		
Tat	14	Regulates viral gene expression [a]
Rev	18	Nuclear transport of viral RNA, stability and utilization factor [b]
Accessory Gene Products		
Nef	25-27	Down regulates cell surface CD4 and MHC-I molecules[c] Augments virus infectivity[c] Modulates signalling pathways of cells[c]
Vpr	15	Nuclear localization of the pre-integration complex (PIC), inhibits cell division, arrest cells in G2/M[d]
Vif	23	Promotes virus infectivity,[e] inhibits APOBEC3G
Vpu	17	Induces CD4 receptor degradation in the endoplasmic reticulum, thus enhance budding[f]
Structural Gene Products		
Gag		
MA	17	Membrane anchoring, env interaction and nuclear transport of viral core[g]
CA	24	Core capsid[g]
NC	7 6	Nucleocapsid, binds RNA Binds Vpr[g]
Pol		
Protease (Pr)	10~12	Cleavage of Gag-pol polyprotein precursor and maturation[g]
Reverse transcriptase (RT)	~117	RNA-dependent and DNA-dependent polymerase activities[g] RNaseH activity catalyses the cleavage of DNA bound RNA via a hydrolytic mechanism[g]
Integrase (IN)	32	mediates the insertion of the HIV-1 proviral cDNA into the genomic DNA[g]
Env		
Gp160	160	Gp120 and gp41 precursor[h]
Gp120	120	CD4 and co-receptor binding[h]
gp41	41	Membrane Fusion[h]

[a] Seelamgari *et al*., 2004. [b] Glushakova *et al*., 2001., Shaheduzzaman *et al*., 2002., Stoddart *et al*., 2003 [c] Zhang *et al*., 2001., Sherman *et al*., 2002., Yao *et al*., 2002., Tungaturthi *et al*., 2003., [d] Montal, 2003. [e] Sheehy *et al*, 2003 [f] Fischer *et al*., 1987., Strebel *et al*., 1987. [g] Kuiken *et al*., 2008 [h] Capon and Ward, 1991., Bernstein *et al*., 1995.

1.1.6. Life cycle of HIV

The typical HIV-1 life cycle comprises several phases i.e. attachment, fusion, reverse transcription, integration, transcription and translation, viral assembly, budding and maturation as shown in Figure 1.4 below.

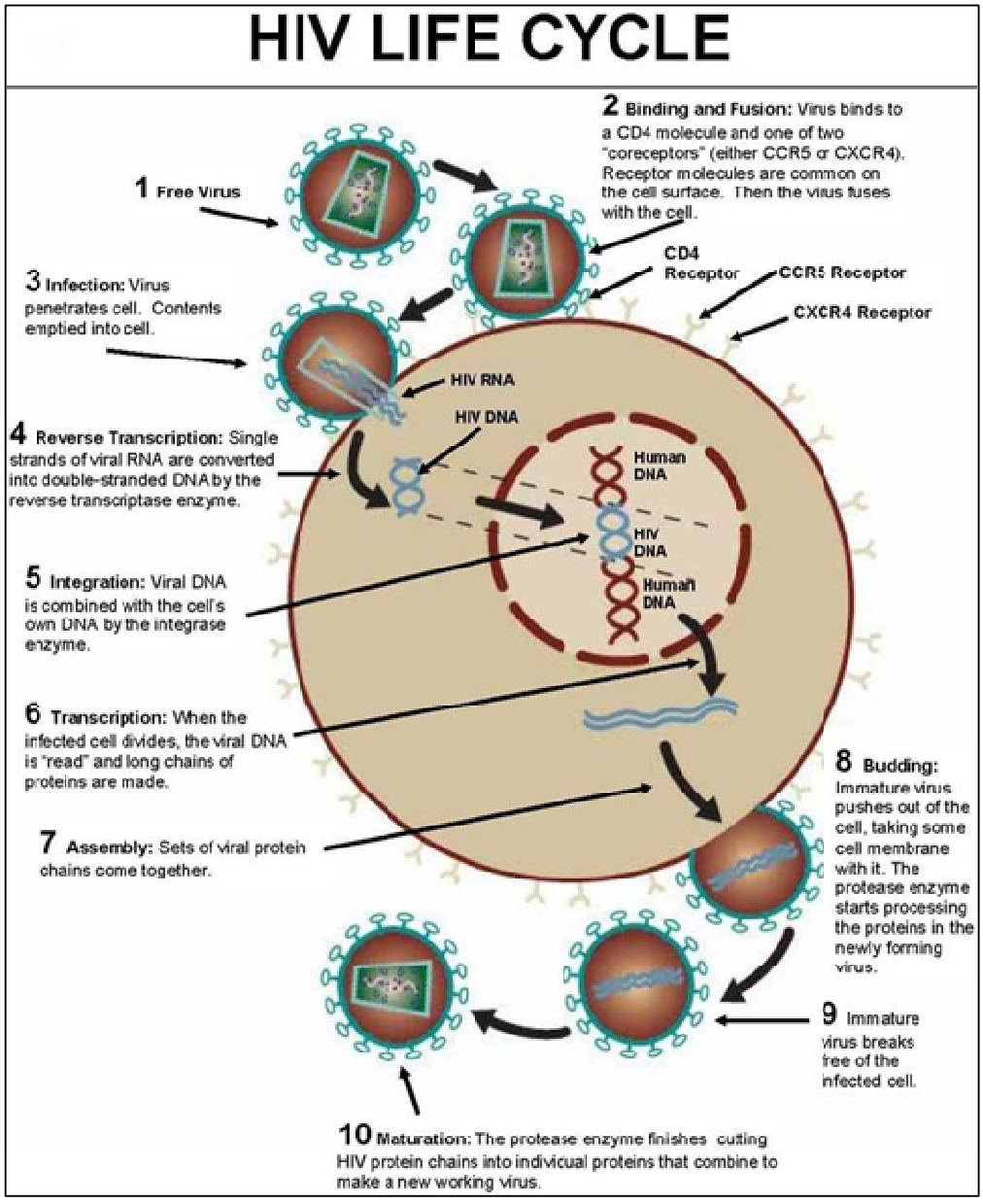


Figure 1.4: A schematic illustration of the typical HIV-1 life cycle. This Figure was taken from Subramani *et al*., 2005.

1.1.6.1. HIV entry and tropism

The life cycle of HIV-1 begins with viral entry into a host cell, as shown in Figure 1.4. Viral entry into a CD4 positive T-lymphocyte is a complex process that consists of a series of coordinated events that are mediated by the gp120 glycoprotein. The gp120 binds to CD4 receptors (attachment) on the surface of the target cell (Samson *et al*., 1996). This interaction introduces conformational changes in the trimeric gp120 molecule, which then leads to co-receptor binding (either CCR5 or CXCR4). The T-tropic (X4) viral strains use the CXCR4 co-receptors and the M-tropic (R5) viral strains use the CCR5 co-receptor for cell entry (Manzoni *et al*., 1990; Lambert and Feltz, 1995; Nakai et *al*., 1996; Berger, 1997 and Berkowitz *et al*., 1998). Dual tropism for the CXCR4 and CCR5 co-receptor has been reported and these viral strains are classified as R5X4 (Berger *et al*., 1998). The M-tropic viral strains are predominant in the early asymptomatic phases of HIV-1 infection and thus CCR5 is regarded as the primary co-receptor for initial HIV-1 infection (Chavis *et al*., 1994; Chavis *et al*., 1995; Nakai *et al*., 1996 and Samson *et al*., 1996). The principal genotypic determinant of co-receptor usage is the V3 variable loop within the gp120 molecule (Hwang *et al*., 1991; Shioda *et al*., 1991; Carrillo *et al*., 1996 and Soulie *et al*., 2008). In addition, modifications in the V1 and V2 variable loops of gp120 have been reported to also introduce variations in HIV-1 co-receptor usage (Groenink *et al*., Groenink *et al*., 1992; Groenink *et al*., 1993; Masciotra *et al*., 1993 and Chesebro *et al*., 1996).

Co-receptor binding further induces exposure of the gp41 ectodomain (Myszka *et al*., 2000 and Salzzwedel & Berger, 2000). The gp41 consists of a cytoplasmic and transmembrane domain in addition to the ectodomain. The ectodomain is made up of four major functional regions; the Tryptophan-rich pretransmembrane domain (PTD), the C-terminal heptad repeat (CHR), the N-terminal heptad repeat (NHR) and a stretch of 15 hydrophobic residues located in the fusion peptide (FP) (Rabenstein *et al*., 1995 and Lev *et al*., 2007). The gp41 adopts a trimeric extended pre-hairpin intermediate (PHI) conformation that allows insertion of the FP into the host cell membrane (Jones *et al*., 1998 and Melikyan *et al*., 2006). The PHI conformation is short-lived (±30 minutes). The PHI later collapses into a low-energy conformation of a six helix bundle (SHB) (Furuta *et al*., 1998 and Munoz-Barroso *et al*., 1998). The

SHB was believed to pull together the viral and cell membranes, but it has been shown that fusion pores on cell membranes form before the complete folding of the SHB (Markosyan *et al*., 2003). Therefore the actual mechanism of fusion is yet to be elucidated (Lev *et al*., 2007). Fusion of the viral membrane with the host cell plasma membrane induces penetration of the viral p24 core into the host cell cytoplasm.

1.1.6.2. Reverse transcription

HIV-1 entry is followed by a poorly understood process of uncoating which modifies the viral core such that reverse transcription can occur (Sarafianos *et al*., 2009). Reverse transcription is a process catalysed by the RT enzyme; a heterodimer consisting of subunits p66 and p51 (DiMarzo-Veronese *et al*., 1986 and Lightfoote *et al*., 1986). The RT enzyme has three catalytic functions: the RNA dependent DNA polymerization, RNaseH activity and DNA dependent DNA polymerization (Goff, 1990). Both the p66 and the p51 exhibit polymerase activity; which is largely dependent on the N-terminal portions of both subunits (Hansen *et al*., 1988); while the RNaseH activity is only associated with the C-terminal portion of the p66 subunit (Hansen *et al*., 1988).

The initiation of reverse transcription occurs at a site in the viral genome known as the primer binding site which consists of eighteen nucleotides complementary to the 3'-terminal end of cellular transfer RNA $(tRNA)^{Lys}$. The partially unwound $tRNA^{Lys}$ initiates reverse transcription by annealing to the 5' region of the primer binding site (Novel, 1994). The viral RT extends the primer tRNA by synthesis of a complementary DNA (cDNA; minus-strand strong-stop DNA) from the 3'OH of the primer to the 5' terminus (van Wamel *et al*., 1998). Synthesis of the cDNA occurs concurrently with the degradation of the RNA template by RNaseH resulting in a single stranded cDNA (Gopalakrishnan *et al*., 1992 and Jacobo-Molina *et al*., 1993). Thereafter, RT generates a polypurine oligonucleotide at the 5' end of the cDNA; which is subsequently copied by extension of the 3' end of the primer into a double stranded cDNA (Jiang *et al*., 1993 and Nouvel, 1994). The methylated adenosine residues and the secondary structure on the primer binding site act as stop signals during synthesis of cDNA (Jiang *et al*., 1993 and Nouvel, 1994).

However, the HIV-1 RT is error prone, and introduces mutations into the viral genome with each replication cycle, resulting in numerous quasispecies within an infected individual.

1.1.6.3. Proviral DNA integration

Subsequent to reverse transcription the cDNA is prepared for insertion into the host genome. This process is catalysed by the 288-amino acid viral integrase enzyme (32kDa). The integrase enzyme consists of 3 independent domains. The N-terminal domain (amino acids 1-49) that carries a $HisX3_{-7}HisX_{23-32}CysX_2Cys$ (HHCC) motif analogous to a zinc finger, and effectively binds Zn^{2+} (Zheng *et al*., 1996). The second domain is the central/catalytic domain (amino acids 50-212) composed of residues 50-212, an RNaseH type fold, and the active site, which is comprised of two Asp residues and one Glu, in a typical DDE motif essential for catalytic activity and viral DNA binding (Engelman *et al*., 1992; Kulkosky *et al*., 1992; van Gent *et al*., 1993 and Johnson *et al*., 2006). The C-terminal domain (amino acids 213-288) is mainly involved in stabilizing the pre-integration complex (PIC) by non-specific binding to viral cDNA (Wang *et al*., 2001 and Chen *et al*., 2000).

Viral DNA integration is a two step process. The first step involves indolytic cleavage of the viral cDNA in the cytoplasm to generate dinucleotide cytosine-adenine (CA) overhangs in a process referred to as 3' processing. The 3' processing step requires a fully functional integrase, the integrity of the last 10-20 base pairs at both ends of the viral cDNA and the presence of specific metallic cationic co-factors such as Zn^{2+} and Mg^{2+} (Lee *et al*., 1997 and Pommier *et al*., 2005).

After 3'-processing, integrase remains bound to the PIC which consists of various viral and cellular proteins in addition to the viral cDNA. The properties of the viral PIC have been attributed mainly to three viral proteins: matrix (MA), nucleocapsid (NC) and Vpr which contribute to the complex nuclear import process of the PIC (Bukrinsky *et al*., 1992). Cellular factors such as Lens Epithelium-Derived Growth Factor/p75 (LEDGF/p75) protein, the DNA flap structure of the viral cDNA, importin-α and importin-β have also been implicated in promoting the translocation of the PIC into nuclei of virally infected cells (Zennou *et al*., 2000; Maertens *et al*., 2003

and Llano *et al*., 2004). Despite extensive research into the area, the mechanism of nuclear import remains unclear (Sherman *et al*., 2002 and Levin *et al*., 2009).

Once in the nucleus, the HIV-1 integrase enzyme catalyses the second step, the strand transfer reaction (Chiu *et al*., 2004). The strand transfer reaction involves the ligation of the 3'-OH cDNA ends generated from 3'-processing, onto the 5'-DNA phosphates of the host chromosome (reviewed by Pommier *et al*., 2005). This process involves a concerted joining of both ends of the viral cDNA and the host genomes' five base stagger which is a result of chromosomal DNA cleavage across the groove (reviewed by Pommier *et al*., 2005; Yoder *et al*., 2000). The final step requires trimming of the last two nucleotides at the 5' ends of the proviral DNA and extension from the 3'-OH chromosomal DNA (Yoder *et al*., 2000). HIV-1 integration into the host chromosome is an reversible process, and there does not appear to be any specificity with respect to integration sites, although active genes are favoured (Yoder *et al*., 2000, Chiu *et al*., 2004, reviewed by Pommier *et al*., 2005).

1.1.6.4. Transcription and translation

The replication phase includes the transcription of the proviral DNA into RNA molecules and their translation into polypeptide precursors (Freed, 2002). The RNA transcription process is initiated by RNA polymerase binding to the proviral DNA and increased levels of the Tat protein that elevates the basal transcriptional activity of the HIV-1 LTR (Dayton *et al*., 1986 and Fischer *et al*., 1986). Tat binds the RNA transactivation response region (TAR) which leads to increased levels of the human positive-transcriptional elongation factor P-TEF-b (Berkhout *et al*., 1989 and Freed, 2002). The P-TEF-b phosphorylates the C-terminal domain of RNA pol II and this stimulates radical transcription (Freed, 2002). Transcription generates a variety of viral RNAs (up to 30 RNA species) that include unspliced RNAs, partially spliced mRNAs (5kb) and small (1.7 to 2.0 kb) multiply spliced mRNAs. The partially spliced mRNAs are translated into Gag and Gag-Pol polyprotein precursors. The partially spliced mRNAs encode the Env, Vif, Vpu and Vpr proteins. The multiply spliced mRNAs are translated into Tat, Nef and Rev (Purcell *et al*., 1993 and Freed, 2002). Some proteins undergo post-translational modifications, such as glycosylation (Env) and myristolation (Nef).

1.1.6.5. Assembly, budding and maturation

HIV-1 maturation requires cleavage of the Gag-Pol polyproteins by the aspartyl protease enzyme to allow maturation of nascent viral particles. (Kohl *et al*., 1988). HIV-1 protease exists as a homodimer with two identical subunits; the active site of the enzyme lies between the subunits. Cleavage of the polyprotein produces four structural proteins which are p24, p17, p7 and p6, whose function includes RNA packaging (Henderson *et al*., 1992). HIV-1 protease cleavage occurs at sites consisting of phenyl-alanine-proline bonds and tyrosine-proline bonds uncommon on mammalian polypeptides (Debouck *et al*., 1992). Proteolytic cleavage introduces morphologic changes in structural proteins followed by assembly of the virion. Viral structural and enzymatic proteins travel to the host plasma membrane where immature virions assemble in cholesterol rich lipid rafts. The Gag p6 recruits components of multivesicular bodies, which facilitate the release of progeny virions from the cell (Overton *et al*., 1990). As new immature virions emerge from the host cell, the host's lipid membranes remain to form the envelope, within which the viral gp120 and the gp41 heterotrimers are embedded (Overton *et al*., 1990). The protease is packaged into virions which allows for continued cleavage of the Gag polyprotein to occur simultaneously with the budding process (Overton *et al*., 1990). Complete processing of the Gag and Gag-Pol in the immature virion yields a mature infectious HIV-1 particle capable of initiating another round of infection.

1.1.6.6. Viral replication

The process of cell infection starts with the attachment of free virions to host cells (Anderson, 2010) as outlined in the life cycle above. Cell infection can also occur in varying mechanisms i.e. cell-to-cell transmission (Mothes *et al*., 2010), mitosis of an infected cell generating two viral-producing progeny cells (Philips, 1994) and through phagocytosis (Noursadeghi *et al*., 2006).

1.2. HIV-1 treatment

The discovery of HIV-1 as the causative agent of AIDS in the early 1980s has since led to considerable success in the development of anti-HIV drugs. To date, there have been more drugs approved for the treatment of HIV-1 infection than any other viral infection (as reviewed in Mehellou and De Clercq, 2009). HIV drugs are currently subdivided into four drug classes, RT inhibitors (i.e. nucleoside inhibitors/NRTIs, nucleotide inhibitors/NtRTIs and non- nucleoside inhibitors/NNRTIs), protease inhibitors (PIs), entry inhibitors (i.e. a fusion inhibitor/FI and a co-receptor antagonist (CRA) and integrase inhibitors (INIs) as summarized in Table 1.2.

1.2.1. RT inhibitors/RTIs

The NRTIs and NtRTIs exhibit anti-HIV RT activity post phosphorylation by cellular kinases into triphosphate/diphosphate metabolites (Balzarrini, 1994 and Hao *et al.*, 1990). The active moieties interact with DNA polymerases allowing incorporation of the nucleoside/tide analogues into a growing DNA chain. Incorporation of RTIs' into a growing DNA chain leads to chain termination due to the active moiety's lack of a 3'-hydroxyl group which prevents further assimilation of nucleotides (Mehellou and De Clercq, 2009).

By contrast, the NNRTIs exhibit anti-HIV RT activity through non-competitive binding to an allosteric site located a short distance (~15 Å) from the catalytic site (De Clerq *et al.*, 2004 and Pauwels, 2004).

1.2.2. Protease inhibitors/PIs

PIs inhibit the proteolytic cleavage of HIV-1 Gag and Gag-Pol polyproteins and this prevents the maturation of virions. Some PI's are transition-state analogues that bind competitively to the active site of the homodimeric enzyme located between monomers, therefore preventing substrate binding (Bannwarth *et al.*, 2007). Other PI's, such as interface peptides, interact with the N- and the C- terminal ends of the dimerized monomers which leads to dissociation of the dimer (Bannwarth and Reboud-Ravaux, 2007).

1.2.3. Entry inhibitors

The approved FI (enfuvirtide) is homologous to a segment on the HR2 region (amino acids 643-678 of the HXB2 sequence) of the gp41. The binding of FI locks the gp41 in a transition state (Wild *et al*., 1993; Wild *et al*., 1994; Chen *et al*., 2002; Kilby *et al*., 2003; LaBonte *et al*., 2003 and Moyle, 2003) and therefore prevents formation of the six-helix bundle hairpin structure necessary for fusion.

The CCR5 antagonist Maraviroc acts by binding directly to the transmembrane CCR5 co-receptor. Maraviroc binds within the 2, 3, 6 and 7 helix near the CCR5 extracellular surface (Dragic *et al*., 2000 and Briz *et al*., 2006), thereby preventing gp120 binding to the co-receptor.

1.2.4. Integrase inhibitors/INs

The recently FDA approved IN inhibitor Raltegravir (Isentress; Merck and co) is a structural analogue of the di-keto acid class of compounds (Hazuda *et al.,* 2000). Raltegravir is a known inhibitor of the strand transfer reaction in HIV-1 integration (Hazuda *et al.,* 2000; Zahm *et al*., 2008) with a concentration required to reduce replication by 50% (IC_{50}) of approximately 10nM (Merck & Co. Inc. Isentress^{TM}, Raltegravir, 2007) and an IC_{95} of 33nM in 50% human serum albumin (Iwato *et al*., 2008).

