THE EFFECTS OF ANTI-HIV NUCLEOSIDE DRUGS ON THE VIRULENCE OF CLINICALLY RELEVANT *CANDIDA* SPECIES

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Degree of Master of Science in Medicine by research only

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Johannesburg, 2006

DECLARATION

I, Bintou Ahmadou Ahidjo, declare that this dissertation is my own work.

It is being submitted for the degree of Master of Science in Medicine to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

…….. (Signature of candidate)

…… day of ……. 2006

DEDICATION

To those never given a choice,

and C.A. Bitti, my pillar of strength

PUBLICATIONS AND PRESENTATIONS

1. B. Ahmadou Ahidjo[1], R. Veale[2], A.G. Dusé[1&3], P. Becker[4] and E. Marais[1&3] . The Effects of Antiretroviral Nucleoside Analogue Drugs on the Virulence of *Candida albicans*. Departments of [1]Clinical Microbiology and Infectious Diseases, [2]Molecular and Cell Biology, University of the Witwatersrand, [3]National Health Laboratory Service and [4]Medical Research Council, South Africa. (Poster presented at the First Conference of The Federation of Infectious Diseases Societies of Southern Africa, Sun City, South Africa, 24-27 July, 2005).
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ABSTRACT

Candida species are opportunistic yeasts that cause infections in immunocompromised individuals such as HIV and cancer patients. Recent studies show that 5-fluorouracil, a nucleoside analogue used for cancer treatment, increases *Candida* cell virulence. The aim of this study is to determine the effects of commonly used anti-HIV nucleoside analogue drugs on the virulence of *Candida albicans*, the predominant species associated with oral candidiasis.

Oral swabs were collected from antiretroviral-naïve HIV-positive individuals. *C. albicans* was characterised from 39 of these swabs using standard microbiological techniques and polymerase chain reaction. The effect of nucleoside reverse transcriptase inhibitors (NRTIs) zidovudine, stavudine, didanosine and lamivudine, at predicted drug peak concentrations in patients, as well as half and double these concentrations on select virulence factors of *C. albicans* isolates were studied. In addition, antifungal susceptibility to amphotericin B was assessed. Not all 39 isolates were used in the assays because of delays in obtaining reagents from respective manufacturers.

Results show no change in the adherence and biofilm formation of 29 isolates upon exposure to NRTIs. In contrast, a steady increase in the number of viable cells was observed upon exposure to double the peak concentration of lamivudine to 23 of the clinical isolates. All 31 isolates tested were susceptible to amphotericin B (MIC≤1μg/ml).

Although these results suggest that NRTIs may have little effect on the virulence of *C. albicans* it is postulated, that, in a dose-dependent manner, cytidine analogues act similarly to 5-FU by activating a signal-transduction pathway which stimulates proliferation.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADP:	Adenosine diphosphate
AIDS:	Acquired Immunodeficiency Syndrome
ART:	Antiretroviral Therapy
ARV:	Antiretroviral
bp:	base pair
CFU/ml:	Colony Forming Units per milliliter
CLSI:	Clinical and Laboratory Standards Institute
DMEM:	Dulbecco's Modified Eagle's medium
DMSO:	Dimethylsulfoxide
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
g:	Relative centrifugal force
HIV:	Human Immunodeficiency Virus
HIV-RT:	HIV-Reverse Transcriptase
hr:	Hour
ITS:	Internal Transcribed Spacer
kb:	Kilo base
MIC:	Minimum Inhibitory Concentration
min:	Minute
NCCLS:	National Committee for Clinical Laboratory Standards
NHLS:	National Health Laboratory Service
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
NADH:	Nicotinamide Adenine Dinucleotide
NRTI:	Nucleoside Reverse Transcriptase Inhibitor
NNRTI:	Non- Nucleoside Reverse Transcriptase Inhibitor
PCR:	Polymerase Chain Reaction
PI:	Protease Inhibitor
SDA:	Sabouraud's Dextrose agar
SDB:	Sabouraud's Dextrose broth
SDS:	Sodium Dodecyl Sulfate

sec:	Second
spp:	Species
TAE:	Tris-acetate EDTA buffer
TE:	Tris EDTA buffer
U:	Units
USA:	United States of America
UV:	Ultraviolet
WHO:	World Health Organisation

CHAPTER 1: LITERATURE REVIEW

Fungi, originally classified with plants, are now classified into a fifth kingdom- the kingdom Fungi, as a result of cell wall differences. They are eukaryotic organisms that contain chitin in their cell walls. These chemoheterotrophic organisms can be either unicellular or multicellular. Based on their distinct morphology, fungi are grouped into yeasts and moulds. An estimated 250,000 species of fungi exist. Of these, only 150 are known to be pathogenic to humans. Most of these fungi are free-living in nature, with the exception of *Candida,* which forms part of the normal flora of humans (Dixon *et al.,* 1999).

1.1 *CANDIDA* SPECIES

Candida is a dimorphic commensal yeast that forms part of the endogenous flora of the mucous membranes of the oral cavity, vagina and gastrointestinal tract of most human beings (Richardson, 1991; Bernhardt *et al.* 2001). This opportunistic yeast causes infections that can either be localized or systemic (Haynes, 2001).

Candidiasis is usually associated with in-dwelling medical devices such as catheters and heart valves, and in the last few decades, a marked increase in the rate of nosocomial candidemia has been observed (Meunier *et al.,* 1992; Beck-Sague and Jarvis, 1993; Pfaller 1996; Pfaller *et al.,* 2000). *Candida* species have been found to be the fourth leading cause of nosocomial infections worldwide (Wenzel, 1995). Most of these infections are due to

Candida albicans, which accounts for 50 - 60% of all nosocomial *Candida* infections (Perea and Patterson, 2002). There has, however, been a 46% increase in the rate of nosocomial infections caused by other *Candida* species (Fridkin and Jarvis, 1996; Abi-Said *et al.*,1997; Levy *et al.*, 1998; Perea and Patterson, 2002; Shin *et al.*, 2002). The most pathogenic *Candida* spp. after *C. albicans* are *Candida parapsilosis, Candida glabrata, Candida tropicalis* and *Candida krusei*, in that order (Kuhn *et al.*, 2002).

Candidiasis is also associated with malnutrition, total parental nutrition, broad-spectrum antibiotics, immunosuppressive therapy, bone marrow therapy and transplantation, amongst others (Richardson, 1991; Murray *et al.*¸1999; Shin *et al.*, 2002; Marais *et al*., 2004). In immunocompromised people, e.g. those infected with the human immunodeficiency virus (HIV), as well as those undergoing cancer treatment, immunosuppressive therapy, long-term antibiotic treatment or in individuals who are organ transplant recipients (Richardson, 1991, Anaissie, 1992; Ueta *et al.*, 2001), candidiasis occurs because immune dysfunction and the suppression of their leukocyte function allows for auto-infection from endogenous flora (Glick, 1994; Ueta *et al.*, 2001) and infection by contact with excretions of mouth, skin, and faeces from *Candida* carriers (Health Canada, 1999). In healthy humans, cell-mediated immunity has been found to play a significant role in recovery from oral infection with *C. albicans*. Data from an experimental murine model suggests that mucosal-associated immunity may play a protective role against oral candidiasis, whilst humoral immunity seems to have no part in this protective mechanism (Farah and Ashman, 2005). In humans however, it appears that cellular factors play a protective role mucocutaneous infection, while humoral factors play a larger role in the prevention of dissemination of Candida infections (Challacombe, 1994).

The ability of *Candida* to cause disease is attributed to its virulence factors. These factors enable this symbiotic yeast to become pathogenic when the host's defense system becomes compromised (Murray *et al.*¸1999; Haynes, 2001).

1.2 *CANDIDA* VIRULENCE FACTORS

Traits that enable infectious microorganisms to invade and colonise a host are known as virulence factors (Cole, 2003). In *Candida* spp. these attributes enable the yeast cells to establish a niche in the host's epithelial surfaces where the host's defense system cannot efficiently eliminate the infection. This niche also allows the *Candida* cells to colonise other tissues, and use the host's available substrates for growth and reproduction (Cole, 2003). Some virulence factors attributed to *Candida* spp. are adherence, biofilm formation and the production of extracellular hydrolytic enzymes.

1.2.1 Adherence

The first step for colonization, infection and subsequent disease causation by an organism is its adherence to a host surface. Adherence to host surfaces is also a prerequisite for subsequent biofilm formation (Cotter and Kavanagh, 2000).

Though little is known about the adherence mechanisms of *Candida* to oesophageal epithelial cells, both specific and non-specific interactions are likely to play an important role in this initial step of oral thrush formation (Cotter and Kavanagh, 2000).

1.2.1.1 Non-specific adherence interactions

Non-specific interactions of electrostatic forces, aggregation and cell surface hydrophobicity, are the primary mechanisms in the process of adhesion. These occur over long distances and are reversible (Cotter and Kavanagh, 2000).

Originally, electrodynamic interactions (van der Waals interactions, and hydrophobicity) were thought to promote adhesion, while the stronger electrostatic ion-ion interactions were thought not to contribute to the adherence of *Candida*. In subsequent years, however, alteration of the electrostatic charge on the surface of *C. albicans* was shown to affect the adherence of yeast (Klotz, 1994), and the chemical groups which gave the overall negative charge of the *Candida* cell wall were thought to be involved in specific adherence mechanisms of the organism (Hobden *et al.*, 1995).

In addition to the electrostatic interactions, hydrophobic interactions of *C. albicans* with the epithelium, as with other organisms, are considered to play an important role in the adherence of this yeast, since the switch from a hydrophilic state to a hydrophobic one renders the commensal yeast pathogenic (Hazen and Hazen, 1992). It was also observed that the hydrophobic *Candida* cells bound more readily to proteins such as fibrinogen than hydrophilic yeast cells (Holl, 1992), and it seems that the cell surface hydrophobicity of *Candida* contributes to other processes affecting disease progression (Masuoka *et al.*, 1997).

The overall effect of these non-specific interactions, however, seem less than those exerted by the specific adherence interactions (Cotter and Kavanagh, 2000).

1.2.1.2 Specific adherence interactions

These interactions, being ligand-receptor interactions, are not reversible. Adherence of *Candida* to epithelial cells is quite specific and evidence shows that mannoproteins, and to a lesser extent phospholipids, sterols and steryl esters, are responsible in mediating this adherence (Cotter and Kavanagh, 2000).

Both the specific and non-specific adherence interactions exerted by *Candida* make the adherence process a complex and multi-component one.