Table 1.2: Classification of currently available anti-HIV drugs. Information was obtained from http://www.thebody.com/content/treat/art5295.html accessed 07-09-2011

RTIs	**Trade name**	**Company**
Azidothymidine	Retrovir/Retrovis	GlaxoSmithKline
Zalcitabine	Hivid	Hoffman LaRoche
Dideoxyinosine	Videx/Videx EC	Bristol Myers Squibb Co
Abacavir	Ziagen	GlaxoSmithKline
Racivir	Coviracil	Pharmasset
Lamivudine	Zeffix/Heptovir/Epivir/	GlaxoSmithKline
Tenofovir	Viread	Gilead Sciences
Efavirenz	Sustiva/Stocrin	Bristol Myers Squibb Co
Etravirine	Intelence	Tibotec Pharmaceuticals
Nevirapine	Viramune	Boehringer Ingelheim
Delavirdine	Rescriptor	Pfizer
Stavudine	d4T/Zerit	Bristol-Myers Squibb
Emtricitabine	Emtriva	Gilead Sciences
PIs		
Saquinavir	Invirase/Fortovase	Roche
Ritonavir	Norvir	Abbott Laboratories
Darunavir	Prezista	Tibotec
Indinavir	Crixivan	Merck
Tipranavir	Aptivus	Boehringer Ingelheim
Fosamprenavir	Lexiva/Telzir	GlaxoSmithKline
Nelfinavir	Viracept	Pfizer/Hoffman LaRoche
Atazanavir	Reyataz	Bristol Myers Squibb Co
Lopinavir	Kaletra/Aluvia	Abbott Laboratories
Amprenavir	Agenerase	GlaxoSmithKline
FIs		
Enfuvirtide	Fuzeon	Roche
CRA		
Maraviroc	Selzentry/ Celsentri	Pfizer
INIs		
Raltegravir	Isentress	Merck

1.2.5. Anti-HIV drugs in development

There are numerous potential drug candidates with anti-HIV activity undergoing phases II and III human clinical trials as shown in Table 1.3. Some of these anti-HIV drug candidates act through previously established inhibition mechanisms. However they represent the next generation of anti-HIV drugs with better resistance and toxicity profiles. Other exciting elements from Table 1.3 include the fact that half of

these candidate anti-HIV drugs belong to relatively new classes such as the entry inhbitors. Agents belonging to drug classes with novel mechanisms of action have enriched existing anti-HIV therapy and will contribute considerably towards delaying the onset of antiretroviral drug resistance if successful (Mehellou and De Clercq, 2010).

Table 1.3: Anti-HIV drugs currently undergoing human clinical trials (Phase II and Phase III). This information was obtained from a review study by Mehellou and De Clercq, 2010.

Drug	Class	Developer	Phase
Apricitabine	NRTIs	Avexa	III
Amdoxovir	NRTIs	RFS Pharm	II
Elvucitabine	NRTIs	Achillion Pharmaceuticals	II
Racivir	NRTIs	Pharmasset	II
BILR 355 BS	NNRTIs	Boehringer Ingelheim	II
(+)-calanolide A	NNRTIs	Sarawak MediChem Pharmaceutics	II
IDX899	NNRTIs	Idenix Pharma	I/II
MIV-150	NNRTIs	Medivir, Chiron	II
RDEA806	NNRTIs	Ardea	II
Rilpivirine	NNRTIs	Tibotec	IIb
AK-602	CRAs	Kumamoto University	III
AMD070	CRAs	AnorMed	II
HGS004	CRAs	Human Genome Sciences	II
Ibalizumab (TNX-355)	CRAs	TailMed Biologics	II
PF-232798	CRAs	Pfizer	II
VCH-286	CRAs	ViroChem Pharm	II
Vicriviroc	CRAs	Schering Plough	III
PRO 140	FIs	Progenics	II
SP01A	EI	Samaritan Pharmaceuticals	III
Elvitegravir	INIs	Gilead Sciences	III

1.2.6. Highly active antiretroviral therapy (HAART)

HIV/AIDS treatment guidelines stress the use of highly active antiretroviral therapy (HAART). HAART was introduced following a rational strategy to delay the emergence of HIV-1 drug resistance through potent virus suppression (reviewed by Carpenter *et al*., 1997). Combination therapy which consists of two or more drugs with varying mechanisms of action provided the required viral suppression to delay the emergence of viral drug resistance (Gupta *et al*., 2008; Mehellou and De Clercq, 2009). Initial HAART regimens resulted in high pill intakes which led to decreased

compliance and adherence rates. The reduced adherence rates led to efforts to design single formulations of HAART by careful selection of regimens that demonstrated synergy as listed in Table 1.4 (Carpenter *et al*., 1997, Mehellou and De Clercq, 2010).

Table 1.4: List of currently FDA-approved anti-HIV drug combinations. This table was adapted from a review study by Mehellou and De Clercq, 2010.

Combination	Components	Manufacturer
Combivir	Zidovudine, Lamivudine	GlaxoSmithKline
Trizivir	Abacavir, Lamivudine, Zidovudine	GlaxoSmithKline
Epzicom/Kinexa	Abacavir, Lamivudine	GlaxoSmithKline
Truvada	Tenofovir, Emtricitabine	Gilead Sciences
Atripla	Tenofovir, Emtricitabine, Efavirenz	Bristol Meyers Squibb, Gilead Sciences

Marked and sustained reductions in HIV/AIDS related mortality and morbidity rates have been observed since the inception of HAART (Palella *et al*., 2002 and Mocroft *et al*., 2003). The use of HAART is associated with prolonged disease-free patient life and reductions in hospitalization rates due to potent viral suppression and CD4+ T-cell repletion (Fleishman and Hellinger, 2003). The success of HAART has been reported numerous times. For instance, a multicentre study in twelve high-income countries has shown a decline of approximately 85% in mortality rates among people living with HIV in comparison to the uninfected population, following the introduction of HAART (Bhasaran *et al*., 2008). More evidence suggests that expansion of HAART programs into resource limited areas has reduced HIV/AIDS mortality rates considerably. For instance, a cohort study in Uganda demonstrated a 95% reduction in HIV/AIDS mortality following introduction of HAART (Mermin *et al*., 2008). An antiretroviral (ARV) scale-up program in Malawi reduced mortality rates among adults by 35% (Jahn *et al*., 2008). ARV scale-up programs in Botswana show that approximately 79% of adult's enrolled for treatment in the early stages of infection were still alive five years after initiation of therapy (Bussmann *et al*., 2008).

South Africa is one country that has also recently began to experience the public health benefits that are associated with the implementation of the government ARV roll-out program which was initiated in April 2004. For example, the Western Cape Province, has since inception of the HAART programme seen its six month mortality rate among patients fall by half (12.7% - 6.6%) between 2001/2002 and the year 2005

(Boulle *et al*., 2008). The Umkhanyakude District in the Kwazulu-Natal Province, has seen mortality rates fall by 22% among woman aged 25-49 and 29% among men from the period 2002/2003 to 2004/2006 (Herbst *et al*., 2009).

In South Africa, the antiretroviral therapy (ART) roll-out programme began slow but has since progressed from the 32895 HIV-1 patients in January 2005 to 871,914 HIV patients on ARVs in 2009 (Rehle *et al.*, 2010). Although successful implementation seemed costly and socially complex (Benatar, 2004; Natrass, 2007); evidence suggests accomplishments would be determined by a better understanding of the wide social, geographical and cultural issues regarding HIV/AIDS (Murray, 2009). For instance, HIV/AIDS in South Africa is associated with racial and ethnic dimensions (De Vos and Baim-Lance, 2002; Gilbert and Walker, 2009).

1.3. Antiretroviral drug resistance

The use of combination therapy has proven remarkably effective in controlling the progression of the HIV epidemic, but the benefits are still compromised by the emergence of ARV drug resistant strains (Palella *et al*., 1998; Lederberger *et al*., 1999 and DeGruttola *et al*., 2000). More than 200 mutations (clinical and *in vitro*) associated with ARV resistance to the four classes of drugs have been characterised and more than ninety of these are associated with HIV RT (Shafer *et al*., 2008).

1.3.1. HIV RT ARV drug resistant strains

ARV drug resistance mutations associated with NRTI's include M184V, thymidine analogue mutations, mutations associated with non-thymidine analogue containing regimens, multi-nucleoside resistance mutations and several recently identified accessory mutations. NRTI's mutations exceed fifty in number and NNRTI drug resistance mutations exceed forty (Shafer *et al*., 2008). Drug resistance to NRTIs may arise through the removal of the chain terminating residue; a kind of repair reaction involving pyrophosphorolysis developed by the mutant RT (De Clercq, 2002). Another NRTI resistance mechanism involves enhanced discrimination of the analogues in favour of authentic nucleotides by the mutant RT (Deval *et al*., 2002; 2004).

NNRTI ARV drug resistance mutations include polymorphic and non-polymorphic accessory mutations.Polymorphisms are mutations that occur without selective drug pressure, and non-polymorphisms occur when virus is exposed to drug (Shafer *et al*., 2007; Shafer *et al*., 2008). Almost all NNRTI resistance mutations are located within the binding site or adjacent to residues within the binding site thus preventing allosteric binding (Sarafianos *et al*., 2004 and Ren *et al*., 2008).

1.3.2. HIV PI ARV drug resistant strains

PI resistance is comprised of more than sixty mutations which include major protease, minor protease, accessory protease and Gag cleavage site mutations (Shafer *et al*., 2008). Many PI mutations are accessory, compensating the replication impairment and susceptibility only in combination with other resistance mutations. Another PI resistance mechanism can be introduced by Gag cleavage site mutations and possibly other parts of the Gag that influence Gag-Pol processing (Cote *et al*., 2001; Dauber *et al*., 2002; Hoffman *et al*., 2003; Prabu-Jeyabalan *et al*., 2004 and van Maarseveen *et al*., 2006).

1.3.3. HIV IN ARV drug resistant strains

More than thirty integrase mutations are associated with ARV drug resistance to the recently approved Raltegravir, and Elvitegravir currently in clinical trials (Shafer *et al*., 2008). Mutations associated with the integrase enzyme are mostly within the binding site vicinity (Lataillade *et al*., 2007). IN mutations are associated with decreased drug susceptibility, loss of enzyme fitness and *vice versa* (Lataillade *et al*., 2007).

1.3.4. HIV entry ARV drug resistant strains

The gp41 protein is associated with fifteen mutations that enhance resistance against enfuvirtide (Shafer *et al*., 2008). Enfuvirtide resistance is associated with mutations in HIV-1 gp41 codons 36 to 45 (according to HXB2 numbering), but is also associated with loss of virulence in mutant isolates (Marcelin *et al*., 2004; Menzo *et al*., 2004; Lu

et al., 2004; Sista *et al*., 2004; Mink *et al*., 2005; Melby *et al*., 2006 and Su *et al*., 2006).

ARV drug resistance mutations against CCR5 antagonists are poorly understood, but dose response curves suggest mutations in gp120 which may lead to enhanced viral binding to the CCR5 receptor in the presence of the CCR5 antagonist (Pugach *et al*., 2007 and Westby *et al*., 2007). CCR5 antagonist ARV drug resistance was recently reported in a patient treated with Aplaviroc (Pfaff *et al*., 2010). These Aplaviroc resistant CCR5 mutations were also observed to introduce a tropism shift toward effector memory cells upon infection of primary CD4+ T cells (Pfaff *et al*., 2010).

1.3.5. Transmission of HIV-1 ARV drug resistant strains

Emergence of ARV drug resistant HIV-1 strains is an acknowledged concern in HIV-1 treatment; but the transmission of drug-resistant (TDR) strains reported exacerbates the existing problem (Grant *et al*., 2002 and Little *et al*., 2002). Reports on TDR strains estimate their prevalence in developed countries where HAART is widely used to be at 10-20% (Grant *et al*., 2002; Ibe *et al*., 2002; Little *et al*., 2002; Simon *et al*., 2002 and Shet *et al*., 2006). TDR has also been reported in regions where HAART usage is less common i.e. Asia and Africa (Barth *et al*., 2008 and Choi *et al*., 2008). These strains often exhibit resistance to several ARV drug classes and cross resistance within a class is also frequent. The emergence of TDR strains further complicates efforts to control the HIV-1 epidemic (Shafer *et al*., 1998; Hertogs *et al*., 2000 and Miller *et al*., 2001); hence the need for continued surveillance for TDR strains, and a continued search for new generation ARV agents.

Assessment of temporal changes in the incidence of ARV drug resistant mutants through analysis of plasma viral load from individuals undergoing treatment suggest drastic decreases in the incidence of new HIV-1 ARV drug resistance (Gill *et al*., 2010). Such decreases were associated with increased numbers of treated patients achieving virological suppression. The high rates of virological suppression over time were associated with the introduction of new HIV drugs and the continual assessment and improvement of HAART (Gill *et al*., 2010). These studies reinforce the ongoing need for novel therapeutic agents.

1.3.6. Challenges in anti-HIV drug development and discovery

Drug discovery and development is a lengthy process of approximately fifteen to eighteen years and with an estimated cost of US$800M to US$880M (Overby *et al*., 2001 and Tollman *et al*., 2001; Cygnus, Business Consulting and Research. Vol 510, October 2005). The drug discovery process is characterised by high attrition rates with only 20% of drug leads entering clinical trials and only 10% of those achieving clinical registration. The attrition rate is mostly due to inadequate ADMET (adsorption, distribution, metabolism, excretion and toxicity properties; Eddershaw *et al*., 2000 and Dimasi *et al*., 2001). Large-scale chemical synthesis, pharmaceutical formulation, chemical stability and undesirable side-effects upon administration *in vivo* are additional factors that lead to increased attrition (Pauwels, 2006). Termination of a drug from the development process is normally due to various factors which are linked. For instance, toxicity might be the decisive reason for termination of a drug candidate from development, but factors such as long-term systematic exposure could be the underlying cause of the toxicity observed (Eddershaw *et al*., 2000). To avoid financial losses in drug discovery and development, pharmaceutical research and development uses rational approaches, with the logic being to reduce attrition in the costly downstream stages by screening agents for ADMET properties in the early stages of the discovery process - a "fail early and fail cheap" strategy (Eddershaw *et al*., 2000; Atterwill *et al*., 2002; Lin *et al*., 2003 and Robert, 2003). This is carried out through introduction of in silico techniques, physicochemical analysis techniques and biological assays that provide reasonable accuracy and preliminary prediction (Yu *et al*., 2003).

1.4. Gold in medicine/Chrysotherapy

Gold is a soft, malleable, lustrous yellow transition metal that can exist in a range of oxidation states, primarily the 0, 1+ and the 3+. The gold (0) state is the most stable, while gold(III)/3+ can be reduced to gold(I)/1+ which is easily reduced to gold(0); (Brown *et al*., 1982, Merchant, 1998).

The putative medicinal properties of gold have been known since antiquity with Arabic, Indian and Chinese physician's prescribing gold preparations for the treatment of diverse ailments (Higby, 1982; Parish, 1992 and Tiekink, 2003). Chrysotherapy (the use of gold for medicinal applications; Fricker, 1996) in modern science was stimulated by Robert Koch's 1890s observation of bacteriostatic effects of gold cyanide toward tubercle bacillus. Gold therapy was effectively introduced in the 1920s following suggestions of tubercle bacillus being the causative agent of rheumatoid arthritis (RA) which was later confirmed otherwise (reviewed by Fricker, 1996). Studies suggest that gold compounds have potential in the treatment of cancer, HIV-1 infection and malaria (Shaw, 1999; Sun *et al*., 2004; Casini *et al*., 2008; Sannella *et al*., 2008).

1.4.1. Rheumatoid Arthritis (RA)

In 1935, Robert Koch's findings inspired Jacque Forestier to further investigate the use of gold compounds in the treatment of various arthritis ailments. These incorporated the treatment of psoriatic arthritis, juvenile arthritis, palindromic rheumatism and discoid lupus erythematosus as described by Champion *et al*., 1990. Early chrysotherapy included the use of sodium aurothiomalate and aurothioglucose to treat RA, a practice that lead to the discovery of the orally bioavailable drug auranofin in 1985 (reviewed by Fricker, 1996). Gold thiolates (Table 1.5) have been the principal gold compounds used in the treatment of arthritis but their mechanism of action is still unclear in part due to the lack of understanding of RA. The three widely used gold drugs, aurathiomalate, aurothioglucose and auranofin, have been shown to inhibit protein kinase C (Mahoney *et al*., 1989). Kinase C is a metallo-enzyme containing Zn^{2+} bound to cysteine (Cys) and histidine (His) residues which are essential for intracellular signal transduction through phosphorylation of serine (Ser) or threonine (Thr) residues on protein surfaces (Herlin *et al*., 1989 and Mahoney *et al*., 1989). Biochemical studies have shown that gold compounds act upon different stages of RA, one possibility was the interaction with peptides presented on the surface of T-helper cells by MHC-II molecules (De Wall *et al*., 2006). Such an interaction would prevent immunologic recognition and phagocytosis since RA is an autoimmune disorder (Best and Sadler, 1996).

Table 1.5: Gold thiolates used medicinally for Rheumatoid Arthritis treatment (modified using information from Sadler, 1976; Brown *et al*., 1980 and Jones *et al*., 1996).

Name	Formula
Myocrisin, Myochrisin, Mychrisis	Sodium gold thiomalate $[Na_2AuSt_m]$, a variable mixture of the mono-($C_4H_4AuNaO_4S$) and disodium ($C_4H_3AuNa_2O_4S$) salts
Solganol	Sodium gold$^+$ thioglucose; gold 4-aminomethylsulphonic-acid-2-mercaptobenzene-1-sulphonic acid
Sanocrysin, Sanochrisin, Crisalbine, Aurothion	Sodium gold thiosulfate $[Na_3Au(S_2O_3)_2]$
Allocrysine, Allochrysin	Sodium gold thiopropanosulfonate
Krysolgan	Sodium gold 4-amino-2-mercaptobenzoic acid
Auranofin	2,3,4,6-tetra-o-acetyl-1-thio-B-D-glucopyranoside; 2,3,4,6-tetra-O-acetyl-1thio-B-D-Pyranosato-S-(triethylphosphine)-Au$^+$

1.4.2. Anticancer activity of gold compounds

The discovery of the anticancer properties of the platinum based drug cisplatin prompted a great deal of interest in the area of metal based cancer chemotherapy (Rosenberg *et al*., 1969). Research on possible chrysotherapy for cancer focused on gold(III) compounds; which led to the testing and discovery of auranofin's anti-cancer activity in HeLa cell lines and P388 leukaemia cell lines *in vivo* (Simon *et al*., 1979; Simon *et al.,* 1981; Simon *et al.,* 1989). This was due to gold(III) compounds isoelectric square planar similarities to cisplatin, and therefore similar antitumor properties were expected. Studies have implicated compound-DNA interactions and inhibition of disease specific thiol-containing, cysteine protease cathepsins as possible mechanisms of action (Gabbiani *et al*., 2007 and Casini *et al*., 2008). In addition, the gold(III) compounds exhibit inhibition of the thioredoxin reductase and the associated

disregulation of the mitochondrial functions, which explains their apoptotic effects during cancer cell growth inhibition (Casini *et al*., 2008). Messori and co-workers have demonstrated the inhibition of cisplatin resistant cancer cell lines by gold(III) compounds (Messori *et al*., 2000); which suggests that gold compounds and cisplatin have a different mechanism of anticancer action (Messori *et al*., 2000, Gabbiani *et al*., 2007).

1.4.3. Antimicrobial activity of gold compounds

Gold compounds have in addition been investigated for activity against various microbial pathogens. For instance Novelli *et al*., 1999 has demonstrated biocidal activity of gold compounds against *Escherichia coli* (*E.coli*) ATCC25922, *Pseudomonas aeruginosa* (*P.aeruginosa*) ATCC 27853, *Staphylococcus aureus* (*S.aureus*) ATCC 25923 and ATCC 6538, and *Staphylococcus epidermidis* (*S.epidermidis*). Moreover, gold compounds also exhibit anti-fungal activity toward *Candida albicans* (*C.albicans*) ATCC 10231, *Aspergillus niger* (*A.niger*) ATCC 16404 and *Trypanosoma cruzi* (*T.cruzi*; Sanchez-Delgado *et al*., 1993 and Novelli *et al*., 1999). Gold compounds have been shown to inhibit malarial pathogen such as *Plasmodium falciparum* (*P.falciparum*) and *Plasmodium berghei* (*P.berghei)*; and some sexually transmitted infections such as *Peptostreptococcus vaginalis* (*P.vaginalis*; Sanchez-Delgado *et al*., 1996; Navaro *et al*., 1997 and Novelli *et al*., 1999).