1.2.2 Biofilm Formation

Biofilms can be defined as structured microbial communities attached to a surface. These layers of cells, embedded within a matrix of extracellular polymeric material, display phenotypes distinct from sessile cells, and are responsible for many human infections (Douglas, 2003). Biofilm infections can either be caused by bacteria or fungi, as well as mixed species or mixed genera biofilms. In the environment, mixed species biofilms have been isolated. These indicate the ability of microorganisms to cohabit within a community in hostile niches. This co-existence can be parasitic e.g. between *C. albicans* and *Pseudomonas aeruginosa* where *P. aeruginosa* formed a biofilm on *C. albicans* filaments, and killed the fungus, or symbiotic e.g. between *C. albicans* and *Staphylococcus epidermidis,* where increased resistance to chemotherapy was observed (Hogan and Kotler, 2002; Adam *et al.*, 2002). In the oral cavity, mixed species biofilms with the commensals *Streptococcus salivarius* and *Streptococcus mitior* reduced candidal adhesion while *Streptococcus mutans* had no significant effect (Samaranayake and McFarlane, 1982). Although the role of bacterial biofilms has been extensively studied, very little is known about fungal biofilms (Ramage *et al.*, 2001; Douglas, 2003).

Candida biofilms have similar properties to bacterial biofilms such as structural heterogeneity, reduced susceptibility to antimicrobial agents, as well as the presence of exopolymeric material (Hawser and Douglas, 1995 (a); Baillie and Douglas, 1998 (b); Baillie and Douglas, 1998). Fungal biofilms, however, are distinct from bacterial ones in that *C. albicans* is dimorphic, and as such has the ability to switch from a yeast form to a

filamentous one. This feature of *C. albicans* confers unique developmental properties to its biofilms (Ramage *et al.*, 2001).

1.2.2.1 Development of *Candida* biofilms

After adherence of the *Candida* cells to the host epithelium or indwelling device, cell proliferation and biofilm formation follow during the course of *Candida* colonization (Ramage *et al.*, 2001).

Initial adherence of the yeast cells occurs within the first two hours of contact, and during the next two hours, the cells germinate and form micro-colonies, which are predominantly budding yeast cells. These budding cells begin to filament, forming pseudohyphae and true hyphae, after approximately 4-6 hrs. This is followed by the development of a monolayer (6-8hrs), where neighbouring yeast hyphae merge to form a network of spatially dispersed filamentous forms. Approximately 8-24 hrs after initial contact, the cells proliferate, and maturation of the biofilm is observed 24-48 hrs later. This final biofilm matrix is multi-layered, and is composed of all fungal morphologies (Ramage *et al.*, 2001; Andes *et al.*, 2004).

1.2.3 Production of extracellular hydrolytic enzymes

Saprophytic organisms secrete extracellular proteinases, whose primary function is to breakdown complex materials into nutrients, or to compete with other environmental organisms (Cunningham and Agard, 2004). Parasitic organisms seem to have modified this property for use during infection (Naglik *et al.*, 2004). These opportunistic organisms hydrolyse host cell membrane proteins. This allows for either adhesion and tissue invasion, or the damage of the host defense system cells, thereby avoiding attack (Klemba and Goldberg, 2002; Peschel, 2002; Rasmussen and Bjorck, 2002; Rosenthal, 2002; Naglik *et al*., 2003).

Candida species have been found to secrete only aspartyl proteinases. Whilst this organism is in its saprophytic stage, these enzymes are essential for growth when protein is the only available nitrogen source. However, in its pathogenic state, these *Candida* enzymes have been associated with other virulence traits such as adherence, phenotypic switching, and hyphal formation (Naglik *et al.*, 2003). Secreted aspartyl proteinases, thus, play an important role in the pathogenicity of *Candida* (Naglik *et al.*, 2004).

1.3 ANTIFUNGAL THERAPY

The marked increase in life-threatening fungal infections since the 1980s has been attributed to several factors, including frequent treatment with broad-spectrum antibiotics and the increase in the number of immunocompromised individuals (Perea and Patterson, 2002).

As a result of this, pharmaceutical companies have developed antifungal agents with systemic activities (Espinel-Ingroff, *et al.* 1999). Today, different classes of antifungal drugs are available for the treatment of fungal infections (Odds *et al.* 2003). Figure 1.1 summarizes the mechanisms of action of each class of antifungal described in Section 1.3.1 (Odds *et al*., 2003).

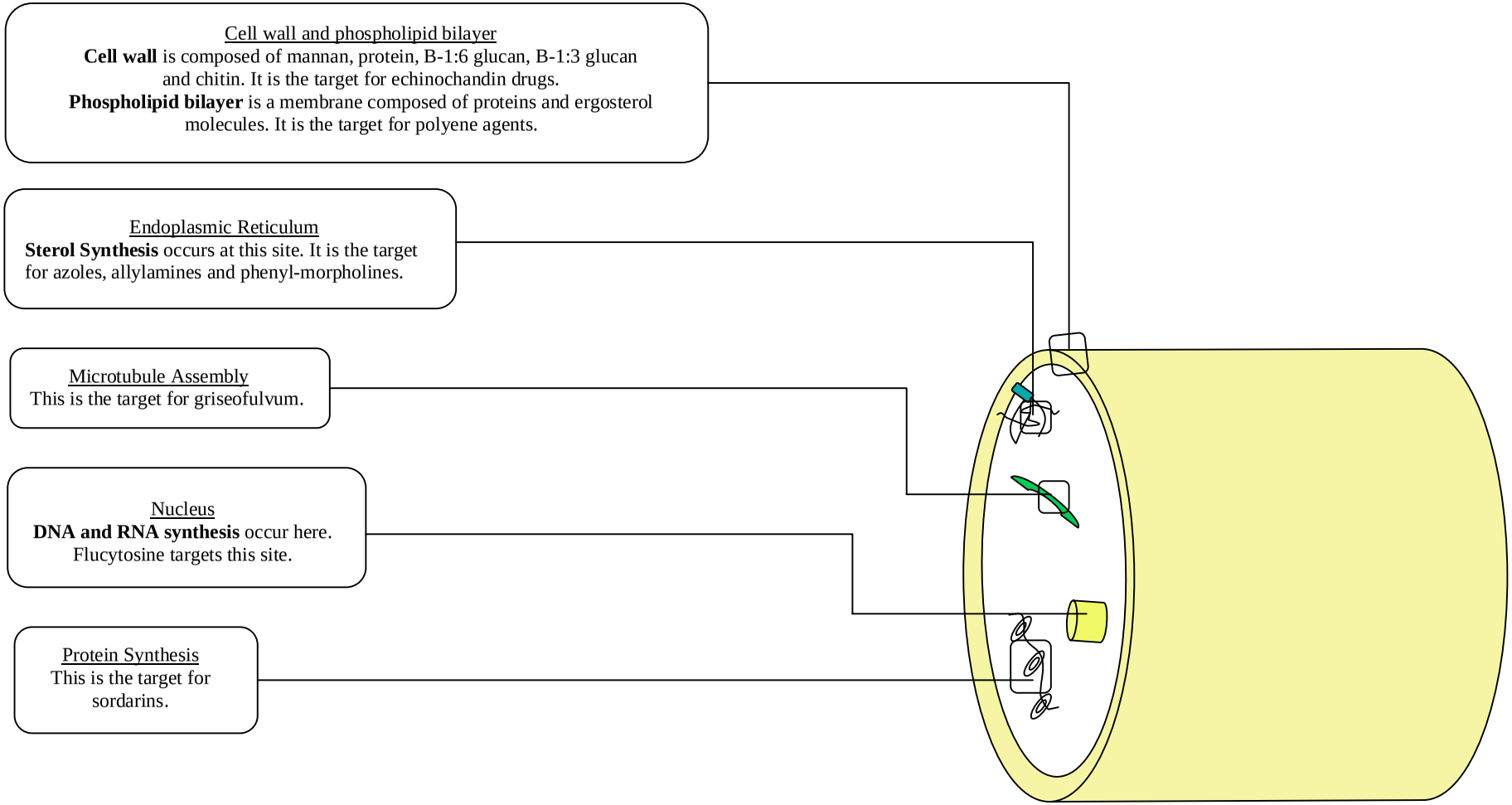


Figure 1.1: Diagrammatic illustration of fungal target areas by antifungal agents.
This cross-section of a fungal hypha shows the sites of action of antifungal drugs currently in use; the cell envelope structure is based on *Candida albicans* data

1.3.1. Antifungal Agents

The different classes of antifungal drugs have been developed to target different areas of fungi. Griseofulvin, isolated from *Penicillium griseofulvum* in 1939, was the first antimicrobial agent targeted specifically against fungi (Odds *et al.* 2003). Its use is limited to the treatment of dermatophyte fungal infections such as ringworm infection and athlete's foot (Odds *et al.,* 2003). It is thought to hinder fungal mitosis by interacting with polymerized microtubules and thus disrupting the fungal mitotic spindle. However, its exact mechanism of action still remains to be elucidated (Odds *et al.,* 2003).

Agents such as flucytosine, the polyenes and the azoles are discussed at a later stage (Sections 1.3.1.1, 1.3.1.2 and 1.3.1.3 respectively).

Another class of antifungal agents developed after flucytosine, polyenes and azoles i.e. the allylamines and phenylmorpholines, act on the ergosterol pathway (Odds *et al.,* 2003). This class of drugs is used for the treatment of few pathogenic yeasts, as not many are susceptible to these agents. These drugs are effective only against a small range of fungi, and have been advocated for the treatment of superficial mycoses (Odds *et al.,* 2003).

The most recent antifungal agents, the echinocandins target proteins responsible for the synthesis of the fungal cell wall polysaccharides. These have not yet been approved for use against *Candida* spp. (Odds *et al.,* 2003), though caspofungin has been approved for use against invasive aspergillosis (Maertens *et al.*, 2000).

1.3.1.1 Flucytosine

This compound, also known as 5-fluorocytosine, (Figure 1.2), is an antimetabolite drug.

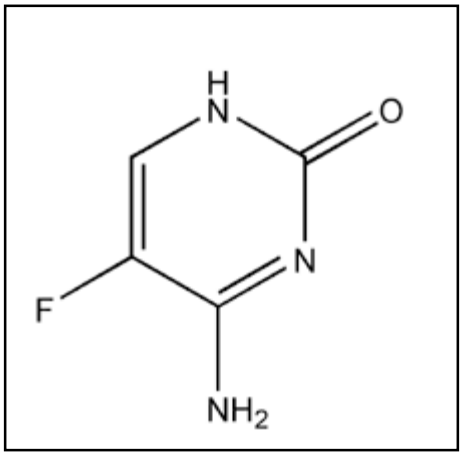


Figure 1.2: Chemical structure of flucytosine.

This pyrimidine is deaminated within the fungal cell to 5-fluorouracil. The latter in turn gets incorporated into RNA, causing chain termination and consequently inhibiting protein synthesis. In addition to this, flucytosine hinders the thymidylate synthetase pathway via 5-fluorodeoxy-uridine monophosphate, thus effectively inhibiting fungal DNA synthesis (Figure 1.3) (Kurtz *et al.*, 2005).