1.5. Potential chrysotherapy of HIV/AIDS

Aurocyanide, a gold compound metabolite, has demonstrated inhibition of HIV-1 proliferation in a cultured T-9 cell line at levels as low as 20nM and was noted for possible synergy in combination with other ARV drugs (Tepperman *et al*., 1994). Other gold compounds reported to exhibit anti-HIV-activities are bisthioglucose)gold(I) (bisAuTG), bis(thiomalate)gold(I) (bis-AuTM), and bis-aurothiolate (bisAuTH). BisAuTG has been reported to prevent HIV-1_{NL4-3} infection in an MT4 cell line with an IC_{50} of 2.6±1.2μM (Okada *et al*., 1993). The current interest in HIV chrysotherapy was stimulated by a report that described the antiviral activity of gold compounds in a patient presented with AIDS and arthritis i.e. Reiter's

syndrome and psoriatic arthritis patient accepting only gold treatment (Shapiro and Masci, 1996). The patient was not accepting antiretrovials but was treated for psoriatic arthritis with the gold-based drug, Auranofin which led to an increase in CD4+ T-cell count of the HIV/AIDS patient. The patient's CD4+ T-cell counts increased from 270cells/mm^3 to 400cells/mm^3 five months after initiation of gold therapy with a CD4/CD8 T cell ratio of 0.47. The patient's viral load measurement after two years following beginning of treatment was 1498 RNA copies/ml (Shapiro and Masci, 1996).

1.5.1. Possible HIV drug targets for gold compounds.

Possible HIV-1 drug targets for gold compounds include inhibition of viral adsorption, inhibition of viral fusion, inhibition of RT by allosteric binding and active site binding (Blough *et al*., 1989; Okada *et al*., 1993). Gold compounds have been observed to inhibit nuclear factor kappa beta (NF-κβ) signalling pathway (Okamoto and Wong., 1986; Yang *et al*., 1995; Traber *et al*., 1998; Jeon *et al*., 2000) but since this pathway is not unique to HIV-1; it is therefore not a good target in that there may be toxicity implications. The mechanism of HIV-1 inhibition by gold compounds could be associated with gold's affinity for proteins. Gold-based compounds have previously been shown to interact with naturally occurring thiols on unpaired cysteines (i.e. Cys^{532} of HIV-1 gp41) and glutathione residues on the surface of proteins (Shaw, 1988; Shaw, 1989).

1.5.2. Viral entry inhibition

Bis(thioglucose)gold(I) has been reported as an HIV-1 entry inhibitor (Okada *et al*., 1993). It was proposed that the compound undergoes gold(I) ligand exchange with thiol groups such as Cys^{532} in the amino terminus of gp41 of HIV-1_{NL4-3}, which are exposed on the surface. This interaction introduces conformational changes in the fusion peptide which releases gp120 from the surface of the virion (Okada *et al*., 1993). Such data directly implicated viral infectivity as a possible anti-HIV-1 drug target for gold-based compounds (Okada *et al*., 1993).

1.5.3. HIV-1 RT inhibition

Aurothioglucose was shown to be an effective inhibitor of HIV-1 RT in cell lysates, inhibiting the initiation and elongation of viral cDNA chains (Blough *et al*., 1989). In addition, an aurothioglucose metabolite bisAuTG was later observed to exhibit anti HIV-1 RT activity (Okata *et al*., 1993). More recently Sun *et al*., 2004 demonstrated *in vitro* HIV-1 RT inhibition by gold(III) porphyrins following reports of *in vitro* HIV-1 inhibition by heme and some synthetic metalloporphyrins (Staudinger *et al*., 1996 and Argyris *et al*., 1999); with $[Au^{III}(TMPyP)]Cl_5$ demonstrating the most potent effects (IC_{50} = 0.31μM) as shown in Table 1.6 (Sun *et al*., 2004). Fonteh *et al*., 2009 has recently reported HIV-1 RT inhibition by gold based compounds at concentrations higher than 10μM.

Table 1.6: Previously reported gold complexes IC_{50}s against HIV-1 RT (Sun *et al*., 2004).

Compound	IC_{50}±STDev (μM)
$[Au^{III}(TMPyP)]$	0.31±0.05
$Na_4[Au^{III}(TPPs)]Cl$	0.57±0.09
$[Au^{III}(TPP)]Cl$	28.4±1.8
$[Au^{III}(salen)]^+$	0.33±0.03
$[Au^{III}(dcbpb)]^+$	0.47±0.09

1.5.4. Gold compounds inhibit the NF-κß signalling pathway

NF-κβ is an inducible cellular transcriptional factor that regulates a wide variety of cellular and viral genes with demonstrated ability to activate HIV-1 gene expression (Baeuerle and Baltimore, 1988; Okamoto *et al*., 1990; 1997 and Hayashi *et al*., 1993). NF-κβ exists in the cytoplasm complexed with an inhibitory molecule inhibitor kappa beta (Iκβ). Upon stimulation by virus/tumour necrosis factor alpha (TNF-α), the inhibitor molecule Iκβ dissociates from the complex allowing NF-κβ translocation to the nucleus where it site specifically binds to DNA (Baeuerle and Baltimore, 1988; Ghosh and Baltimore, 1990; Barnes *et al*., 1997 and May *et al*., 1998). In latently infected cells, NF-κβ binding to DNA induces the transcription of HIV-1 genes including *tat*, which leads to an explosive increase in viral replication (Okamoto and Wong, 1986 and Okamoto *et al*., 1989). The gold compound aurothioglucose has been

shown to inhibit the dissociation of the NF-κβ/Iκβ complex in OM101 and Ach2 cell lines latently infected with HIV-1 (Traber *et al*., 1998). Other studies have demonstrated inhibition of the DNA-binding ability of NF-κβ through a gold compounds redox mechanism and by blocking the Iκβ kinase enzyme known to phosphorylate the Iκβ (Yang *et al*., 1995 and Jeon *et al*., 2000).

1.5.5. Gold compounds as potential pro-drugs

Gold(III) thioglucose has been reported as an HIV RT inhibitor although it's been observed not to readily enter cells. Gold compounds are largely known to form metabolites such as $[Au(CN)_2]^-$ which enhance their cellular (in H-9 cells) uptake, and as a result sufficient gold accumulates for viral inhibition (Roy *et al*., 1994). Studies based on in vitro experiments monitoring intestinal uptake of auranofin have demonstrated that it is a metabolite of the drug that crosses the intestinal wall (Tepperman *et al*., 1984). Other studies identified dicyanogold(I)/$[Au(CN)_2]^-$ as the primary metabolite in patients accepting treatment with sodium gold thiomalate and gold thioglucose (Elder *et al*., 1993). These gold compounds are known to enter cells through transmembrane transport of their apparent metabolites, which are probably formed as a result of their binding to serum proteins to which their affinity is evident. For instance, gold compounds have been reported to interact with the soft ligands i.e. thiolates and phosphines on human serum albumin (HSA), horse heart cytochrome c, bovine ubiquitin, hemoglobin and metallothionein (Shaw, 1989; Best and Sadler, 1996 and Casini *et al*., 2006). Moreover, 80% of gold is found bound to HSA subsequent to exposure (Casini *et al*., 2006). These protein-drug interactions are possibly the cause of gold compound breakdown which creates the inhibitory gold metabolites. Therefore, such data suggests a pro-drug effect, and offers a possible mechanism of action.

1.6. Summary

In South Africa, the country most affected by the HIV/AIDS pandemic, greater emphasis and research efforts should be focused on developing the next generation and/or novel anti-HIV agents for use locally. This study uses a short gun approach to elucidate gold compounds as potential inhibitors of HIV-1. Theoretical approaches are used to assess bioavailability of test compounds and *in vitro* tests are carried out to identify inhibitors and their biological properties. Structures of HIV-1 inhibitors identified will later be used as a base to rationally design second generation inhibitors with improved biological profiles.

1.7. Hypothesis

Theoretical and experimental analysis of a collection of gold-based compounds will aid identify good anti-HIV-1 inhibitors and their mechanism of action.

1.8. Objectives

Gold-based compounds were previously observed to inhibit HIV-1 replication, therefore it was decided that a blind chemical screen would be carried out on our existing collection. The overall objective of this study is to systematically analyse gold-based compounds as inhibitors of HIV-1, and this will be accomplished through fulfilment of the following aims:

- To theoretically analyse all compounds within the library through on-line software and via the Lipinski Rule of Five (RO5).
- To experimentally determine and evaluate the solubility, cytotoxicity and antiretroviral activity of each of the compounds.
- To narrow down the mechanism of action for compounds with antiretroviral activity.
- To closely correlate all data with compound structures, and provide commentary on useful characteristics.

CHAPTER 2

MATERIALS AND METHODS

2.1. Synthetic chemical compounds and reagents used in this study

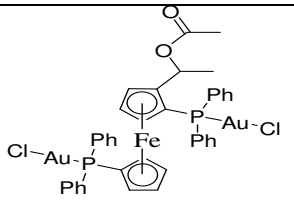
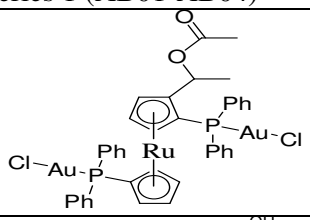
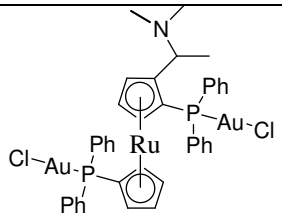
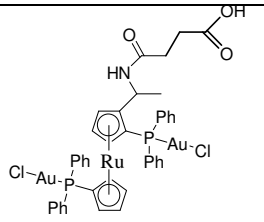
2.1.1. Synthetic chemical compounds investigated

From an in-house collection of 150 gold compounds available at Mintek, 45 gold-based compounds were selected for further analysis in this study. These 45 compounds arise from 5 compound families (see Tables 2.1 to 2.5 below) and were selected as a representative sample of the 150 gold compounds. The compounds exhibit structural similarity within the 5 compound families but are structurally diverse between compound families.

2.1.1.1. Chemical structures of gold compounds Autek Biomed (AB) 01-AB04 (Series 1)

This Series consist of four gold(I) triphenylphosphine complexes from metallocene derivatives (Fe and Ru), obtained from Professor James Darkwa's laboratory at the University of Johannesburg, South Africa (Table 2.1).

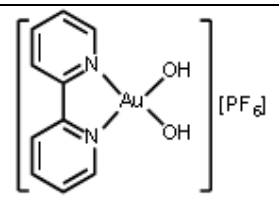
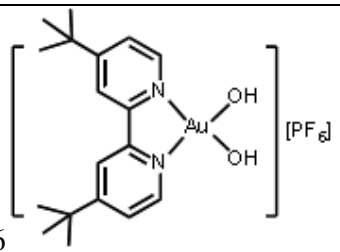
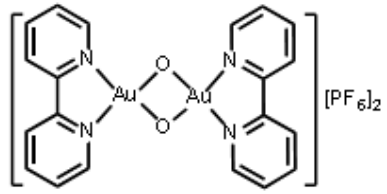
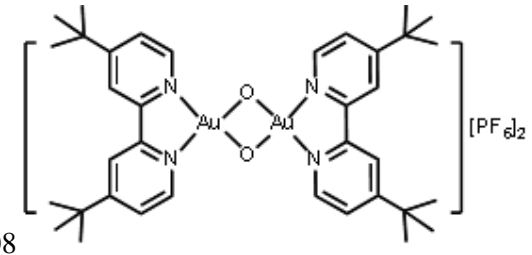
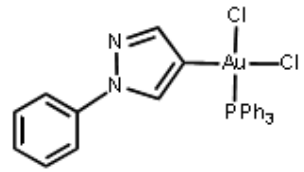
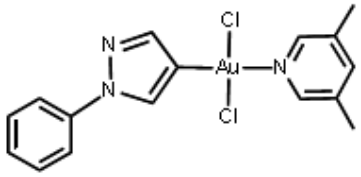
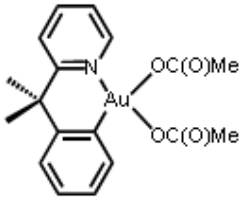
Table 2.1: Chemical structures of gold compounds in Series 1 (AB01-AB04)

AB01 	AB02 
AB03 	AB04 

2.1.1.2. Chemical structures of gold compounds AB05-AB11 (Series 2)

The seven compounds were a kind gift from Professor Maria A. Cinellu of the Universita di Sassari, Italy (Table 2.2). This Series of compounds contain NNOO coordinated gold(III) complexes. These complexes are PF_6^- salts (with the exception of AB09, AB10 and AB11), known to confer low solubility in ionic liquids.

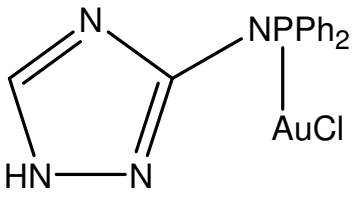
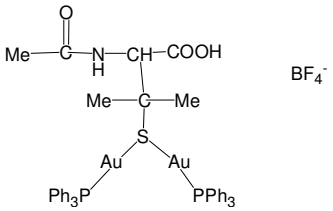
Table 2.2: Chemical structures of gold compounds in Series 2 (AB05-AB11)

 AB05	 AB06
 AB07	 AB08
 AB09	 AB10
 AB11	

2.1.1.3. Chemical structures of gold compounds AB12-AB13 (Series 3)

The two compounds AB12 and AB13 used in this study were synthesised in Professor Antonio Laguna's Laboratory at the Universidad de Zaragoza, Spain (Table 2.3). They are a small gold(I) phosphine and a thiolate complex, with Chlorine as coordinated counter ion and triflate and tetrafluorborate salts.

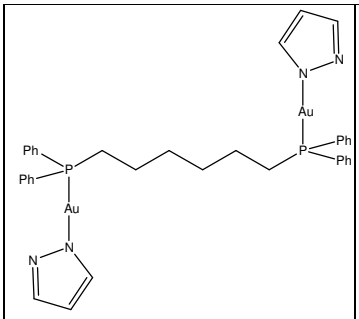
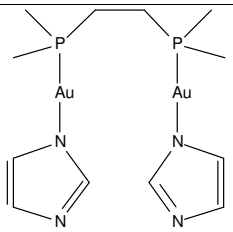
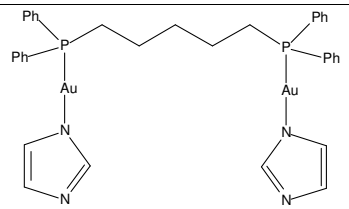
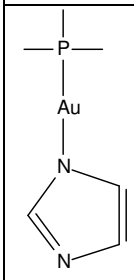
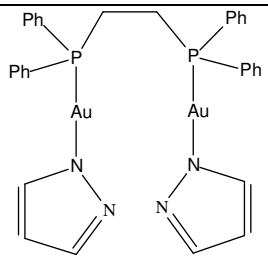
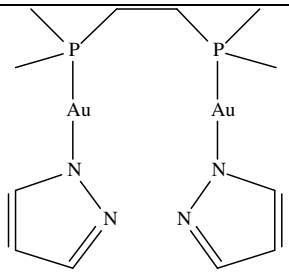
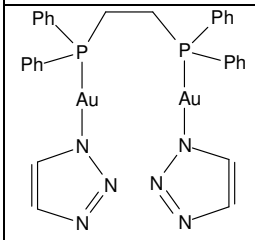
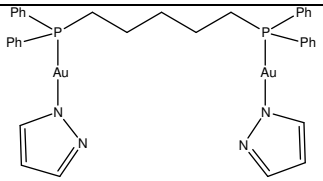
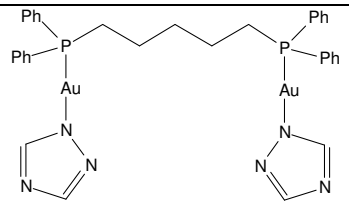
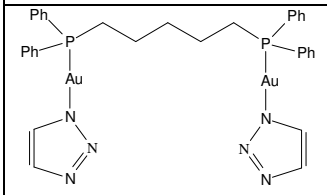
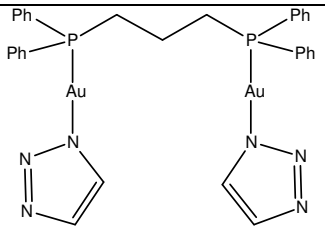
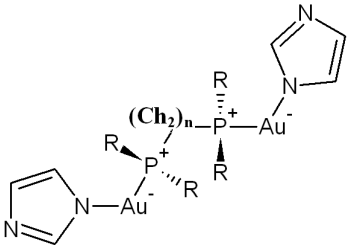
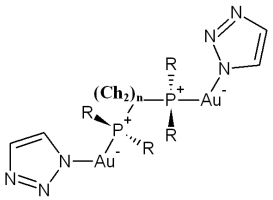
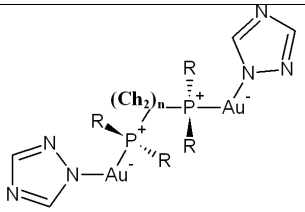
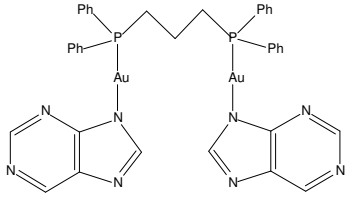
Table 2.3: Chemical structures of gold compounds in Series 3 (AB12-AB13)

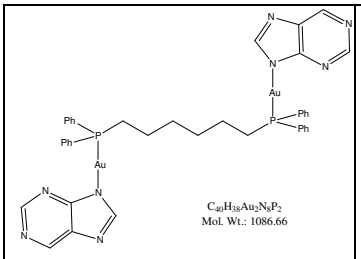
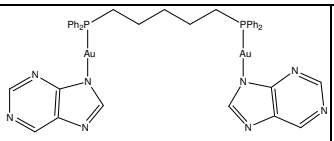
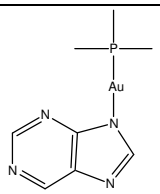
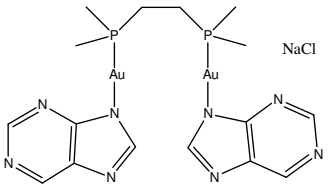
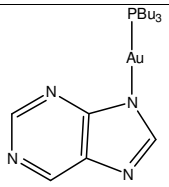
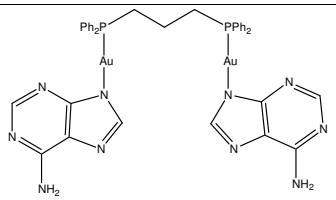
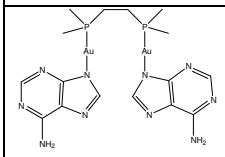
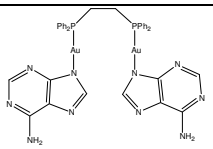
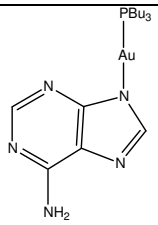
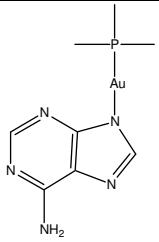
 AB12	 AB13

2.1.1.4. Chemical structures of gold compounds AB14-AB38 (Series 4)

This Series of 26 compounds (AB14-AB38) were synthesised in Prof H.G. Reubenheimer's Laboratory at the University of Stellenbosch, South Africa, and they consist of some mono- and bridge- phosphine and nitrogen-based gold(I) complexes (Table 2.4). These compounds vary in the length of the hydrocarbon bridge, the nature of the phosphine substituent and nitrogen-rich heterocycle (purines, azoles).

Table 2.4: Chemical structures of gold compounds in Series 4 (AB14-AB38)

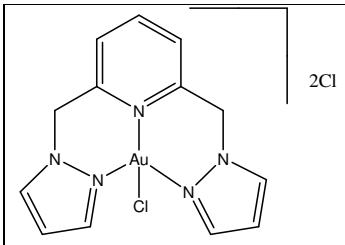
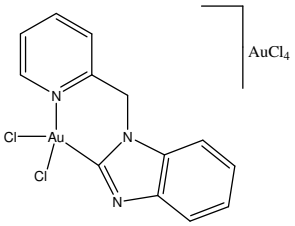
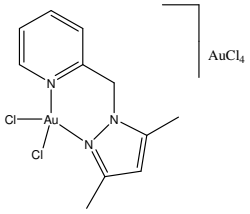
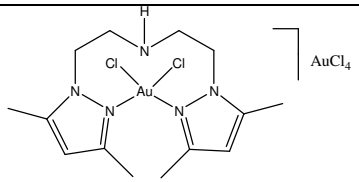
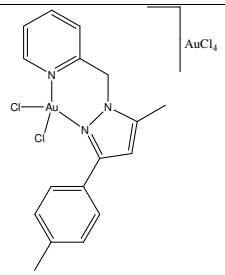
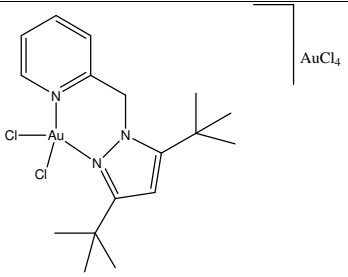
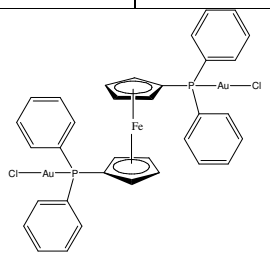
 AB14	 AB15	 AB16
 AB17	 AB18	 AB19
 AB20	 AB21	 AB22
 AB23	 AB24	 AB25
 AB26	 AB27	 AB28

 AB29	 AB30	 AB31
 AB32	 AB33	 AB34
 AB35	 AB36	 AB37
 AB38		

2.1.1.5. Chemical structures of gold compounds AB39-AB45 (Series 5)

This Series of seven compounds was synthesised in Professor James Darkwa laboratory at the University of Johannesburg, South Africa, and consists of gold(III) complexes of substituted pyrazol and pyridine (as bidentated nitrogen donors) linked with a methylene or ethylene bridge (Table 2.5); and a ferrocene diphosphine gold(I) complex has also been included (AB45).