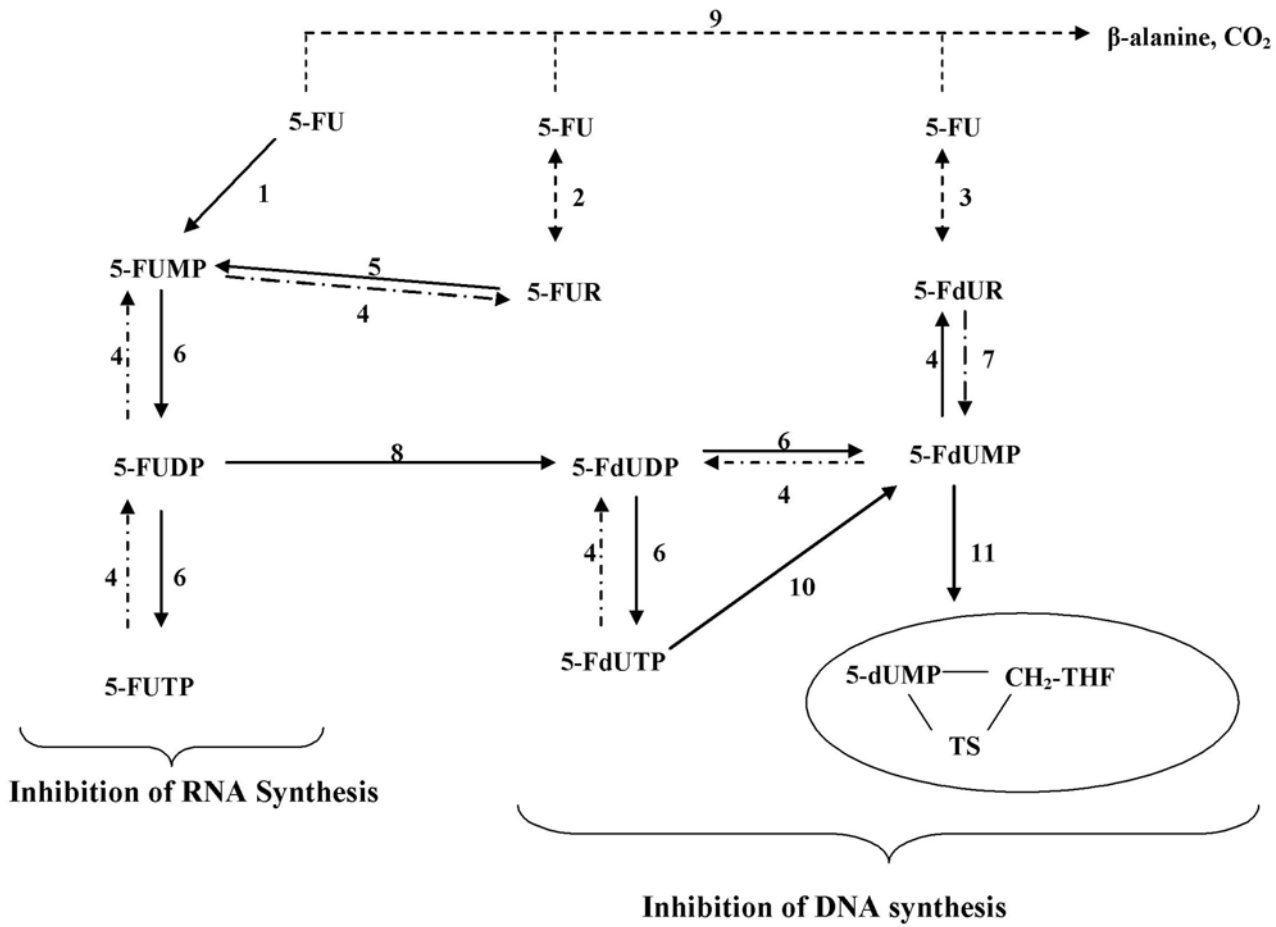


Figure 1.3: 5-Fluorouracil (5-FU) activation pathways to nucleotides in humans (black arrows) and yeasts (broken arrows).
T1: uracil/orotate phosphoribosyltransferase; 2: uridine phosphorylase; 3: thymidine phosphorylase; 4: 5' nucleotidases or phosphatases; 5: uridine kinase; 6: nucleoside monophosphate/diphosphate kinase; 7: thymidine kinase; 8: ribonucleotide reductase; 9: dihydropyrimidine dehydrogenase; 10: dUTP phosphorylase; 11: thymidylate synthase. Legend: 5-FUMP: 5-fluorodeoxy-uridine-5'-monophosphate; 5-FUDP: 5-fluorodeoxy-uridine-5'-diphosphate; 5-FUTP: 5-fluorodeoxy-uridine-5'-triphosphate; 5-FUR; 5-fluorouridine; 5-FdUR: 5-fluorodeoxyuridine; 5-FdUMP: 5-fluoro-2'-deoxyuridine-5'-monophosphate; 5-FdUDP: 5-fluoro-2'-deoxyuridine-5'-diphosphate; 5-FdUTP: 5-fluoro-2'-deoxyuridine-5'-triphosphate.

1.3.1.2 Polyenes

This class of antifungal drugs are responsible for fungal membrane alteration (Odds *et al.*, 2003). The members of this class, nystatin, natamycin and amphotericin B, are all products of *Streptomyces* species (Dixon and Walsh, 1996).

Amphotericin B, which was discovered in 1956, remains the mainstay antifungal agent today due to its broad spectrum of activity against most systemic fungal infections (Espinel-Ingroff, *et al.* 1999). It is an amphoteric compound composed of a hydrophilic polyhydroxyl chain and a lipophilic polyene hydrocarbon chain (Figure 1.4).