Table 2.5: Chemical structures of gold compounds in Series 5 (AB39-AB45)

 AB39	 AB40
 AB41	 AB42
 AB43	 AB44
 AB45	

2.1.2. Cell lines, viral isolates, and recombinant enzymes used in this study

The following reagents were obtained through the NIH AIDS Research and Reference Reagents Program, Division of AIDS, NIAID, NIH: PM1 cells from Dr. Marvin Reitz (Catalogue # 3038); HIV-1_{HXB2} reverse transcriptase (p66>Q/p51) from Dr Stuart Le Grice (Catalogue # 2897); HIV-1_{HXB2} reverse transcriptase/ M184V from Dr. Vinayaka Prasad (Catalogue # 3195); HIV-1_{Ba-L} from Dr Suzanne Gartner (Catalogue # 510); and the HIV-1_{NL4-3} from Dr Malcolm Martin (Catalogue # 2479)

(https://www.aidsreagent.org). The purified recombinant integrase enzyme utilised in this study was prepared within our laboratory from the expression vector pINSD.His.Sol (Catalogue # 2958).

2.2. Theoretical screening of synthetic compound collection

2.2.1. Osiris Molecular Property Explorer

The gold compounds described in section 2.1.1 were screened theoretically using the Osiris Molecular Property Explorer (JAVA-based sketch program) available online at http://www.organic-chemistry.org/prog/peo/ accessed 07-09-2010. The compounds were assessed for several chemical, physicochemical and toxicity parameters through analysis of chemical structures. Specifically, these included water-octanol partitioning coefficiency (cLogP), aqueous solubility (LogS), molecular weight, tumourigenicity, mutagenicity, irritancy, possible effects on reproduction, fragment-based drug likeness and the overall drug-like score. The LogP evaluation uses a reference training set of approximately 368 different atom types to evaluate properties such as atomic number and ring membership thus predicting hydrophilicity; and approximately 5000 experimentally determined logP values. The compound LogS prediction uses approximately 2000 experimentally determined and atomic properties (this excludes ring membership) used in prediction of LogP as an optimizing training set. The assessment of toxicity uses 3300 clinically approved drugs and a set of pre-computed structural fragments from the Registry of Toxic Effects of Chemical Substances (RTECS) as a reference database. Predicted outcomes are valued and colour coded, the red colour represents high risk toxicity fragments and the green is interpreted as drug conform behaviour. Drug-like properties for the 45 compounds were evaluated using a reference database of 3300 approved drugs and 15000 non-drug-like Fluka chemicals by measuring occurrence frequency of specific fragments in the compounds chemical structure. The final drug score combined all parameters assessed to predict the compounds overall drug potential. Following the Osiris Property Explorer assessment of compound structures the drug-likeness scores were analysed using Table 2.6 below.

Table 2.6: Analysis of Osiris Molecular Property Explorer results

Drug-likeness score	Interpretation of Score
1	Desired score
0.7-0.99	Good drug-likeness score
0.4-0.69	Intermediate drug-likeness score
≤0.4	Poor drug-likeness score

2.2.2. Lipinski's Rule of Five (Ro5)

All 45 gold compounds were assessed theoretically in accordance with the four rules of the Lipinski's Ro5. Lipinski's Ro5 is a rule of thumb used to predict the drug-likeness of chemical compounds.

The Rule of Five requires:

: Compounds hydrogen bond donors less than 5 (OH and NH groups)

: Compounds hydrogen bond acceptors less than 10 (N and O atoms)

: Compounds molecular weight less than 500g/mol

: Compounds partition coefficient (LogP) less than 5

The compounds were analysed by visual inspection to determine the number of hydrogen donors and acceptors while the molecular weight and the LogP values were predicted by application of the Osiris Property Explorer detailed in Section 2.2.1. All rules are weighed equally; with a score value of one, thus the score value of each compound is out of a total of four.

The scores were interpreted in accordance with the NIAID – Division of AIDS, HIV / OI / TB Therapeutics Database (http://chemdb.niaid.nih.gov/ accessed 07-09-2010) as in Table 2.7 below.

Table 2.7: Analysis of Osiris Molecular Property Explorer results according to the NIH

Score = Number of Parameters	Interpretation of Score
4	Good
3-1	Intermediate
0	Poor

2.3. Aqueous solubility of synthetic compounds

The aqueous solubility was adapted from a protocol by Millipore Corporation, Billerica, MA USA ("Determination of aqueous compound solubility using a 96-well filter plate to remove precipitated solids prior to UV/VIS Spectroscopic analysis" available at http://millipore.com/techpublications/tech1/pc2445en00 accessed 07-09-2011). Compounds were initially prepared at a stock concentration of 10mg/ml in dimethyl sulfoxide (DMSO; Sigma Aldrich, St Louis MO, USA) and a standard curve for each compound was established using concentrations of 500µM, 200µM, 50µM, 12.5µM and 3.13µM. Chloramphenicol (Sigma Aldrich, St Louis MO, USA) was included as a control compound. All compound concentrations were prepared in a filtered 80:20 (universal buffer, pH7.4: acetonitrile; ACN) solution in 2.2ml 96-deep-well Master-blocks (Greiner Bio-One, Germany) keeping the DMSO concentration below 5% per sample. The universal buffer was prepared using high purity water, 45mM ethanolamine, 45mM potassium dihydrogen phosphate and 45mM potasium acetate; which were respectively purchased from Sigma Aldrich, St Louis MO, USA; Associated Chemical Enterprises Pty Ltd, South Africa; Sigma Aldrich, St Louis MO, USA and Merck, Germany. The assay was left on a shaker (100-300 rpm) for 30 minutes prior to transfer into Greiner UV-Star™ analysis plates and spectrometric detection (X-Mark™ Microplate Spectrophotometer, Bio-Rad Laboratories, Inc. USA) at 260nm to 500nm; from which a standard curve for each compound was determined.

After the standard curve was drawn, compounds (500µM) were prepared in universal buffer (pH7.4) in 200µl volumes in Millex®-LCR filter units, and kept on a shaker (100-300 rpm) for ninety minutes. The samples were vacuum filtered into deep-well Master-Blocks, and subsequently transferred (160µl) to the UV-Star™ analysis plates; wherein 40µl/per sample ACN was added. The assay was quantified using spectrophotometric (X-Mark™ microplate spectrophotometer) detection at each compound's optimal wavelength. The determined optimal absorbance was divided by the slope of the corresponding compound's standard curve, and then multiplied by the dilution factor (1.25) of ACN as shown in the formula below.

$$\text{Aqueous solubility} = (A_{max} \text{ filtrate} / \text{ Slope}) \times 1.25$$

Low aqueous solubility was defined as gold compound concentrations less than 200µM that had dissolved into medium; concentrations between 200µM to 500µM were considered moderate and good solubility was compound solubility greater/equal to 400µM.

2.4. Cytotoxicity assays of synthetic compounds

2.4.1. Gold compounds cytotoxicity in a PM1 cell line

Cytotoxicity testing for all 45 compounds was carried out in PM1 cell lines at a concentration of $1x10^5$ cells/ml. The cell concentration was determined using the trypan blue exclusion (Gibco/Invitrogen, Mowbray, South Africa) method. Cells were initially prepared in RPMI complete media supplemented with 10% foetal calf serum (FCS), 0.2% gentamycin and 0.2% streptomycin/penicillin (Sigma Aldrch, St Louis MO, USA); and were stabilized (100µl/well) for an hour in a 37°C, 5% CO_2 incubator. Eight serial dilutions (400-3.125µM) of each compound were prepared in RPMI complete media and plated at 100µl/well to the incubated cells. After 96 hours of incubation at 37°C and 5% CO_2, cell viability was assayed by addition of 10µl CellTiter 96 Aqueous One Solution Assay to each well (Promega, USA). The assay was read at an absorbance wavelength of 490nm on a multi-plate reader (BP800, BioHIT, Finland) after two hours. Cytotoxic concentration (CC_{50}) was determined as

the concentration of the test substance required to reduce cell viability by 50% using Origin 6.1 software (Origin Lab Corporation, Northampton, MA). Each experiment was carried out in triplicate.

2.4.2. Cytotoxicity assays in peripheral blood mononuclear cells (PBMCs)

The following assay was carried out at the Southern Research Institute, Birmingham, USA, following submission to the NIAID AIDS Research and Reference Reagent Program. Only compounds AB05-AB11 were screened using the following protocol because they had demonstrated considerable direct RT inhibition (see section 2.5.2). Fresh human blood was obtained from the Biological Specialty Corporation (Colmar, Pennsylvania, USA) from donors screened seronegative for HIV and HBV. PBMC isolation was carried out using the standard Ficoll-Hypaque density gradient (Lymphocyte separation medium, Cell Grow #85-072-CL, density 1.078 ± 0.002mg/ml). For the standard PBMC cytotoxicity assay, PHA stimulated cells were pooled from at least two donors. The PBMCs were prepared to a final concentration of $1x10^6$ cells/ml and 50µl/well ($5x10^4$ cells/well) PBMCs were seeded in ninety-six well microplates (round-bottom). Compounds were evaluated using a 200µM high-test concentration with 8 additional serial half-log dilutions. The assay was incubated for 7 days in a 37°C, 5% CO_2 incubator. Cytotoxicity studies included a control NRTI, azidothymidine (AZT; 200µM-0.193µM) in addition to the AB05-AB11 test compounds, plus media without cells control. Compound cytotoxicity and cell viability were measured using the Molecular Devices SPECTRAmax plate reader at 490/650nm following addition of 20µl-25µl MTS (CellTiter 96 Reagent, Promega. USA) and 4 hours of incubation at 37ºC.

2.5. Direct enzyme activity of synthetic compounds

All 45 gold compounds were tested in a range of assays to determine if they exhibited direct enzyme activity against HIV-1 IN and RT.

2.5.1. Direct HIV-1 integrase strand transfer assay

The direct HIV-1 IN strand transfer assay was set up and performed to determine whether any of the 45 test compounds were capable of preventing the incorporation of donor DNA into target DNA by IN.

The donor DNA (biotin 5'- ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCA-3' and 5'-ACTGCTAGAGATTTTCCACACTGACTAAAAG-3'; Inqaba Biotech Pretoria, South Africa) was annealed by heating to 95ºC for 5 minutes and then cooled to room temperature. The working concentration of donor DNA (0.15µM) was prepared in buffer A (10mM Tris-HCl, 0.1M $NaCl_2$; pH 7.2); before transfer (100µl) into streptavidin coated microplate (MP) wells. The reaction was sealed and incubated for an hour at room temperature (shaking at 50rpm). Subsequently, the supernatant was aspirated and wells were washed three times with 300µl 1XPBS buffer. Recombinant integrase (1µM) prepared in buffer B (20mM Hepes, 75mM $NaCl_2$, 10mM $MgCl_2$, 2µM $ZnCl_2$, 10mM $MnCl_2$, 1% glycerol; pH 6.8) was subsequently transferred into each well, sealed and incubated for thirty minutes (shaking at 50rpm). The wells were washed 2 times with buffer B, and subsequently 90µl of each of the gold compounds (10µM) prepared in buffer B were transferred into duplicate or triplicate wells. The assay was sealed and incubated for thirty minutes at 37°C. A concentration of 2.5µM target DNA (5'-TGACCAAGGGCTAATTCACT-3' Fluorescein and 5'-AGTGAATTAGCCCTTGGTCA-3' Fluorescein; Inqaba Biotech, Pretoria, South Africa) was prepared, added at 10µl/well and then incubated for an hour at 37°C. The wells were washed three times (ten minutes each at 37°C) with 300µl buffer C (0.03M sodium citrate, 0.3M $NaCl_2$; pH 7.0). Monoclonal anti-FITC-alkaline phosphatase sp (Sigma Aldrich, St Louis MO, USA) working solution was prepared 1:10 000 in buffer D (50mM Tris-HCl, 150mM $NaCl_2$, 0.05% Tween 20; pH 7.4) and added at 200µl per well. The plate was incubated for two hours at 25°C; and then washed three times as before in buffer D (without 0.05% Tween 20). A Sigma FAST™ p-Nitrophenylphosphate substrate prepared according to manufacturer's (Sigma Aldrich, St Louis MO, USA) instructions was added to the wells (200µl). The assay was incubated for thirty minutes in 37°C and quantified using a multiplate reader at a wavelength of 405nm (xMARK™, Bio-Rad Laboratories Inc).

2.5.2. Direct HIV-1 RT assay

The direct HIV-1 RT assay was performed to determine whether any of the 45 test compounds were inhibitors of the polymerase activity of HIV-1 RT.

The 45 gold compounds were tested in a direct HIV-1 RT assay at 10µM according to manufacturer's instructions (Roche Diagnostics. Reverse Transcriptase Assay, colorimetric, version May 2006). Compounds that showed good inhibition at 10µM were then tested at 1µM; and where good inhibition was retained further testing was performed at 0.5µM and subsequently 0.1µM. Briefly, HIV-1 RT was initially reconstituted in 250µl autoclaved high purity water to a final concentration of 2ng/µl, and stored at -20°C in aliquots until used. The reaction mixture was prepared by the addition of 430µl autoclaved high purity water to the template, followed by addition of 1ml incubation buffer (ready-to-use solution) to a separate vial of nucleotides. A 100µl template solution was added to the prepared nucleotide solution to make up the final reaction mixture. The enzyme-linked immunosorbent assay (ELISA) protocol began with the preparation of solution mixture which consisted of the following: 20µl of each of the 45 gold compounds (at the desired final concentration), 20µl reaction mixture (10µM dUTP/dTTP, template/primer hybrid) and 20µl HIV-1 RT enzyme (2ng/µl) which was incubated for an hour at 37°C. After incubation, the samples were transferred into the wells of micro-plate (MP) modules, covered with foil and then incubated at 37°C for an hour. During the second incubation anti-digoxigenin-peroxidase (DIG-POD) working solution was prepared to a final concentration of 200 milliunits per milliliter (mU/ml); e.g. 50µl antibody solution and 4.95ml of conjugate dilution buffer). After one hour incubation, the solutions were removed from the wells and the wells were washed five times with 250µl washing buffer (prepared by the addition of 225ml autoclaved high purity water to stock solution) per well. Thereafter 200µl anti-DIG-POD working dilution was added to each well, covered with foil and incubated for an hour at 37°C. During incubation an 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) substrate solution was prepared by dissolving an ABTS tablet in 5ml substrate buffer. Following a one hour incubation the wells were washed as described previously and 200µl of ABTS substrate solution was added to each well. Photometric detection was performed using a multiplate reader (BP800, BioHIT, Finland) at a wavelength of 405nm with a reference

wavelength of 490nm after a 15 minute incubation. This assay was carried out with a positive control (20µl lysis buffer, 20µl RT and 20µl reaction mixture), a negative control (40µl lysis buffer and 20µl reaction mixtures) and NNRTI Efavirenz was used as a control compound. The assay was performed in triplicate and Origin 6.1 software was used to determine inhibitory concentrations (IC_{50}) required to inhibit 50% of HIV-1 RT activity.

2.5.3. Direct RT assay: 10% FCS

Testing of the influence of 10% FCS on potency of the selected compounds AB05-AB08 was carried out at 10µM using the HIV-1 RT assay according to manufactures instructions, as described in section 2.5.2 with specific modifications. In particular; FCS (Highveld Biological, Johannesburg, South Africa) was prepared to 10% of the final sample volume (60µl) within the 20µl drug compound mixture. The 10% FCS concentration was used as it represents the serum protein concentration used in cell (PBMCs) based antiviral assays (see section 2.6). The rest of the assay was carried out as before in duplicate experiments of triplicates; and included the provided positive control (20 µl lysis buffer, 20µl RT and 20µl reaction mixture), the provided positive control containing 10% FCS and a negative control (40µl lysis buffer and 20µl reaction mixture).

2.5.4. Direct HIV RT assay: 4.5mg/ml human serum albumin (HSA)

Compounds AB05-AB08 were investigated at concentrations of 10µM, 1µM, 0.5µM and 0.1µM or 100µM, 75µM, 50µM, 25µM, 10µM, 0.5µM and 0.1µM depending on potency using the HIV-1 RT assay as determined in section 2.5.2 with specific modifications. In particular, the addition of 4.5mg/ml HSA (Sigma Aldrich, St Louis, MO, USA) to a final sample volume of 120µl was prepared with the test drug compound solution, RT and reaction mixture. The 4.5mg/ml HSA concentration was used as it represents 10% of the serum protein concentration normally found in the plasma of AIDS patients (Cobertt *et al*., 1999). The rest of the assay was carried out as before (section 2.5.2) in triplicate. The change (*n*-fold) in 50% inhibition of RT

activity ($FCIC_{50}$) relative to the HSA-free assay was used to determine the effect of the serum protein on compound inhibition.

2.5.5. Direct RT: Inhibition of polymerase (p66 subunit) activity

Testing of four compounds (AB05-AB08) for HIV-1 p66 inhibition was carried out at a 10µM concentration using the direct HIV-1 RT assay as described in section 2.5.2 with some modifications. Specifically, this involved the addition of the RT mutant [(subtype B HIV-1_{HXB2} reverse transcriptase (p66>Q/p51)] which lacks RNaseH activity. The use of this specific enzyme requires the addition of substrate enhancer to ABTS substrate prior to photometric detection at wavelengths 405/490nm after thirty minutes using a multiplate reader (xMARK^{TM}, Bio-Rad laboratories Inc). These assays were carried out in triplicate experiments with a positive control (20µl lysis buffer, 20µl wild type RT and 20µl reaction mixture) and a negative control (40µl lysis buffer and 20µl reaction mixture).

2.5.6. Inhibition of a 3TC/Lamivudine resistant mutant

The four compounds (AB05-AB08) were also investigated for activity against drug resistant RT at a 10µM concentration using the direct RT protocol that was described in section 2.5.2 with special modifications. The specific modifications included the use of the HIV-1_{HXB2} reverse transcriptase/ M184V mutant. This mutant HIV-1 RT is known to demonstrate 3TC/lamivudine resistance. The assay was quantified using photometric detection at 405/490nm by a multiplate reader (xMARK^{TM}, Bio-Rad Laboratories Inc) after 15 minutes. The assays were carried out in triplicate experiments; with a positive control comprising of 20µl lysis buffer, 20µl RT and 20µl reaction mixture and a negative control (40µl lysis buffer and 20µl reaction mixture).

2.6. Anti-HIV activity of gold compounds AB05-AB11 in PBMCs

The antiviral assays for the seven compounds (AB05-AB11) were carried out at the Southern Research Institute, Birmingham, USA following submission to the NIAID AIDS Research and Reference Reagent Program. PBMCs were isolated as described

in section 2.4.2. The standard antiviral PBMC assay was performed as follows: PHA stimulated PBMCs were pooled from at least two HIV/HBV seronegative donors. PBMCs were prepared to a final concentration of $1x10^6$ cells/ml and were pre-treated with viral stock at a multiplicity of infection of 0.1. Viral isolates used in each assay were the HIV-1 subtype B HIV-1_{Ba-L} and the HIV-1_{NL4-3} isolates. The infected cells were then seeded at 50µl/well ($5x10^4$ cells/well) in 96 well microtiter plates; and the compounds were evaluated using a 200µM high-test concentration with 8 additional serial half-log dilutions. Each assay contained AZT as a positive control antiviral measure (1µM) and cells plus virus as a negative control; and was maintained for 7 days in a 37°C, 5% CO_2 incubator. The antiviral assay was quantified using an RT assay adapted from Buckheit *et al*., 1991. Briefly, a reaction mixture was prepared by mixing titriated thymidine triphosphate (125µl 1.0M DTT, 40µl 1.0M $MgCl_2$, ^{3}H-TTP, 80Ci/mmol, NEN; Pharmacia, Inc.) with a primer solution (150µl poly Ra (20mg/ml), 0.5 oligo Dt (20units/ml) and 5.35ml sterile dH_2O) and transferred (10µl) into round bottom plates. Fifteen microliters of virus-containing supernatant was subsequently added to the reaction mixture and incubated at 37°C for an hour. Thereafter, reaction mixtures were spotted onto DE81 filter-mats (Wallac, Turku, Finland) and washed five times in 5% sodium phosphate buffer or 2X saline-sodium citrate (SSC) (Life Technologies, Carlsbad, USA) for five minutes each; and two times for one minute each in dH_2O, followed by a two times wash for a minute in 70% ethanol prior to drying. Incorporated radioactivity (counts per minute, CPM) was quantified using standard scintillation techniques.

2.7. ^{1}H NMR measurement of protein-synthetic gold compound interactions in physiological medium

The compounds AB05-AB11 were selected for investigation following their successful inhibition of RT (Section 2.5.2). The final concentration of each test compound included DMSO-*d6* (Sigma Aldrich, St Louis MO, USA) of maximum 5% in deuterated PBS (dPBS). The dPBS (pH7.4) contained: 3.2mM Na_2HPO_4, 0.5mM KH_2PO_4, 1.3mM KCl and 135mM NaCl dissolved in deuterated oxide (Sigma Aldrich. St Louis, USA). The pH was adjusted to pH7.4 using deuterated chloride (Sigma chemical. St Louis, USA). The ability of HSA to interact with each compound was investigated at 1mM in a final volume of 500µl PBS and test compound (1mM).

Spectra were recorded at 300K using the 400MHz Bruker Avance Spectrometer equipped with a broadband inverse (BBI) 5mm probe. Proton chemical shifts were in parts per million (ppm), and were referenced to residual solvent resonances. Protein signals were almost undetectable due to their relatively low concentrations and the T2-filter contained in the pulse program (stdwsrf.ak, avance version 00/02/07). Water suppression was achieved by a Watergate W5 pulse sequence with gradients using double echo (Liu *et al*., 1998). The intensities of the DMSO signal in all the references spectra recorded (^{1}H NMR) were matched in each sample, in order to make signal change comparable. Any changes in ^{1}H NMR spectra of compounds were monitored using the reference spectra (off-resonance) of the saturation transfer difference (STD). Possible interactions between HSA and the gold compounds were investigated using NMR's STD technique.

2.8. Measurement of synthetic gold compound stability in biological medium

The synthetic gold compounds AB05-AB08 were prepared at 1mM in sterile 10% RPMI-1640 complete (RPMI, 10% FCS, 0.002% gentamycin and 0.002% streptomycin/penicillin) media supplemented with 10% deuterated oxide (D_20; Sigma Aldrich, St Louis MO, USA) in final volume of 440µl. Thereafter, the samples were transferred to UV-irradiated (thirty minutes) NMR tubes prior to NMR spectral reading at 298K using the 400MHz Bruker Avance Spectrometer provided with a BBI 5mm probe. Proton chemical shifts were analysed in ppm referenced to residual solvent resonances.

Observation of changes in each compounds spectra required water suppression due to the aqueous composition of the sample. Thus, several water suppression methods such as Watergate, ID water suppression by pre-saturation and ID water suppressed nuclear overhauser effect (NOE) with gradients were used.

2.9. Statistical Analysis

All experiments were conducted in at least triplicate with bioligal duplicates ($n \geq 6$) and the analysis of data was carried out with the use of Microsoft (MS) Office Excel^{TM} 2003, version 11. Averaged results were evaluated for inaccuracy by the

determination of standard deviations by using MS Office Excel. The Origin 6.1 software was used in the determination of CC_{50}s and IC_{50}s. Statistical analysis was carried out by the application of the Students t-test from simple interactive statistical analysis (SISA) at a 95% confidence interval (CI) available online at http://home.clara.net/sisa/ accessed 07-09-2011.

CHAPTER 3
RESULTS

3.1. Theoretical screening of synthetic compounds

Overall, theoretical screening results as measured through the Osiris Property Explorer were obtained for 34 compounds, and included predictive analysis of drug compound lipophilicity, aqueous solubility, toxicity, drug-likeness and drug-like score. In addition, Lipinski's Ro5 predicted oral bioavailability data for all 45 compounds tested.

3.1.1. Osiris Property Explorer

Results from the Osiris Property Explorer for 34 of the 45 compounds are shown in Table 3.1. The remaining 11 compounds, AB05-AB08, AB13, and AB40-AB45 were not evaluated as the Osiris program could not recognize their cationic structures.

An overall acceptable cLogP value below 5.0 was observed for 88% (n=30) of the compounds screened; such ccompounds are known to have a good probability of cellular absorption (http://www.organic-chemistry.org/prog/peo/cLogP.html accessed 07-09-2011). Approximately 79% (n=27) of compounds were predicted to be lipophilic while the remaining were predicted to be hydrophilic (high LogP values represent low hydrophilicities; http://www.organic-chemistry.org/prog/peo/cLogP.html accessed 07-09-2011). Most of the gold compounds were predicted to have LogS values below -4 (which means that compounds were predicted to have low aqueous solubility; http://www.organic-chemistry.org/prog/peo/cLogP.html accessed 07-09-2011) with the exception of AB10 and AB11. Low solubility is associated with poor cellular absorption and distribution characteristics.

The compounds in Series 1 were predicted to consist of fragments associated with mutagenic, tumourigenic and reproductive effects as shown in Table 3.1. Series 2 & 3 was predicted to consist of fragments known to induce tumourigenic and reproductive effects, with only AB10 and AB11 thought to be irritants. In Series 4, AB14 was associated with mutagenic and irritant effects, while AB16 and AB17 were only considered irritants. AB18 and AB19 consisted of fragments known to induce mutagenic effects. AB20 to AB34 and AB36 were all predicted to consist of fragments associated with irritant effects with AB22 containing an additional mutagenic fragment. Compound AB39 from Series 5 was associated with irritant effects.

Assessment of the compounds by Osiris Property Explorer indicated that the presence of ethyl acetate and phenylphosphine fragments contributed to the irritancy while pyrazoles were suggested to contribute to mutagenecity. The structures of ethyl acetate, phenylphosphine and pyrazoles are shown in Figure 3.1.

Overall, 31 of the 34 gold compounds screened showed low drug scores with the exception of AB10, AB11 and AB17 which exhibited intermediate drug likeness scores.

Table 3.1: Osiris Property Explorer assessment of 34 synthetic gold compounds.

Compound	cLogP	LogS	M.W. g/mol	Drug-Likeness	Muta-genic	Tumor-igenic	Irritant	R.E.	Drug score
Raltegravir	-1.05	-1.18	444.0	5.95					0.81
Series 1									
AB01	4.01	-10.84	1105.39	-33.63					0.09
AB02	4.01	-10.84	1105.39	-31.52					0.09
AB03	3.72	-10.00	1135.56	-25.83					0.09
AB04	3.11	-10,72	1207.58	-27.80					0.09
Series 2									
AB09	3.65	-7.09	673.34	-31.38					0.09
AB10	3.44	-3.28	518.22	1.15					0.43
AB11	3.81	-2.05	511.35	0.33					0.50
Series 3									
AB12	1.13	-5.74	500.67	-29.76					0.29
Series 4									
AB14	4.87	-10.49	982.61	-42.954					0.061
AB15	-0.87	-7.7	678.21	-11.43					0.15
AB16	5.91	-12.8	968.57	-39.7					0.06
AB17	-0.25	-4.11	340.11	-6.22					0.4
AB18	3.01	-9.403	926.50	-37.55					0.08
AB19	-2.373	-5.104	678.21	-12.409					0.162
AB20	1.853	-9.381	928.47	-37.55					0.10
AB21	4.401	-10.214	970.0	-40.264					0.07
AB22	4.015	-10.967	970.55	-40.624					0.07
AB23	3.47	-6.26	970.55	-47.67					0.16
AB24	3.25	-10.19	982.61	-40.62					0.098
AB25	5.72	-11.58	982.61	-42.03					0.07
AB26	3.06	-8.95	984.58	-42.95					0.1
AB27	3.83	-9.73	984.58	-42.03					0.07
AB28	4.77	-14.75	1044.60	-37.69					0.08
AB29	6.16	-15.56	1086.66	-42.95					0.06
AB30	5.7	-15.29	1072.66	-40.62					0.07
AB31	-0.36	-5.35	392.00	-7.28					0.31
AB32	-1.08	-10.18	825.44	-12.4					0.13
AB33	3.96	-7.66	1018.00	-15.8					0.17
AB34	4.28	-15.95	1072.66	-45.14					0.08
AB35	-1.56	-11.38	810.00	-19.92					0.13
AB36	3.82	-15.68	1004.00	-45.02					0.09
AB37	3.71	-8.27	420.00	-23.2					0.16
AB38	-0.6	-5.95	407.00	-14.82					0.28
Series 5									
AB39	3.68	-9.73	1018.00	-45.53					0.09

AZT. Azidothymidine No negative effect. Negative effect. R.E. Reproducible effects

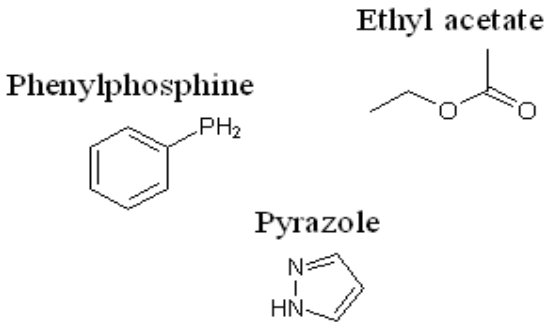


Figure 3.1: Fragments identified through Osiris Property Explorer present in the gold compounds assessed known to induce cytotoxicity.

3.1.2. Lipinski's Ro5 of synthetic compounds

All 45 compounds screened met the following Lipinski's Ro5 requirements: they consisted of hydrogen bond acceptors less than five and had less than ten hydrogen donors (Table 3.2). Most of the gold compounds screened had high molecular weights (MW) i.e. MW>500g/mol with the exception of AB17, AB31 and AB38. Compounds screened met the required solubility criteria i.e. partition co-efficiencies were predominantly less than five with the exception of AB16, AB25, AB29 and AB30. Only AB17, AB31, and AB38, had good Lipinski's Ro5 scores (4/4) whereas the rest of the compounds exhibited intermediate scores (3/4 or 2/3). Some of the gold compounds namely AB39-AB45, were not screened for cLogP because partitioning co-efficiencies could not be obtained from the Osiris Property Explorer; therefore these compounds were assessed using only the information that could be accumulated on them. The Lipinski's Ro5 scores are represented as percentages in Figure 3.2. Overall, only 7.3% (n=3) of the gold compounds investigated are possible orally bioavailable drug candidates by the Lipinski's Ro5. A good portion (86%, n=39) of compounds were predicted to exhibit intermediate oral bioavailability while the remaining compounds showed low Lipinski's Ro5 scores.

Table 3.2: Evaluation of 45 synthetic gold compounds using the Lipinski's Ro5.

Compound	Rule 1	Rule 2	Rule 3	Rule 4	Score
Raltegravir	√	√	√	√	4/4
Series 1					
AB01	√	√	x	√	3/4
AB02	√	√	x	√	3/4
AB03	√	√	x	√	3/4
AB04	√	√	x	√	3/4
Series 2					
AB05	√	√	x	√	3/4
AB06	√	√	x	√	3/4
AB07	√	√	x	√	3/4
AB08	√	√	x	√	3/4
AB09	√	√	x	√	3/4
AB10	√	√	x	√	3/4
AB11	√	√	x	√	3/4
Series 3					
AB12	√	√	x	√	3/4
AB13	√	√	x	√	3/4
Series 4					
AB14	√	√	x	√	3/4
AB15	√	√	x	√	3/4
AB16	√	√	x	x	2/4
AB17	√	√	√	√	4/4
AB18	√	√	x	√	3/4
AB19	√	√	x	√	3/4
AB20	√	√	x	√	3/4
AB21	√	√	x	√	3/4
AB22	√	√	x	√	3/4
AB23	√	√	x	√	3/4
AB24	√	√	x	√	3/4
AB25	√	√	x	x	2/4
AB26	√	√	x	√	3/4
AB27	√	√	x	√	3/4
AB28	√	√	x	√	3/4
AB29	√	√	x	x	2/4
AB30	√	√	x	x	2/4
AB31	√	√	√	√	4/4
AB32	√	√	x	√	3/4
AB33	√	√	x	√	3/4
AB34	√	√	x	√	3/4
AB35	√	√	x	√	3/4
AB36	√	√	x	√	3/4
AB37	√	√	x	√	3/4
AB38	√	√	√	√	4/4
Series 5					
AB39	√	√	x	ND	2/3
AB40	√	√	x	ND	2/3
AB41	√	√	x	ND	2/3
AB42	√	√	x	ND	2/3
AB43	√	√	x	ND	2/3
AB44	√	√	x	ND	2/3
AB45	√	√	x	ND	2/3

Rule 1: Not more than 5 hydrogen bond acceptors. Rule 2: Not more than 10 hydrogen donors. Rule 3: A molecular weight under 500g/mol. Rule 4. A partition coefficient log *P* less than 5. X: Does not adhere to rule. √: Adheres to rule. ND: Not done. AZT. Azidothymidine. Score: Lipinski's Ro5 Score

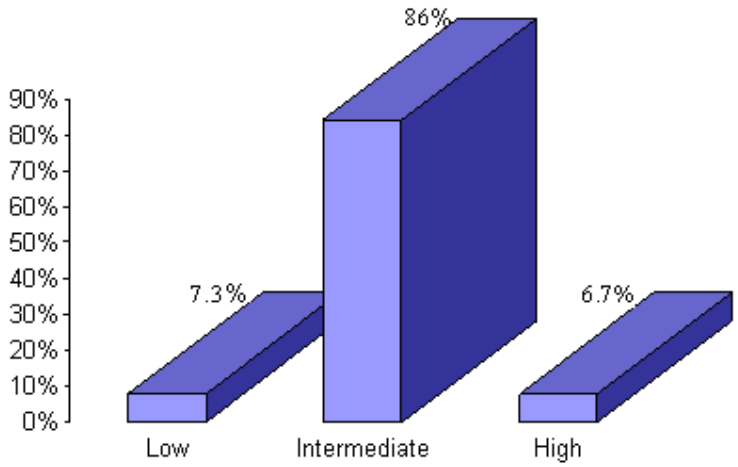


Figure 3.2: Overall Lipinski's Ro5 scores of the 45 investigated gold compounds.

Based on the combined results from the Osiris Property Explorer and Lipinski's Ro5, gold compounds AB10, AB11, AB17, AB31 and AB38 are theoretically good drug candidates.

3.2. Aqueous solubility of synthetic gold compounds

To determine the aqueous solubility of each synthetic gold compound, a standard curve for each compound was first obtained. Figure 3.3 shows the representative standard curve for which the chloramphenicol control is the test compound. From the standard curve of each synthetic gold compound (results not shown), aqueous solubility was determined. The compounds demonstrated varied aqueous solubility as shown in Table 3.3. The synthetic gold compounds with a value of >400µM were considered soluble. Overall, compounds AB17, AB31, AB32, and AB37 were observed to be soluble at concentrations between 400µM and 500µM in aqueous medium, while AB13, AB15, AB19, AB35, AB36 and AB38 were soluble at concentrations above 500µM but below 800µM. The overall solubility levels for the 45 synthetic gold compounds are depicted graphically in Figure 3.4. It was observed that 36% (n= 16) of the gold compounds were soluble in aqueous media, 40% (n=18) showed poor solubility and approximately 24% were observed to exhibit moderate aqueous solubility. The compounds AB01-AB04 exhibited low/no solubility and were observed to precipitate out of solution. The compounds in Series 5 (AB39-AB45) were observed to be moderately soluble to soluble but the assay results were not

reproducible for these 7 compounds; which mean that the compounds precipitated out of solution at varying rates.

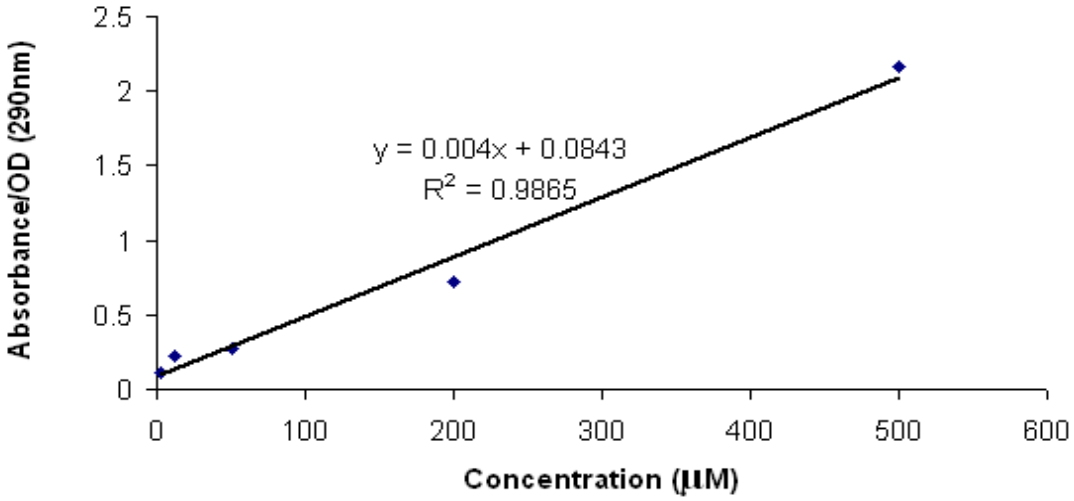


Figure 3.3: The representative standard curve of aqueous solubility in which chloramphenicol was the test compound.

Table 3.3: Aqueous solubility of the 45 synthetic gold compounds and the chloramphenicol control compound.

Compound	Absorbance (nm)	Slope(M)	R^2	Solubility (µM)/S ± Std dev
Chloramphenicol	290	0.003917	0.986	406.635 ± 59.746
Series 1				
AB01	NS	NS	NS	*NS
AB02	NS	NS	NS	*NS
AB03	NS	NS	NS	*NS
AB04	NS	NS	NS	*NS
Series 2				
AB05	320	0.006	0.898	323.437 ± 16.026
AB06	320	0.0039	0.922	266.025 ± 13.042
AB07	320	0.8058	0.894	4.884 ± 0.006
AB08	320	0.0064	0.917	306.884 ± 49.934
AB09	320	0.006	0.863	332.586 ± 18.480
AB10	320	0.0035	0.971	394.583 ± 2.875
AB11	320	0.0063	0.961	283.283 ± 0.773
Series 3				
AB12	270	0.0031	0.993	195.766 ± 16.478
AB13	270	0.0019	0.983	526.644 ± 0.909
Series 4				
AB14	290	0.0018	0.944	62.538 ± 3.002
AB15	290	0.0007	0.934	814.285 ± 6.860
AB16	290	0.0011	0.888	71.875 ± 6.625
AB17	290	0.0002	0.964	430.208 ± 13.906
AB18	290	0.0046	0.962	24.818 ± 3.725
AB19	290	0.002	0.994	769.583 ± 8.188
AB20	290	0.0022	0.991	39.867 ± 6.923
AB21	270	0.0006	0.881	147.916 ± 40.991
AB22	270	0.0009	0.897	99.845 ± 67.917
AB23	270	0.0009	0.943	95.370 ± 24.277
AB24	270	0.0008	0.942	122.916 ± 18.541
AB25	270	0.0013	0.941	65.865 ± 96.873
AB26	270	0.0006	0.860	238.425 ± 3.076
AB27	270	0.0009	0.997	103.472 ± 10.196
AB28	270	0.0063	0.945	362.946 ± 33.816
AB29	270	0.0054	0.999	219.444 ± 25.782
AB30	270	0.0056	0.996	260.732 ± 42.538
AB31	270	0.0049	0.997	426.934 ± 43.694
AB32	270	0.0064	0.962	482.541 ± 23.845
AB33	270	0.0063	0.995	103.670 ± 2.273
AB34	270	0.0031	0.947	141.061 ± 34.624
AB35	270	0.0063	0.911	650.948 ± 150.193
AB36	270	0.0065	0.734	575.144 ± 34.527
AB37	270	0.0013	0.924	493.269 ± 11.576
AB38	270	0.0046	0.990	524.184 ± 1.408
Series 5				
AB39	270	0.0006	0.910	241.319 ± 44.676
AB40	270	0.0013	0.999	534.054 ± 49.609
AB41	270	0.0012	0.996	563.454 ± 269.
AB42	270	0.006	0.999	525.781 ± 793.138
AB43	270	0.0017	0.999	531.433 ± 14.295
AB44	270	0.003	0.999	522.222 ± 39.682
AB45	270	2.00E-05	0.815	4513.889 ± 32.632

*NS=Not soluble

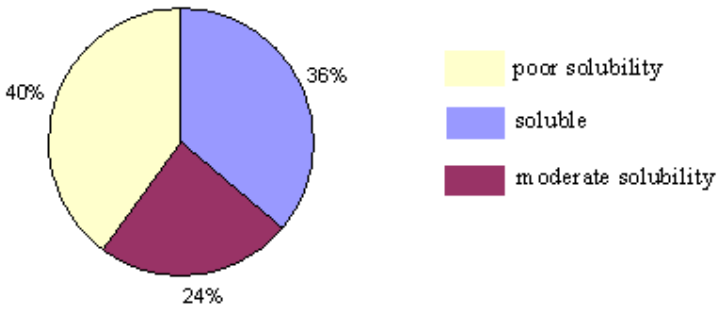


Figure 3.4: Proportion of the 45 synthetic gold compounds exhibiting aqueous solubility.