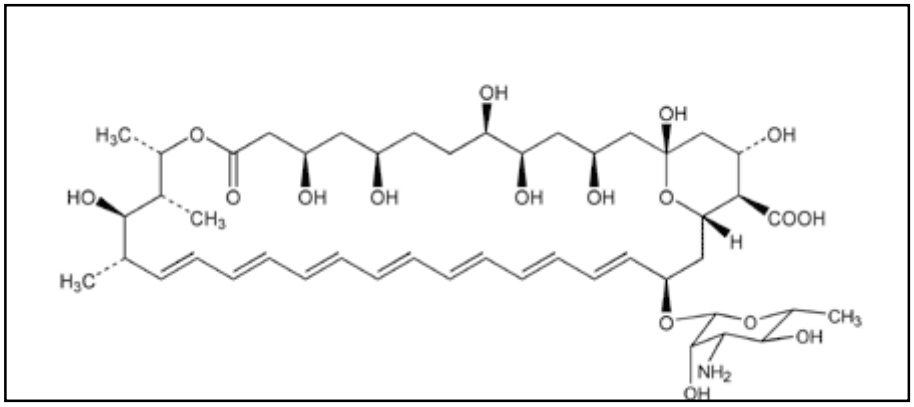


Figure 1.4: Chemical structure of amphotericin B

Amphotericin B, unlike other antimicrobials, binds to sterols and not enzymes. Its main binding site is ergosterol, the primary fungal cell membrane sterol. This interaction disrupts the osmotic balance of the membrane, leading to leakage of intracellular

potassium and magnesium ions as well as sugars, metabolites, resulting in cell death. The exact nature of this fungicidal activity, however, still remains to be elucidated (Odds *et al.*, 2003).

1.3.1.3 Azoles

The members of this class are composed of five-membered organic rings containing two or three nitrogen molecules, the imidazoles and triazoles, respectively. Fluconazole and itraconazole are the most clinically applied azoles (Figure 1.5) (Dixon and Walsh, 1996).

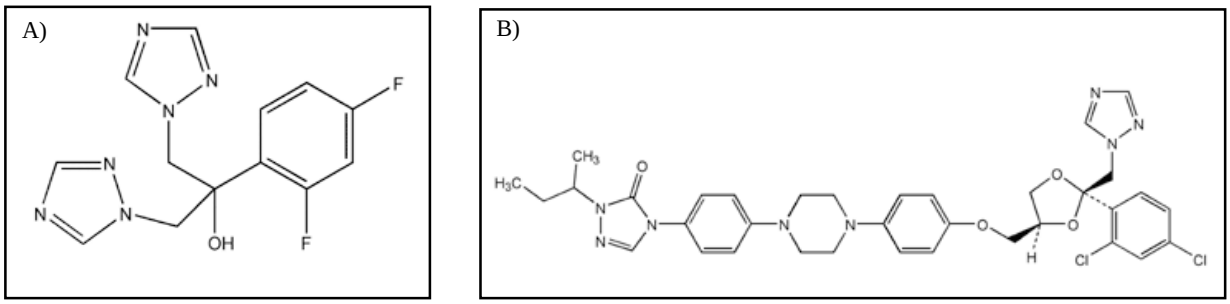


Figure 1.5: Chemical structure of the triazoles A) fluconazole and B) itraconazole

The main mechanism of action of all azoles is the inhibition of cytochrome P450 14α-demethylase, the enzyme responsible for the demethylation of lanosterol to ergosterol in the erogosterol pathway (Sheehan *et al.,* 1999; Odds *et al.,* 2003). This leads to the depletion of ergosterol, and replacement with other sterols, thereby resulting in the alteration of the permeability and fluidity of the fungal membrane (Odds *et al.*, 2003).

The primary target of the azoles is cytochrome P450-Erg11, which catalyses the oxidative removal of the 14α-methyl group of lanosterol by P450 14α-demethylase activity. The P450 protein contains an iron protoporphyrin moiety at its active site, to which the azoles bind via a nitrogen atom of the imidazole or triazole ring. The other portion of the azole molecule then binds to the apoprotein according to its individual structure (Odds *et al.*, 2003).

1.3.2 Antifungal Resistance in *Candida* spp.

Antifungal resistance is defined as the relative insensitivity of fungi to an antifungal agent as tested *in vitro* and compared with other fungi of the same species (Loeffler and Stevens, 2003). Primary resistance, which is observed in organisms never exposed to antifungal drugs, has been observed in *C. krusei* with regards to fluconazole. Most other *Candida* species, however, develop secondary resistance to antifungal agents (Loeffler and Stevens, 2003).

Secondary resistance of *Candida* species has been documented mostly with the azoles (Loeffler and Stevens, 2003). This has been attributed to factors such as alteration of drug efflux, overexpression of lanosterol demethylase (the azole target), altered sterol $\Delta^{(5,6)}$ desaturase, and alterations in the plasma membrane composition which affects membrane fluidity and asymmetry, thus leading to a decrease in the uptake of the drug, (Ghannoum and Rice, 1999; Loeffler and Stevens, 2003).

In HIV-positive patients a high prevalence of antifungal drug resistance in *Candida* species has been observed (33% fluconazole resistance as opposed to 11% fluconazole resistance from HIV-negative isolates) (Law *et al.,* 1994). Johnson and co-workers (1995) attributed azole resistance mainly to the prolonged fluconazole therapy in the treatment of recurrent oral candidiasis. Subsequent studies examined the role of *Candida* virulence traits with respect to antifungal resistance. Increased adherence, germ tube formation, levels of secreted aspartyl proteinases and extraphospholipase activity of fluconazole resistant *C. albicans* was observed when compared to sensitive isolates *in vitro,* and the virulence of a fluconazole-resistant strain proved to be higher than that of a fluconazole-sensitive strain *in vivo* (Fekete-Forgács *et al.,* 2000).