3.3. Measurement of cell cytotoxicity

The CC_{50} for the synthetic gold compounds were determined in PM1 cell lines for all compounds and PBMCs (AB05-AB11) using auranofin (Figure 3.5) and AZT as the respective controls. The cytotoxicity was determined and the CC_{50} values exhibited varying cytotoxicity among test agents as summarised in Table 3.4.

The cytotoxicity in PBMCs for compounds AB05-AB11 was comparable to that of the PM1 cell line, with the exception of AB10 and AB11. The cytotoxicity of AB10 in the PBMCs (CC_{50}=56.5µM) was approximately half that observed in the PM1 cell line (CC_{50} = 107.412 ± 4.099µM); and AB11 was approximately 4 fold more cytotoxic in PBMCs.

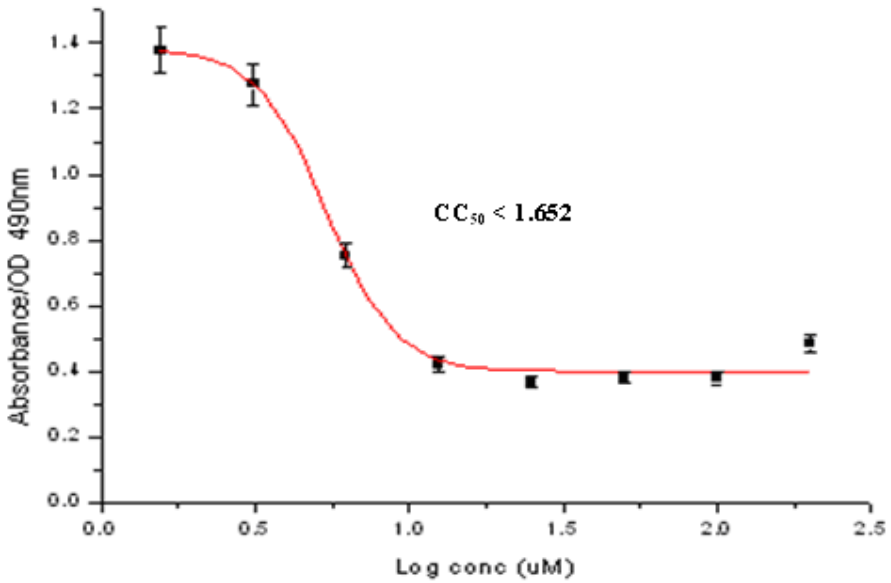


Figure 3.5: A representative graph that demonstrates the relationship between PM1 cell viability and auranofin concentration. The auranofin concentration required to reduce cell viability by 50% was determined to be less than 1.652. The Y-axis represents optical density at a wavelength of 490nm. The X-axis represents the Log values of varying compound concentrations.

Table 3.4: Evaluation of gold compounds cytotoxicity in PM1 cells and PBMCs.

Compound	CC_{50} (µM) ± Std dev	CC_{50} (µM) ± Std dev
	PM1 cell line	**PBMCs**
Control compound	<1.562 (Auranofin)[a]	>1000nm (AZT)[b]
Series 1		
AB01	$10.741 \pm 2.143 \times 10^{-16}$	-
AB02	>100	-
AB03	>100	-
AB04	>100	-
Series 2		
AB05	49.403 ± 0.130	40.6
AB06	$11.276 \pm 3.467 \times 10^{-16}$	6.28
AB07	$22.771 \pm 1.515 \times 10^{-15}$	18.7
AB08	$3.423 \pm 8.485 \times 10^{-18}$	2.94
AB09	3.054 ± 0.275	2.45
AB10	107.412 ± 4.099	56.5
AB11	33.847 ± 6.201	8.30
Series 3		
AB12	$9.655 \pm 7.130 \times 10^{-15}$	-
AB13	$0.994 \pm 1.250 \times 10^{-17}$	-
Series 4		
AB14	<1.5625	-
AB15	72.8 ± 9.298	-
AB16	<1.5625	-
AB17	4.625 ± 1.661	-
AB18	<1.5625	-
AB19	<1.5625	-
AB20	<1.5625	-
AB21	1.590 ± 0.551	-
AB22	1.250 ± 0.070	-
AB23	<1.5625	-
AB24	<1.5625	-
AB25	3.100 ± 0.565	-
AB26	<1.5625	-
AB27	2.366 ± 0.550	-
AB28	2.125 ± 0.671	-
AB29	<1.5625	-
AB30	<1.5625	-
AB31	3.05 ± 0.777	-
AB32	34.140 ± 3.592	-
AB33	1.780 ± 0.254	-
AB34	<1.5625	-
AB35	<1.5625	-
AB36	2.800 ± 0.144	-
AB37	<1.5625	-
AB38	<1.5625	-
Series 5		
AB39	2.190 ± 4.444x10-16	-
AB40	$116.493 \pm 4.177 \times 10^{-15}$	-
AB41	$48.827 \pm 1.221 \times 10^{-15}$	-
AB42	$31.855 \pm 3.177 \times 10^{-15}$	-
AB43	$54.697 \pm 4.233 \times 10^{-15}$	-
AB44	31.125	-
AB45	>100	-

In general 64.4% (n= 29) of gold compounds were observed to be cytotoxic in the PM1 cell line (CC_{50} values ≤10µM). Approximately 22.2% (n=10) of test compounds showed moderate cytotoxicity (CC_{50} values between 10µM and 100µM) while 13.3% (n=6) exhibited low cytotoxicity (CC_{50} values > 100µM).

3.4. Direct enzyme activity

In order to establish a mechanism of HIV-1 inhibition, gold compounds were screened for inhibition of the integrase enzyme and the reverse transcriptase enzyme.

3.4.1. Direct HIV-1 integrase inhibition

At a concentration of 10µM, the 45 synthetic gold compounds were mostly observed to exhibit low levels of integrase inhibition, as shown in Table 3.5. AB04 and AB39 exhibited IN inhibition of approximately 52.8% and the rest of the compounds did not reach the cut-off of 50% inhibition at 10µM. The remaining compounds demonstrated negligible IN inhibition. Overall, strand transfer inhibition assays showed that only two of the gold compounds inhibit IN mediated strand transfer, meaning that at 10µM these gold compounds reduce the activity of IN from 100% to approximately 48%.

Table 3.5: The direct inhibition of HIV-1 strand transfer activity by each of the 45 synthetic gold compounds.

Compound	10μM Percent inhibition (%)
Chicoric Acid	98.759
Series 1	
AB01	NI
AB02	NI
AB03	NI
AB04	52.800
Series 2	
AB05	NI
AB06	NI
AB07	NI
AB08	NI
AB09	NI
AB10	NI
AB11	NI
Series 3	
AB12	NI
AB13	NI
Series 4	
AB14	43.294 ± 3.580
AB15	32.337 ± 2.567
AB16	NI
AB17	41.183 ± 2.089
AB18	NI
AB19	NI
AB20	18.373 ± 4.157
AB21	NI
AB22	19.376 ± 5.522
AB23	NI
AB24	28.405 ± 0.596
AB25	24.920 ± 1.635
AB26	NI
AB27	NI
AB28	27.771 ± 2.630
AB29	40.496 ± 0.085
AB30	20.863 ± 1.484
AB31	30.517 ± 1.483
AB32	22.333 ± 9.487
AB33	27.032 ± 8.195
AB34	NI
AB35	44.852 ± 0.479
AB36	36.536 ± 6.445
AB37	NI
AB38	NI
Series 5	
AB39	52.842
AB40	NI
AB41	NI
AB42	NI
AB43	NI
AB44	NI
AB45	NI

NI - no inhibition of HIV-1 integrase strand transfer

3.4.2. Direct RT assay

All 45 gold compounds were successfully tested for direct HIV-1 RT inhibition at a concentration of 10μM. Depending on their potency they were subsequently tested at 1μM, followed by 0.5μM and 0.1μM (Table 3.6). Overall, 12 of the 45 compounds exhibited direct inhibition of RT activity at concentrations above 50% at a 10μM concentration. In addition, the IC_{50} values of 10 of 11 compounds which showed direct RT activity at the lower concentrations tested was determined, and ranged from 0.46 to 50.39μM.

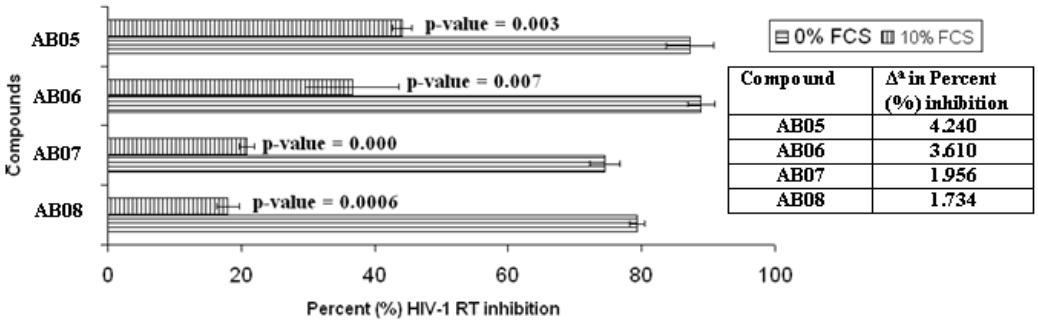
Table 3.6: Gold compounds HIV-1 RT direct inhibition.

Compound	10µM % inhibition	1µM % inhibition	0.5µM % inhibition	0.1µM % inhibition	IC_{50}[a](µM)± Std dev
AZT-tp	98.346	-	-	-	-
Neviripine	99.676	-	-	-	-
Series 1					
AB01	16.302	-	-	-	-
AB02	15.210	-	-	-	-
AB03	14.117	-	-	-	-
AB04	9.803	-	-	-	-
Series 2					
*AB05	75.966	78.033	43.968	14.405	0.502 ± 0.018
*AB06	72.212	76.761	44.446	13.716	0.461 ± 0.053
*AB07	74.677	79.207	52.036	14.388	0.503 ± 0.042
*AB08	76.526	86.839	46.224	21.726	0.660 ± 0.000
*AB09	77.815	30.870	-	-	1.381 ± 0.220
*AB10	75.686	52.886	-	-	1.054 ± 0.001
*AB11	73.557	44.863	-	-	8.796 ± 0.016
Series 3					
AB12	9.747	-	-	-	-
AB13	18.151	-	-	-	-
Series 4					
AB14	<0	-	-	-	-
AB15	<0	-	-	-	-
AB16	<0	-	-	-	-
AB17	14.864	-	-	-	-
AB18	11.261	-	-	-	-
AB19	14.513	-	-	-	-
AB20	4.391	-	-	-	-
AB21	4.902	-	-	-	-
AB22	<0	-	-	-	-
AB23	<0	-	-	-	-
AB24	<0	-	-	-	-
AB25	<0	-	-	-	-
AB26	20.253	-	-	-	-
AB27	22.626	-	-	-	-
AB28	14.100	-	-	-	-
AB29	19.932	-	-	-	-
AB30	9.117	-	-	-	-
AB31	10.534	-	-	-	-
AB32	13.770	-	-	-	-
AB33	9.940	-	-	-	-
AB34	18.694	-	-	-	-
AB35	74.393	7.809	1.756	-	-
AB36	15.863	-	-	-	-
AB37	<0	-	-	-	-
AB38	11.711	-	-	-	-
Series 5					
*AB39	70.726	40.361	-	-	10.900 ± 1.979
AB40	48.446	24.698	-	-	-
AB41	67.924	37.148	-	-	50.390 ± 0.636
*AB42	60.266	33.935	-	-	13.350 ± -0.777
AB43	51.664	40.736	-	-	-
AB44	17.230	-	-	-	-
AB45	<4.533	-	-	-	-

*****Compounds identified to exhibit considerable inhibition of HIV-1 RT. AZT-tp:3´-Azido-3´-Deoxythymidine-5´- Triphosphate

3.4.2.1. Effect of 10% FCS on direct HIV-1 RT inhibition

The four synthetic gold(III) compounds with good IC_{50} values in Series 2 (AB05-AB08; Table 3.6) were retested at the 10µM concentration in the direct RT assay in the presence of 10% FCS, and percentage inhibition values were compared (Figure 3.6). The presence of foetal calf serum was shown to reduce the anti-HIV-1 RT activity of compounds AB05-AB08 significantly (*p* value <0.05). The percentage inhibitions were reduced from 75.9 to 17.9%, 72.20 to 20%, 71.6 to 36.6% and 76.5 to 44.1% for AB05, AB06, AB07 and AB08, respectively.



Compound	Δ[a] in Percent (%) inhibition
AB05	4.240
AB06	3.610
AB07	1.956
AB08	1.734

Figure 3.6: Effect of 10% foetal calf serum on the ability of four selected synthetic gold(III) compounds (10µM; Series 2) to directly inhibit HIV-1 RT activity. The p-values which represents significance in changes were obtained using the Student t-test which compared the original percentage inhibitions of the compounds with those in assay modified with of 10% FCS. [a] The fold difference in percentage inhibition in the assay following introduction of 10% FCS.

3.4.2.2. Effect of 4.5mg/ml HSA on direct HIV-1 RT inhibition

The presence of 4.5mg/ml HSA in the direct HIV-1 RT assay reduced the potency of AB05, AB06, AB07 and AB08 across the range of concentrations tested, and thus increased IC_{50} values significantly ($p<0.05$) as shown in Table 3.7.

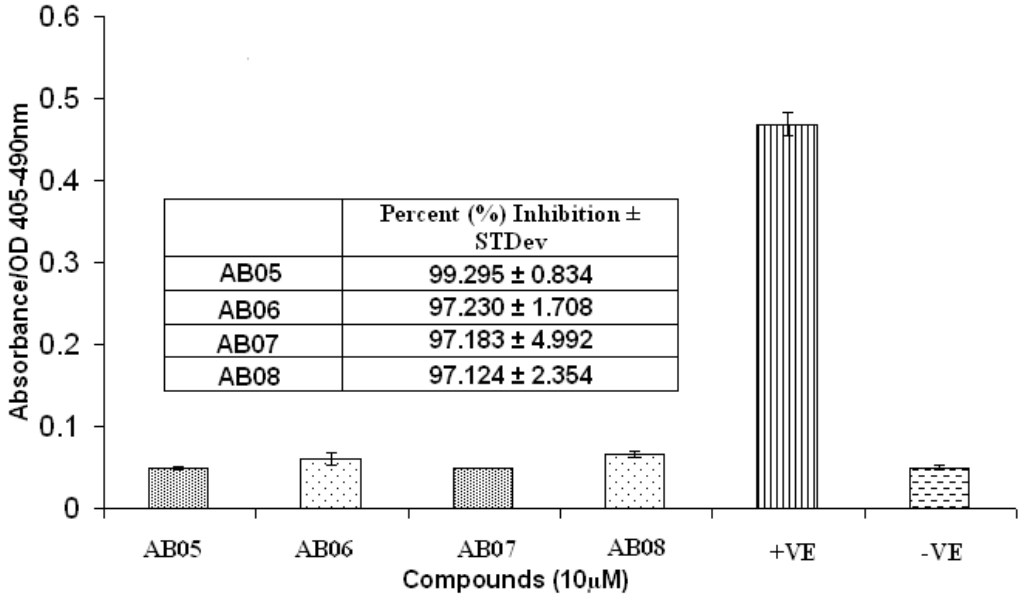
Table 3.7: The effect of 4.5mg/ml HSA on selected Series 2 gold(III) compounds direct HIV-1 RT inhibition

	IC_{50}(µM) in the absence of HSA	**IC_{50}(µM) in the presence of HSA**	**P-value[a]**	**$FCIC_{50}$[b]**
AB05	0.502±0.018	15.941±0.439	0.0003	31.754
AB06	0.461±0.053	16.157± 6.610	0.0027	35.047
AB07	0.503±0.042	9.575 ±1.570	0.009	19.035
AB08	0.660±0.000	5.328 ± 0.963	0.004	8.072

[a]The p-value was obtained using the Student t-test which compared the compound original IC_{50} values shown in Table 3.6 and those obtained in the 10% HSA (4.5mg/ml) reaction.[b]The $FCIC_{50}$ demonstrates the fold difference in IC_{50}'s.

3.4.2.3. Direct HIV-1 RT inhibition of HIV-1_{HXB2} reverse transcriptase (p66>Q/p51) mutant which lacks RNase H activity

The four selected gold(III) compounds (AB05-AB08; Series 2) were evaluated for inhibition of a mutant p66 at a concentration of 10µM. Residual RT activity (as measured by A_{405}) in the presence of each of the four compounds is shown in Figure 3.7. As shown earlier, these values were used to determine the percentage direct RT inhibition for each compound (reported in the Table within Figure 3.7). The compounds exhibited exceptional inhibition of the p66 RT subunit; with percentage inhibitions from a low of 97.124% (AB08) to 99.295% (AB05).



	Percent (%) Inhibition ± STDev
AB05	99.295 ± 0.834
AB06	97.230 ± 1.708
AB07	97.183 ± 4.992
AB08	97.124 ± 2.354

Figure 3.7: Direct RT inhibition of the HIV-1_{HXB2} reverse transcriptase (p66>Q/p51) mutant which lacks RNaseH activity. The test compounds exhibited inhibitions that ranged from 97.125% (AB08) to 99.295% (AB05). The positive control contains the HIV-1 RT without inhibitors. The negative control does not contain HIV-1 RT.

3.4.2.4. Direct HIV-1 RT Inhibition of a 3TC/Lamivudine resistant mutant

The inhibition of the 3TC/lamivudine resistant mutant HIV-1 RT by the four selected Series 2 compounds was determined at a 10µM concentration. Residual RT activity (as measured by A_{405}) in the presence of each of the four compounds is shown in **Figure 3.8.** These values were used to determine the percentage direct RT inhibition for each compound (reported in the Table within Figure 3.8).

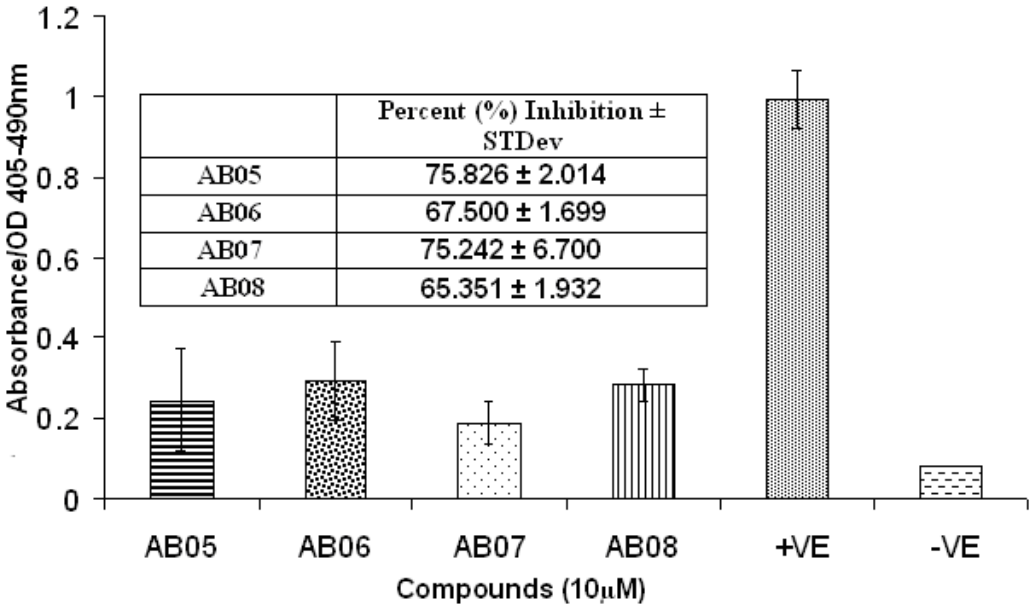


Figure 3.8: Direct RT inhibition of the HIV-1_{HXB2} reverse transcriptase/M184V mutant which is known to be resistant to 3TC/lamivudine by 10μM gold-based compounds. . The test compounds exhibited inhibitions in the ranges 65.351% (AB08) to 75.826% (AB05). The positive control contains the HIV-1 RT and reaction mixture. The negative control contains the reaction mixture.

3.5. Antiviral activity in PBMCs

Series 2 synthetic gold(III) compounds showed low/no cytotoxicity (Table 3.4) and direct RT inhibition with IC_{50} values below 10μM (Table 3.6), and were therefore selected for *in vitro* phenotypic inhibition assays. The results from the testing of compounds AB05 to AB11 against growth of HIV-1 subtype B isolates BaL (CCR5-utilizing) and NL4-3 (CXCR4-utilizing) in PBMCs are summarised in Table 3.7.