Biofilm formation of *Candida* is also thought to be implicated in antifungal resistance. The mechanism of action of this increase in resistance of biofilms to antifungals, both *in vitro* and *in vivo* has yet to be elucidated (Hawser and Douglas, 1995; Andes *et al.* 2004). This increase in antifungal resistance was previously attributed to low growth rate, matrix production or unique biofilm-associated patterns of gene expression (Kumamoto, 2002). Experimental data however suggested that low growth rate was not solely responsible for antifungal resistance (Baillie and Douglas, 1998). Results from another study by the same group indicated that production of the biofilm's exopolymeric matrix also did not constitute a significant barrier to the diffusion of antifungal agents and the presence of this extensive matrix did not enhance resistance (Baillie and Douglas, 2000). Though a few studies have provided clues of the genetic basis for increased antifungal resistance of cells in a biofilm, this mechanism still remains to be elucidated (Ramage *et al.,* 2002; Lupetti *et al.,* 2002; Micheli *et al.,* 2002).

1.4 *CANDIDA* AND THE AIDS PANDEMIC

The Acquired Immunodeficiency Syndrome (AIDS) pandemic has already caused the death of over 20 million people globally, and an estimated 39.4 million people are living with HIV (UNAIDS, 2004). *Candida*, the main causative agent of fungal infections in HIV-positive individuals (Jin *et al.*, 2003), causes oral candidiasis which has been observed in approximately 90% of HIV-infected individuals (Repentigny *et al.*, 2004).

Candidal infections are associated with reduced immune function and lowered numbers of CD_4+ lymphocytes (Patton *et al.*, 1999). Oral candidiasis is one of the first indications of HIV infection in most individuals (Samaranayake *et al.*, 2002), and it usually presents in its pseudomembranous form, as smooth white plaques, in the oral cavity of these individuals (Figure 1.11)

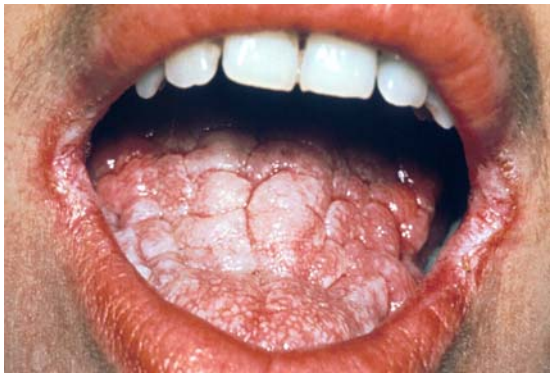


Figure 1.6: Chronic oral candidiasis of the tongue in an adult with an underlying immune deficiency showing the characteristic white pseudomembrane

Candida albicans is the most frequent causative agent of oral candidiasis (Repentigny *et al.*, 2004). It is part of the oral flora of more than 60% of all human beings (Cassone and Cauda, 2002), and as such, the most common source of the oral candidiasis is the patient. Immunocompromised individuals can also be exogenously infected with *C. albicans* through nosocomial transmission, sexual transmission and oral transmission (Dromer *et al.*, 1997; Repentigny *et al.*, 2004).

An increase in the rate of *C. albicans* carriage as well as an increase in the frequency of oral candidiasis is usually observed in HIV-positive individuals. It has been suggested that these increases could be due not only to the compromised immune systems of these

individuals but may also be attributed to possible alterations in the quality of the host oral environment and mucosal cells, as well as *Candida* virulence traits (Jin *et al.,* 2003).

Previous studies comparing adherence (the prerequisite of colonisation), of yeasts from HIV-positive and HIV-negative patients to mucosal surfaces generated variable data (Sweet *et al.*, 1995; Pereiro *et al.* 1997; Tsang *et al.* 1999). In 2003, Jin and co-workers proposed that the increase in carriage and frequency of *C. albicans* in HIV-positive individuals could be in part due to the enhanced ability of the colonising *C. albicans* to produce biofilms on the oral mucosal surfaces. They found no significant differences in the amount of biofilm produced by isolates obtained from HIV-positive and HIV-negative individuals. Using a linear statistical model, they found an associated decrease in *Candida* biofilm formation in patients younger than 35 years, as well as patients who had CD_4+ counts of greater than 350 cells/l, and attributed an increase in biofilm formation to the use of zidovudine at the time of sampling (Jin *et al.,* 2003).

Tsang *et al.* (1999) also showed similar results of increased adherence of *C. albicans* to buccal epithelial cells *in vitro* in the presence of zidovudine, which was attributed to zidovudine-related xerostomia, anaemia, neutropenia, and increase in host cell receptivity for yeasts. Based on this and their own data, Jin and co-workers suggested that the use of this antiretroviral nucleoside analogue drug was associated with increased virulence of *C. albicans* (Jin *et al.,* 2003).

Historically amphotericin B was the recommended drug of choice for a variety of fungal infections in HIV/AIDS patients attending state hospitals in South Africa. However, in the

last five years, as part of a Diflucan partnership programme with the South African Ministry of Health, Pfizer Inc. has made fluconazole available to state hospitals at no cost. As yet, resistance to fluconazole has not been observed, while an 8.4% resistance to amphotericin B has been reported (Blignaut *et al.*, 2002(a)).