Acceptable IC_{50} values at a range 0.41μM to 40.8μM were observed. Antiviral activity observed was isolate dependent. HIV-1_{BaL} was more easily inhibited than HIV-1_{NL4-3} by all the synthetic gold compounds, but more resistant to inhibition by AZT. However, the compounds exhibit cytotoxicity at CC_{50} concentrations that are slightly higher than the compounds IC_{50}, resulting in low SI values. These results indicate that the compounds in their current form are poor drug candidates to continue to the preclinical development phase. The cytotoxicity and antiviral graph curves (results not shown) were also observed to be paralleled which was an indication of loss of viral replication due to cell death by the gold compounds.

Table 3.8: Inhibition of HIV-1 by gold(III) compounds (Series 2) in PBMCs.

Compounds	HIV-1 Isolate	IC_{50}(μM)	*CC_{50}(μM)	Selective Index (SI)[a]
AB05	Ba-L	14.1	40.6	2.87
	NL4-3	37.2		1.09
AB06	Ba-L	3.66	6.28	1.71
	NL4-3	4.43		1.42
AB07	Ba-L	8.22	18.7	2.27
	NL4-3	13.0		1.43
AB08	Ba-L	1.83	2.94	1.61
	NL4-3	3.15		<1.00
AB09	Ba-L	0.41	2.45	5.95
	NL4-3	1.12		2.19
AB10	Ba-L	23.5	56.5	2.41
	NL4-3	40.8		1.38
AB11	Ba-L	1.80	8.30	4.60
	NL4-3	7.86		1.06
AZT	Ba-L	2.34nM	>1000nM	>427
	NL4-3	1.30nM		>834

[a]The SI was determined by division of the CC_{50} with the IC_{50} values obtained. *PBMC cytotoxicity data obtained from Table 3.4

3.6. ^{1}H NMR analysis of synthetic gold compound interactions with HSA

The saturation transfer differences (STD) were obtained for compounds AB05-AB11. Figure 3.9 depicts the interaction of each compound with HSA, as determined through subtraction of spectral intensities of ligands before and after binding. Overall, all compounds, except AB07 and AB09, showed STD peaks which means that these compounds interact with the protein. For instance, AB05 peaks were observed at 8.0-9.0ppm, AB06 and AB08 peaks where observed between 2.0-1.0ppm, AB10 and AB11 showed STD peaks between 3.0-2ppm and 3-1ppm respectively. The lack of STD signal of AB07 and AB09 could be attributed to two factors: the lack of binding or alternatively high affinity binding (false negative).

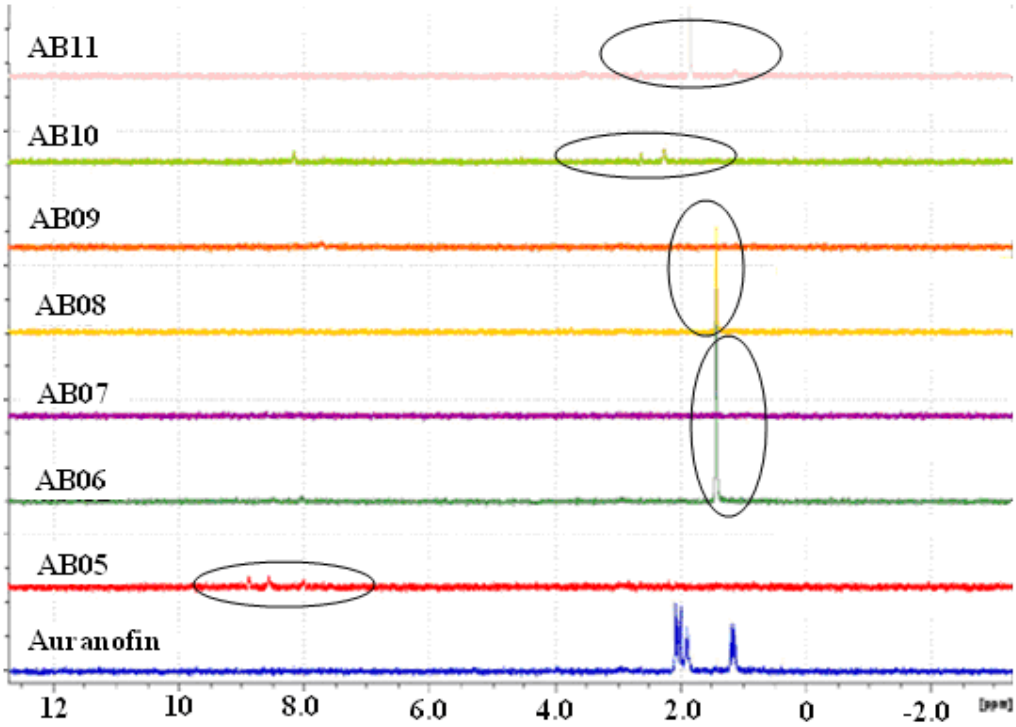


Figure 3.9: STD spectra of theAB05-AB11 Series of compounds to HSA (1:0.01mM) in 5% DMSO-*d6 deuterated* PBS, using auranofin as a reference. The black circles depict STD peaks which represent interaction of protein and compound.

Competitive binding assays were then carried out using compound AB05 to establish where on the serum protein HSA the compounds bind. Auranofin was used as a control as it is known to bind the Cys^{34} on the surface of HSA (Figure 3.10). Figure 3.10 shows the three signals obtained from the STD technique for auranofin with HSA, and auranofin, AB05 and HSA with (a) unsaturated protein, (b) protein saturation (with water suppression), and (c) the difference in signals between a and b, which is a result of a binding ligand that exhibit changes in the intensities of signal subsequent to interaction with a saturated protein. STD peaks observed at 2-1ppm are a demonstration that AB05 displaces auranofin therefore implies a common binding site for both compounds.

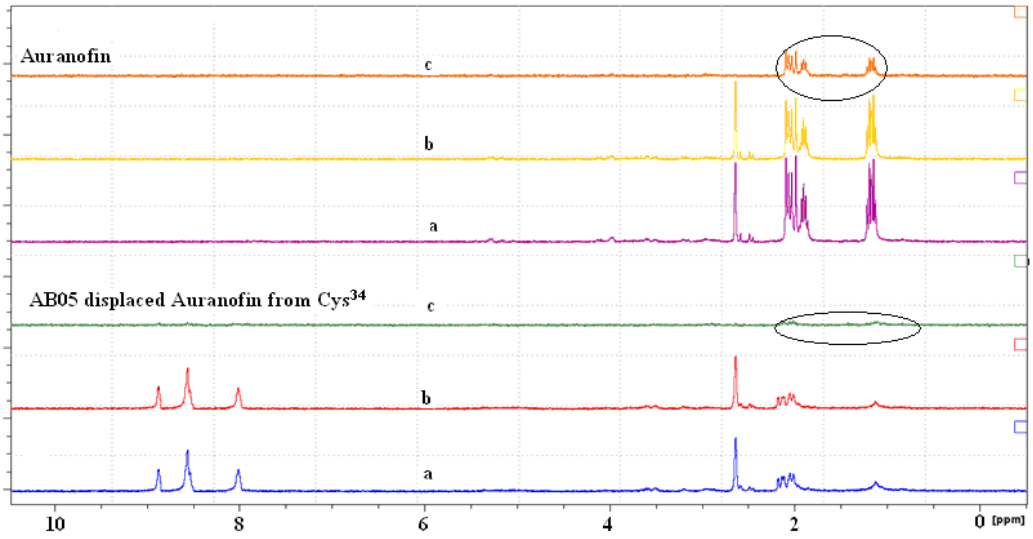


Figure 3.10: Auranofin binding to HSA (1: 0.01 mM) with 5% DMSO-d_6 in PBS (D_2O). The circled peaks are the highlighted STD which represents interaction of protein and compound. (a) unsaturated protein, (b) protein saturation (with water suppression), and (c) the difference in signals between a and b.

3.7. Compound stability in 10% RPMI complete media over time

STD NMR was also used to evaluate the compounds AB05-AB08 stability in biological media. AB05 was observed to lose signal intensity at 8.5-9.0ppm and at ~7.5ppm and 8ppm (Figure 3.11). AB06 STD was observed to lose intensity at ~1.4ppm over 72hours. AB07 showed no changes in STD signal until the 72nd hour when a signal was observed at ~8.5ppm, and AB08 exhibited loss of signal intensity at approximately 1.4ppm. Loss of signal intensities shows loss in chemical structure of the gold compounds due to interactions with serum proteins in 10% FCS. This implies the compounds are not stable in biological media.

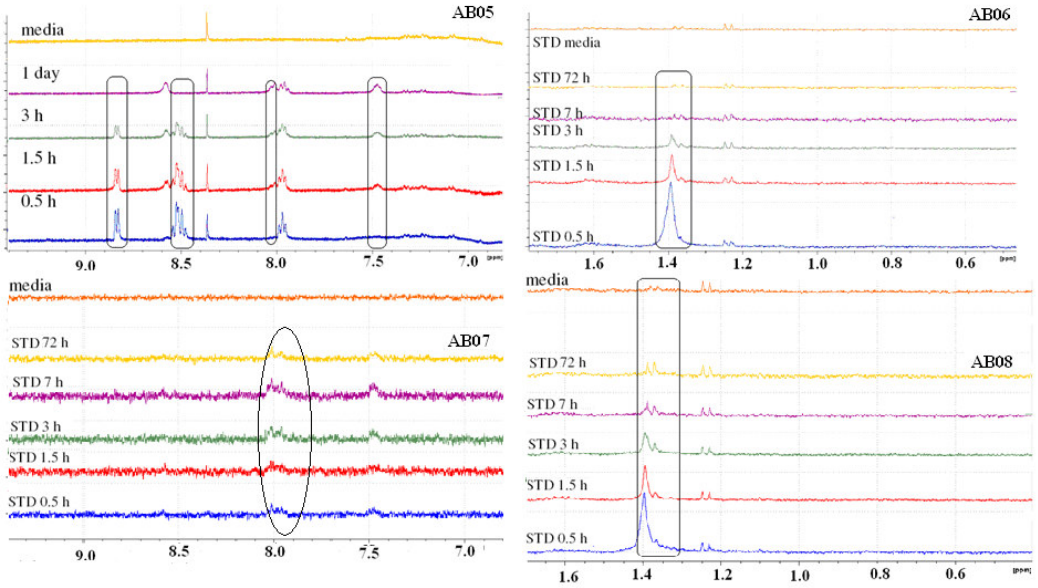


Figure 3.11: Stability of AB05-AB08 in 10% complete RPMI media at 37°C using STD NMR. AB05 stability was read after 0.5h, 1.5h, 3h and 24h; while compounds AB06-AB08 were read after 0.5h, 1.5h, 3h, 7h and 72h. The black circles depict STD peaks which represent the stability of the compounds at the given times.

3.8. Summary of theoretical and experimental data obtained for the 45 gold compounds evaluated in this study

Table 3.9 below summarises all quantitative data obtained in this study. This excludes the NMR results since that work was not analysed numerically.

Table 3. 9: Summary of all theoretical and experimental data obtained for the 45 gold compounds evaluated in this study.

Compound	Osiris Property explorer score	Lipinki's Ro5 score	Solubility	Cytotoxicity in PM1 cells	Cytotoxicity in PBMCs	IN strand transfer inhibition	RT direct inhibition (10uM)	RT direct inhibition IC_{50}	Antiviral activity Ba-L (SI)	Antiviral activity NL4-3 (SI)
AZT	-	-	-	-	>1000nM	-	-	-	2.34(>427)	1.30(>834)
Ral	0.81	4/4	-	-	-	-	-	-	-	-
Series 1										
AB01	0.09	3/4	*NS	10.741	-	NI	16.302	-	-	-
AB02	0.09	3/4	*NS	>100	-	NI	15.210	-	-	-
AB03	0.09	3/4	*NS	>100	-	NI	14.117	-	-	-
AB04	0.09	3/4	*NS	>100	-	52.800	9.803	-	-	-
Series 2										
AB05	-	3/4	323.437	49.403	40.6	NI	75.966	0.502	14.1(2.87)	37.2 (1.09)
AB06	-	3/4	266.025	11.276	6.28	NI	72.212	0.461	3.66 (1.71)	4.43 (1.42)
AB07	-	3/4	4.884	22.771	18.7	NI	74.677	0.503	8.22 (2.27)	13.0 (1.43)
AB08	-	3/4	306.884	3.423	2.94	NI	76.526	0.660	1.83 (1.61)	3.15 (<1.00)
AB09	0.09	3/4	332.586	3.054	2.45	NI	77.815	1.381	0.41 (5.95)	1.12 (2.19)
AB10	0.43	3/4	394.583	107.412	56.5	NI	75.686	1.054	23.5 (2.41)	40.8 (1.38)
AB11	0.50	3/4	283.283	33.847	8.30	NI	73.557	8.796	1.80 (4.60)	7.86 (1.06)
Series 3										
AB12	0.29	3/4	195.766	9.655	-	NI	9.747	-	-	-
AB13	-	3/4	526.644	0.994	-	NI	18.151	-	-	-
Series 4										
AB14	0.061	3/4	62.538	<1.5625	-	43.294	<0	-	-	-
AB15	0.15	3/4	814.285	72.8	-	32.337	<0	-	-	-
AB16	0.06	3/4	71.875	<1.5625	-	NI	<0	-	-	-
AB17	0.4	4/4	430.208	4.625	-	41.183	14.864	-	-	-
AB18	0.08	3/4	24.818	<1.5625	-	NI	11.261	-	-	-
AB19	0.162	3/4	769.583	<1.5625	-	NI	14.513	-	-	-

Compound	Osiris Property explorer drug score	Lipinki's Ro5 score	Solubility	Cytotoxicity in PM1 cells	Cytotoxicity in PBMCs	IN strand transfer inhibition	RT direct inhibition (10uM)	RT direct inhibition IC50	Antiviral activity BaL	Antiviral activity NL4-3
AB20	0.10	3/4	39.867	<1.5625	-	18.373	4.391	-	-	-
AB21	0.07	3/4	147.916	1.590	-	NI	4.902	-	-	-
AB22	0.07	3/4	99.845	1.250	-	19.376	<0	-	-	-
AB23	0.16	3/4	95.370	<1.5625	-	NI	<0	-	-	-
AB24	0.098	3/4	122.916	<1.5625	-	28.405	<0	-	-	-
AB25	0.07	2/3	65.865	3.100 ±	-	24.920	<0	-	-	-
AB26	0.1	3/4	238.425	<1.5625	-	NI	20.253	-	-	-
AB27	0.07	3/4	103.472	2.366	-	NI	22.626	-	-	-
AB28	0.08	3/4	362.946	2.125	-	27.771	14.100	-	-	-
AB29	0.06	2/4	219.444	<1.5625	-	40.496	19.932	-	-	-
AB30	0.07	2/4	260.732	<1.5625	-	20.863	9.117	-	-	-
AB31	0.31	4/4	426.934	3.05	-	30.517	10.534	-	-	-
AB32	0.13	3/4	482.541	34.140	-	22.333	13.770	-	-	-
AB33	0.17	3/4	103.670	1.780	-	27.032	9.940	-	-	-
AB34	0.08	3/4	141.061	<1.5625	-	NI	18.694	-	-	-
AB35	0.13	3/4	650.948	<1.5625	-	44.852	74.393	-	-	-
AB36	0.09	3/4	575.144	2.800	-	36.536	15.863	-	-	-
AB37	0.16	3/4	493.269	<1.5625	-	NI	<0	-	-	-
AB38	0.28	4/4	524.184	<1.5625	-	NI	11.711	-	-	-
Series 5										
AB39	0.09	2/3	241.319	2.190	-	52.842	70.726	10.900	-	-
AB40	**-**	2/3	534.054	116.493	-	NI	48.446	-	-	-
AB41	**-**	2/3	563.454	48.827	-	NI	67.924	50.390	-	-
AB42	**-**	2/3	525.781	31.855	-	NI	60.266	13.350	-	-
AB43	-	2/3	531.433	54.697	-	NI	51.664	-	-	-
AB44	-	2/3	522.222	31.125	-	NI	17.230	-	-	-
AB45	-	2/3	4513.889	>100	-	NI	<4.533	-	-	-

Desired biological properties.

CHAPTER 4
DISCUSSION

The early stages of drug discovery have become progressively complex since the addition of development hurdles aimed at reducing the failure rate of candidate compounds further down the pipeline. These development hurdles include profiling of drug candidates using *in vitro* and *in vivo* techniques early in the development process; notably the evaluation of ADMET parameters which would typically require advanced preclinical studies. However, early drug candidate profiling which includes virtual analysis of compounds using in silico techniques is expected to result in synthesis of compounds with the highest drug-like properties (Gleeson, 2008). This study was designed to fit the currently adopted process of drug discovery and development by early profiling of 45 available gold compounds using theoretical screening and *in vitro* assessment techniques.

Overall, theoretical screening identified six compounds AB10, AB11, AB17 (Osiris; Tables 3.1 and 3.9) and AB17, AB31 and AB38 (Lipinski Ro5; Tables 3.2 and 3.9) as best possible drug candidates. By contrast, *in vitro* phenotypic inhibition testing identified compounds AB05 to AB08 as best possible drug candidates (Tables 3.6 and 3.9), albeit with high (AB08) to moderate cytotoxicity (Tables 3.4 and 3.9). The *in vitro* assessment of compounds HIV-1 inhibition, virtual screening of the compounds probability as drugs, and the evaluation of compounds ADMET properties such as solubility, plasma protein binding, stability and cytotoxicity allowed us to gather sufficient information to aid in the future rational design of gold-based HIV-1 inhibitors, thus achieving the study objectives.

4.1. Theoretical analysis

Theoretical analysis of compounds involved the application of the Osiris Property Explorer (which predicted drug-like properties of the gold compounds) and Lipinski's

Rule of Five (used to assess the probability of compounds as orally bioavailable drugs). These studies were carried out to gather information on the properties of the compounds to be tested such as drug-likeness and identify their shortcomings in order to aid future design of new gold based drug-like chemical entities.

According to the Osiris Property Explorer, six parameters should be taken into consideration to generate hits for early stage drug discovery: toxicity risks, cLogP, aqueous solubility, molecular weight, drug-likeness and overall drug likeness score. A toxicity risk assessment identifies fragments known to induce mutagenicity, tumourigenicity, irritancy and reproductive effects. The cLogP is a measure of a compounds hydrophilicity or lipophilicity in which desired hydrophobicity is defined by low values (cLogP<5) for reasonable probability of absorption. Aqueous solubility (LogS) is also known to be associated with compound absorption, and a high aqueous solubility is desired for systematic transportation while some lipophilicity is necessary for cell membrane diffusion to reach target site of action (Jing *et al*., 2009). Optimizing compounds for efficacy within the drug development pipeline is almost always associated with increased MW, so it's necessary to start with compounds that have low MW. High MW is also associated with decreased absorption and reduced potential of reaching the target site. Approximately 80% of all traded drugs have MW less than 450g/mol (http://www.organic-chemistry.org/prog/peo/mw.html accessed 07-09-2011) therefore compounds with MW ≤450g/mol have a higher chance of success within the drug development pipeline than higher MW inhibitors. The fragment based drug-likeness sums up the fragments within a compound and a desired positive value represents drug-like properties frequently observed in traded drugs. The overall drug-likeness score sums all assessed parameters to judge a compound's overall potential to qualify as a drug.

In total 75.55% (n=34) of the gold compounds could be assessed using the Osiris Property Explorer. The remainder contain cationic structures that the Osiris Property Explorer could not account for during analysis. Overall the compounds were not drug-like and the Osiris scores were low. Of the compounds screened 21% (n=7) were predicted as non-toxic while the remaining gold compounds were considered toxic. The predicted toxicity in Series 1 and AB12 (Series 3) was associated with fragments such as ethyl acetate/phenylphosphines and pyrazoles which are considered tumour

inducing mutagens and irritants respectively (illustrated in Figure 3.1). Therefore future rational design of gold-based anti-HIV compounds should minimize the use of these fragments to circumvent their toxic effects. The gold compounds were predicted to be mostly lipophilic with 9% (n=3) of them predicted to have cLogP values greater than five which meant they were highly lipophilic. This lipophilicity suggests these compounds would be less soluble in aqueous medium. The high lipophilicity further predicts low permeability through lipophilic cell membranes (Kellogg *et al*., 2005). Approximately 80% of FDA approved drugs have a LogS value greater than -4 (Osiris Property Explorer, http://www.organic-chemistry.org/prog/peo/logS.html accessed 07-09-2011) and only 6% of the gold compounds tested in this study were predicted to fall within this desired solubility range. This implies that rational drug design of future gold compounds should use fragments associated with aqueous solubility specifically. In addition, 80% of the FDA approved drugs have a MW less than 450g/mol (Osiris Property Explorer) whereas only 12% of the gold compounds had MW within the desired range. Reduction of molecular weights would require identification of chemical structure involved in inhibition and thereafter modifications can be made. However, this may not be feasible as the gold element itself is large (196.97g/mol) which then adds to the existing MW of the compounds; as a result gold compounds will inherently be bulky. Another parameter that led to reduced drug-likeness (overall drug-likeness< 0.5) is the gold element within chemical structures since only a few metal based drugs are traded and are not likely to be part of the drug-likeness reference database.

The Lipinski's Rule of Five predicts good oral absorption and bioavailability when compounds consist of hydrogen bond (H-bond) donors less than five, H-bond acceptors less than ten, compound MW is less than 500g/mol and LogP is less than five (Lipinski *et al*, 1995). The Rule of Five suggests that if two out of the four parameters are out of the given range, poor absorption or permeability is possible (Lipinski *et al*, 2001). The overall intermediate score (3/4 and 2/3) observed suggest that gold compounds have a good probability of oral bioavailability which is above the acceptable 50% bioavailability. The data obtained could be improved through rational design of gold compounds with LogP below five and molecular weights below 500g/mol. Studies have demonstrated that increases in MW results in decreased absorption, permeation and bioavailability (Camenisch *et al*., 1998; Chae *et al*., 2005).

Only three compounds exhibited a 100% Lipinski's score (as shown in Table 3.2), which predicts good permeability (intestinal and blood brain barrier; Navia *et al*., 1996, Testa *et al*., 1996) by the gold compounds on a whole.

The fragments that contribute toward good oral bioavailability should be considered during design and development of future gold based therapeutic agents. In addition, sufficient understanding of structures identified to limit bioavailability can be used to facilitate drug design. Taken together, the compounds were clearly not well designed in line with theoretical predictions. As predictions are indicators of drug candidates these compounds were not good candidates for drug discovery. Based on theoretical screening, compound fragments identified to frequent good drug-likeness (AB10 and AB11) and good oral bioavailability (AB17, AB31 and AB38) could be used to rationally optimize second generation HIV-1 inhibitors. However, theoretical techniques used in this study cannot be used to predict whether a compound will have anti-HIV activity.

4.2. Aqueous solubility

All the gold compounds were subsequently screened for aqueous solubility to confirm the compounds preference to interact with polar environments containing hydrogen bond donors or acceptors (Kellogg *et al*., 2005). In concept, aqueous solubility of a solute is the concentration (mol/l) of its saturated aqueous solution; which is affected by MW and the amount of energy required to dissociate from crystalline structure into solution (Kellogg *et al*., 2005; Wang *et al*., 2007). In this study no correlation could be made between the gold compound aqueous solubility and MW (data not shown). A possible reason for this is that a small number of compounds were investigated.

In general the gold compounds exhibited moderate solubility with 9% (n=4) of the gold compounds being water insoluble which suggests potential problems for *in vitro* testing, because this could lead to poor reproducibility and false positives in bioassays (Mondal *et al*., 2009; Di and Kerns, 2006). In total 27% (n=12) of gold compounds exhibited favourable intermolecular adhesive interactions with water molecules therefore resulting in aqueous solubility above 500µM. AB13 was isolated as a salt that contain a carboxyl moiety, therefore the good solubility it exhibited was

attributed to these properties which supports findings by Starr and King, 1992. They observed that the solubility of carboxylic acid in organic solvents increased sharply as water concentration in the solvents increased (Starr and King, 1992). The aqueous solubility of AB15 and AB19 was attributed to the imidazoles (which is known to be miscible in water at a concentration of 663g/L: http://www.inchem.org/documents/sids/sids/288324.pdf accessed 07-09-2011) that form part of their chemical structures. The good solubility of compounds AB31, AB32 and AB37 was attributed to their constituent adenine moieties previously reported to be soluble in pure water (Krzaczkowska *et al*., 2004). No correlation could be drawn between the predictive analysis (cLogP and LogS) and the aqueous solubility study. The lack of correlation between parameters was associated with the limited number of gold based drugs that could be used to form databases for in silico drug discovery programs such as the Osiris Property Explorer. The compounds from Series 1, Series 2, Series 3, Series 4 and Series 5 (except AB45) are classified gold(I); and the remaining are gold(III). Overall better aqueous solubility was observed by gold(I) when compared to gold(III). For instance, gold(I) compounds AB13, AB17, AB19, AB31, AB32, AB35-AB38 and AB40-AB44 exhibited solubility ≥400µM. Future design of HIV-1 inhibitors in our laboratory will consider structural fragments in gold compounds observed to be aqua soluble while minimizing fragments that were frequently found in low solubility compounds.

4.3. Compound cytotoxicity in the PM1 cell line and PBMCs

The cytotoxicity of gold compounds was evaluated in PM1 cell lines and PBMCs (Table 3.4). An overall high cytotoxicity was observed and was possibly due to the fact that gold compounds are known to induce increased levels of reactive oxygen species (ROS) (Kudrin *et al*., 2000) resulting in oxidative stress (Omata *et al*., 2006) through destructive interactions with cellular molecules (DNA, proteins and lipids) (Thannickal and Fanburg, 2000). Other reasons include gold compounds varying oxidation states which could lead to undesired redox properties in biological media (Shaw, 1999); and the associated ligands (the ethyl acetate, phenylphosphine and pyrazole fragments) were predicted to induce toxicity by the Osiris Property Explorer which was discussed in section 4.1. The gold(I) compounds were observed to be more cytotoxic than the gold(III), although all compounds that exhibited cytotoxicity

≥100µM were gold(I) (Table 3.4). Gold(III) cytoxicity was associated with the compounds reduction to gold(I), while gold(I) cytotoxicity was associated with ligand stability. The gold(I) was exposed while gold(III) was embedded within the chemical structure and this made the chemical structure of gold(III) more stable, because it was not as exposed to interactions with proteins. The compounds in Series 1 exhibited no cytotoxicity in PM1 cells but since these were not soluble in DMSO or aqueous media, the absence of cellular uptake and compound precipitation out of solution could explain the lack of cytotoxicity observed. The cytotoxicity of Series 2 compounds in PBMCs and the PM1 cell line were comparable and less cytotoxic when compared to the control auranofin. The cytotoxicity of auranofin in PM1 cells observed in this study was comparable to that reported by Vergara (CC_{50} = 1.25 ± 0.5/CC_{50} = 1.5 ± 0.5) in ovarian carcinoma cell lines sensitive (A2780/S) or resistant to cisplatin (A2780/R; Vergara *et al*., 2010). AZT exhibited minimal cytotoxicity and the gold compounds fared poorly in comparison. Ultimately, it is understood that future drug candidates need to match or improve on the cytotoxicity profiles exhibited by currently utilized drugs. The cytotoxicity studies will aid future drug design and development because chemical structures frequently associated with cytotoxicity would be excluded. Those fragments associated with low cytotoxicity would be theoretically coupled with those associated with aqueous solubility and anti-HIV-RT and IN inhibition to produce derivatives with highly drug like properties.

4.4. Direct HIV-1 enzyme inhibition by gold compounds

The compounds were initially tested for HIV-1 IN inhibition. In total 2 compounds exhibited inhibition greater than 50% (namely, AB39 and AB04) and another 8% demonstrated inhibition between 40%-50% at a single dose of 10µM (Table 3.5). A 10µM concentration was used as a cut-off concentration because beyond this inhibition was likely to be associated with non-specificity of compounds. Greater degrees of inhibition were expected because HIV-1 integrase is known to consist of 8 cysteine residues. The N-terminal domain of integrase has a His_2Cys_2 motif with two cysteines vital to enzyme activity. Mutations to this His_2Cys_2 have been reported to induce IN paralysis (Cai *et al*., 1997). The zinc binding motif within the amino-terminal domain is also known to contain Cys moieties essential for zinc atom coordination (Cys40 and Cys43; Marchand *et al*., 2009). Other Cys residues essential

for integration have been identified within the catalytic core; namely Cys56, Cys65 and Cys130 (Carayon *et al*., 2010). Because of the knowledge of gold compounds affinity for cysteine residues, and the fact that the IN enzyme consists of eight exposed cysteines, considerable inhibition was expected; however, this was not observed. Structural alterations to these compounds would include structure activity relationships to enhance their drug-likeness, solubility and anti-HIV-1 IN potency. The information obtained from the moderate HIV-1 IN inhibition by AB39 (Series 5) and AB04 (Series 1) would be useful for future gold based drug design of HIV-1 IN inhibitors. The structures can be used to design derivatives with high docking scores against HIV-1 IN in silico, which are likely to induce potent inhibition *in vitro* subsequent to their synthesis.

All gold compounds were tested for HIV-1 RT inhibition at a concentration of 10µM and 82% of compounds showed a varying degree of activity as shown in Table 3.6. In total 24% (n=11) of the compounds (AB05-AB11, AB35 AB39, AB41, and AB42) tested showed good HIV-1 RT inhibition (60.26% to 77.8%). The compounds were further assessed for RT inhibition at concentrations 1µM, 0.5µM and 0.1µM in which a concentration dependent inhibition was observed. The compounds AB05-AB11 demonstrated good anti-HIV RT activity in particular AB05, AB06, AB07 and AB08 which exhibited nanomolar scale inhibition. Such inhibition at nanomolar concentration will reduce compound toxicity because it implies the use of less compound *in vivo*. This anti-HIV RT activity observed supports that of other gold compounds previously reported in literature (IC_{50} values determined ranged from 0.31µM to 28.4µM which were comparable to those in this study; Sun *et al*., 2004), and implies that such compounds be considered in future design and development of HIV-1 RT inhibitors. Sun *et al* evaluated gold porphorins for *in vitro* inhibition of HIV-1 RT and in combination with AZT. When tested in combination with AZT, gold porphorins demonstrated synergistic inhibitory effects of the HIV-1 RT (Sun *et al*., 2004). Assessment of structure activity relationships for compounds AB05-AB11 could lead to the design of drug-like second generation gold based HIV RT inhibitors, and the current study provides a start for the development of such inhibitors. The relevant chemical structure for RT inhibition requires identification; then thereafter the information from the Osiris Property Explorer and the Lipinski's Rule of Five

analyses would apply in the development of highly drug-like compounds with sufficient bioavailability.

Since only gold(III) compounds were able to inhibit HIV-1 RT, this suggested that their physical structures were stable for a duration that allowed the induction of inhibition. To exonerate the inhibitors of their cytotoxic effects while retaining their anti-HIV-1 RT efficacy, these gold(III) HIV RT inhibitors could be modified to gold(I) while retaining their core structure. These modifications would reduce undesired factors such serum binding, instability and cytotoxicity.

The compounds were initially tested for inhibition of a mutant HIV-1 RT that lacked RNaseH activity as illustrated in Figure 3.7. This inhibition was an indication that these gold compounds inhibit the polymerase activity of the enzyme thereby excluding interaction with RNaseH as a mechanism of action; due to the wide structural differences between the two sub-domains (Budihas *et al*., 2005). The gold compounds were further investigated against a 3TC resistant mutant HIV-1_{HXB2} Reverse Transcriptase/M184V. The compounds AB05-AB11 demonstrated potent inhibition of this mutant enzyme (Figure 3.8). In addition, the fact that these compounds are not nucleotides/nucleosides analogues excluded a NRTI/NsRTI mechanism of action as a possibility. This information therefore suggested that second generation rational drug design should focus on the p66 subunit of HIV-1 RT as the target for gold-based compounds. The potent inhibition of HIV-1 RT by AB05, AB06, AB07 and AB08 inspired further investigations into their mechanism of action. Gold compounds are known to undergo ligand exchange with cysteine-rich peptides on protein surfaces (Shaw, 1989). Since two cysteinyl residues (C38 and C280) are exposed on the HIV-1 RT surface (Ratner *et al*., 1985), interaction with these would likely contribute to the inhibition observed. The mechanism of action could be through active site binding and/or binding of the C38 and C280 essential for RT's catalytic function or both. Compound AB39 showed moderate RT and integrase inhibition therefore it would be necessary to exclude possible non-specific inhibition by testing the compounds for anti-RNaseH activity since this RT subdomain is closely related to integrase in structure (Marchand *et al*., 2008).

4.5. Gold compound HIV-1 inhibition in PBMC's

Subsequent to direct HIV-1 RT inhibition of the compounds AB05-AB11, the compounds were submitted to the NIH (USA) for evaluation of antiviral inhibition in PBMCs. PBMCs were used because they are the major target cells of HIV-1, and express the CD4 receptor, which is the major receptor for primary HIV-1 isolates (Sattentau and Weiss, 1988), as well as the CCR5 and CXCR4 co-receptors (Bleul *et al*., 1997). The inhibition observed in direct RT assays was not observed against the macrophage-tropic HIV-$1_{Ba\text{-}L}$ and T-cell tropic HIV-1_{NL4} viral isolates in PBMCs (Chekmasova and Strayer, 2006; Abstract), as illustrated in Table 3.8. The HIV-$1_{Ba\text{-}L}$ and HIV-1_{NL4} respectively utilizes the CCR5 and the CXCR4 receptors which are the principal co-receptors for HIV-1 infection. HIV-1 entry is therefore an exceptional therapeutic target, but the lack of inhibition of either viral isolate observed in this study tentatively excludes tropism associated inhibition by gold compounds as a possibility. The compounds were observed to exhibit HIV-1 RT inhibition at nanomolar concentrations in cell free assays as discussed in section 4.4. As micromolar concentrations were utilized in the PBMCs for antiviral assay, inhibition was expected even if not equivalent to that in direct assays but none was observed. The altered cell based inhibition was attributed to several reasons i.e. serum protein binding, bioavailability and cytotoxicity. Compound cytotoxicity was associated with the absence of viral proliferation, meaning the IC_{50} values observed were an indication of cell death therefore the virus could not replicate. This was indicated through cytotoxicity curves that paralleled the antiviral curves. Other factors that could have prohibited antiviral inhibition in PBMCs include serum protein binding which was shown to alter bioavailability of inhibitor to the target (refer to Figure 3.9). Thus, the second generation compounds AB05-AB11 should be rationally designed for optimal binding affinity against the desired target (HIV-1 RT) while minimizing their affinity for unwanted targets such as serum proteins. A targeted drug delivery system could be considered during development of gold based drug design; or the compounds should be designed such that the gold component is protected to reduce binding with serum proteins. Reduced serum binding would add to compound stability and reduced cytotoxicity due to compound breakdown.

4.6. Effect of serum proteins on direct HIV-1 RT inhibition by gold compounds

Serum protein binding was assessed as a possible parameter that led to loss of antiviral efficacy in PBMCs. To assess serum protein binding, the direct HIV-1 RT assay was modified to contain 10% foetal bovine serum and compound efficacy was observed to have reduced considerably for all four compounds (AB05, AB06, AB07 and AB08) tested. Albumin is the predominant extracellular protein in blood and has been reported to bind gold compounds. For this reason, the effect of HSA was evaluated separately using 4.5mg/ml HSA which is the typical concentration in HIV-1 infected individuals (Corbett *et al*., 1999). The addition of HSA into the assay also introduced considerable fold-change reductions in anti-HIV-1 RT efficacy. Protein-drug interactions are known to present widespread ADMET implications such as the reduced/loss of direct inhibition observed (Pavey *et al*., 2001; Kratochwil *et al*., 2002).

The loss of potency in PBMCs (anti-HIV assay) may thus be attributed to the presence of serum proteins in the culture media, which binds most of the compound leaving a minimal free fraction which cannot elicit a considerable pharmacological response (reviewed in Gleeson, 2008). Serum protein binding of drugs has previously been correlated to MW of drug agents, where increased MW led to increased plasma protein binding (Abraham, 1993; reviewed in Gleeson, 2008). Approximately 87% of the gold compounds tested have molecular weights greater than 500g/mol. Therefore, the high MW and the Cys residues from serum proteins enhanced the probability of blood plasma binding. These proteins (HSA)-drug (AB05-AB11) binding studies were assessed using STD NMR techniques and a level of interaction was observed. AB07 and AB09 were an exception since no STD signal was observed, which either indicates high affinity binding or lack of binding. A strong STD NMR signal confirms binding of compound to protein while the lack of an STD NMR signal is consistent with the notion that compound is solvent-exposed and is not in close proximity with the protein surface (Haselhorst *et al*., 2006).

AB05 was used as a representative compound in competitive binding studies in which the mechanism of albumin binding was evaluated. The compound was used with the aim of defining the meaning of the slightly visible STD NMR, as either high affinity

binding or lack of binding. AB05 was observed to displace auranofin which has been known to bind Cys^{34} (Shaw, 1999) on the surface of HSA, which implicates a common binding site for the gold-based compounds. Since Auranofin is known as a high affinity binder to HSA, its displacement indicates that gold compounds are likely to bind Cys^{34} with comparable affinity.

Serum binding and its effect were further evaluated through compound stability studies in media containing 10% FCS. The gold compounds were observed to metabolize in 10% complete culture media. This was observed from the gradual changes in STD spectra. A diminishing STD signal was observed for AB05, AB06 and AB08 at approximately 9.2ppm's, 1.4ppm's and 1.41ppm's respectively. The metabolisation was shown to be immediate after the addition of compound into media, since changes were observed in the first thirty minutes and continued until compounds were completely metabolized after twenty-four (AB05) to seventy-two hours (AB06 and AB08). AB07 was observed to exhibit an increase in STD spectral intensity at 9.5ppm's which implicated gold metabolite formation. The metabolite formed was shown to interact with serum proteins in culture media as described in section 4.6. The fact that such compounds interact with serum proteins and subsequently their anti-HIV-1 RT efficacy is reduced, excludes them from classification as pro-drugs.

4.7. Summary

Due to the severity of the HIV-1 pandemic and the development of antiretroviral drug resistance to currently approved FDA drugs, it is essential to continue with the development of novel anti-HIV drugs with improved clinical and pharmacological profiles. Thus the aim of this study was to analyse 45 available gold based drugs with the intention of obtaining data that could be used when designing second generation gold-based inhibitors of HIV-1 replication. The gold-based compounds were assessed for the following parameters: theoretical drug-likeness, aqueous solubility, cytotoxicity, serum binding, cell based inhibition and direct enzymatic activity. The Osiris Property Explorer predicted low drug-likeness scores and drug scores for all gold compounds. The Lipinski's Rule of Five predicted an overall intermediate oral bioavailability for gold compounds with an exception of a few which exhibited high scores. The theoretical data obtained could not be used to filter gold compounds

because conclusions on HIV-1 inhibition could not be drawn from them. Compounds were grouped according to similarities in chemical structure therefore a correlation existed between each group and the biological properties such as the hydrophilicity of the group AB01-AB04, the high cytotoxicity within the group AB14-AB38 and direct inhibition of HIV-1 RT within the group of compounds (AB05-AB11); which was diminished in PBMC inhibition assay studies. Gold compounds were observed to undergo chemical changes through STD NMR due to metal centre reduction during interaction with serum proteins. Serum proteins were also observed to have an antagonistic effect on the anti-HIV-1 potency of the gold compounds, and as a result reduced anti-HIV-1 RT activity was observed in the PBMC assay. Gold compounds were shown to inhibit the polymerase activity of HIV-1 RT while strand transfer was implicated a possible mechanism of action for integrase inhibition. The test compounds were not rationally designed, but inhibition of HIV-1 RT and IN by gold (III) compounds provides an opportunity for rational design of second generation inhibitors with improved biological profiles.

4.8. Concluding remarks

The findings of this study demonstrated the value of predictive screening coupled with experimental analysis of synthetic compounds. The theoretical techniques predicted low drug scores for the gold-based compounds but predicted good drug-likeness and drug scores for Raltegravir. The low theoretical drug scores were validated *in vitro* by undesired cytotoxicity, solubility and protein binding studies although compounds within Series 2 exhibited good HIV-1 RT inhibition. Inhibition of HIV-1 alone proved not to equal good drug-likeness as other biological properties are equally important for predicting drug-likeness like with Raltegravir. In conclusion, sufficient data was generated to considerably aid the rational design of anti-HIV-1 gold-based compounds. Since compounds within Series 2 were structurally related, tailoring of second generation inhibitors using their core chemical structure for improved biological properties and HIV-1 RT inhibition is possible.

4.9. Future Prospects

Gold compounds AB05, AB06, AB07 and AB08 have demonstrated potential as HIV-1 inhibitors therefore are classified as drug discovery HITS. Further studies are required to fully develop these HIT compounds into more effective LEAD candidates. Other compounds that were promising drug discovery HIT candidates included AB09, AB10 and AB11 suitable for anti HIV-RT activity. Furthermore, AB04 was a promising inhibitor of HIV-1 IN while AB39 was a promising candidate for dual inhibitors of HIV-1 RT and HIV-1 IN.

For future work the following is recommended:

- Rational design of compounds AB05-AB11 derivatives with high docking/drug-likeness scores and the evaluation of adherence to the Lipinski's Rule of Five.
- Evaluation of AB39 and AB10 as possible HIV-1 RT RNaseH inhibitors.
- Rational design of AB39 and AB10 compound derivatives with high docking against HIV-1 integrase and high drug scores.
- Chemical synthesis of theoretically successful derivatives of AB05-AB11.
- Evaluation of direct and cell based HIV-1 RT inhibition and ADMET properties.
- In depth assessment of the anti-HIV RT mechanism of action by the gold based compound derivatives of AB05-AB11.
- Development of a targeted drug delivery system to reduce serum binding of compounds and increase efficiency of uptake into infected cells.

CHAPTER 5
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