1.4.1 Antiretroviral Therapy

The implementation of Highly Active Antiretroviral Therapy (HAART) has revolutionised the treatment of HIV and has considerably improved the quality and length of life of infected individuals (Field and Laughlin, 1999).

HAART consists of either two nucleoside reverse transcriptase inhibitors and one non-nucleoside reverse transcriptase inhibitor, or two nucleoside reverse transcriptase inhibitors and one protease inhibitor. In South Africa two antiretroviral (ARV) regimens have been instituted in the management of patients on the national “roll out” programme (Table 1) (Simelela, 2004). The second line regimen is given to patients only when toxicity or treatment failure occurs with the first line regimen, and regimen 1b is given to women who can and/or who want to fall pregnant (Simelela, 2004).

Table 1: The current South African antiretroviral roll out regimens

Line	Regimen	Prescribed Drugs
1st line	1a	d4T, 3TC and efavirenz
	1b	d4T, 3TC and nevirapine
2nd Line	2	AZT, ddI and lopinavir/ritonavir

Key: d4T-stavudine; 3TC-lamivudine; ddI -didanosine; AZT- zidovudine

1.4.1.1 *Nucleoside reverse transcriptase inhibitors (NRTIs)*

Nucleoside reverse transcriptase (RT) inhibitors are nucleoside analogues that lack a 3' hydroxyl group required for DNA synthesis. Due to this unique chemical structure, they inhibit the reverse transcription of viral RNA to DNA by terminating DNA strand elongation and consequently preventing the completion of the HIV replication cycle (Weissbrich *et al.*, 2002). NRTIs also compete for the binding site of the reverse transcriptase thus competing with the authentic substrate for the catalytic site (Weissbrich *et al.*, 2002).

The NRTIs currently in clinical use are zidovudine (AZT) and stavudine (d4T), which are both thymidine analogues; zalcitabine (ddC), and lamivudine (3TC), which are cytidine analogues and didanosine (ddI), which is an inosine analogue (Gibbon, & Swanepoel, 2000) (Figure 1.7). In these forms, the NRTIs have no anti-HIV activity. Only after phosphorylation by a host kinase or nucleotidase to their 5'-triphosphate forms do these drugs exert anti-HIV activities.

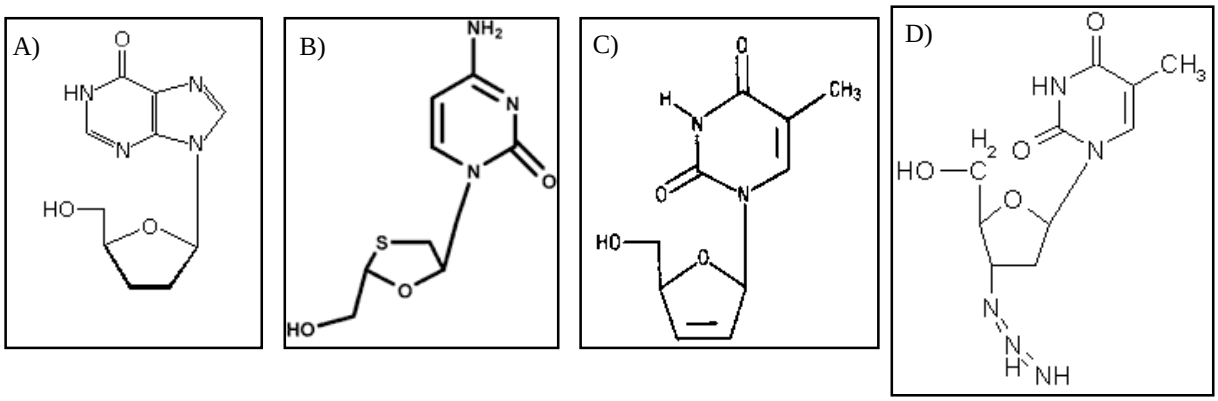


Figure 1.7: Structures of the NRTIs A) ddI, B) 3TC, C) d4T and D) AZT

1.4.1.2 *Non-nucleoside reverse transcriptase inhibitors (NNRTIs)*

Non-nucleoside reverse transcriptase inhibitors are composed of a wide variety of different chemical classes, and differ structurally from the NRTIs by not being nucleoside or nucleotide analogues (Weissbrich *et al.*, 2002). Members of this class include nevirapine and efavirenz (Figure 1.8)

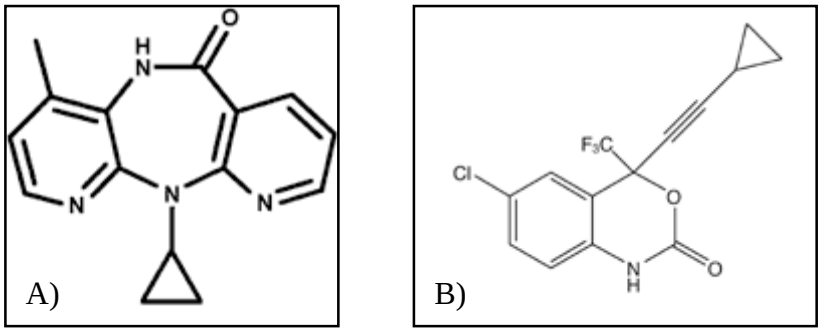


Figure 1.8: Structures of the NNRTIs A) nevirapine, and B) efavirenz

NNRTIs which are specific only to HIV-1 reverse transcriptase inhibit reverse transcription in a non-competitive manner. They are highly selective allosteric inhibitors, and unlike the NRTIs, do not need to be activated in the cell (Srinivas and Fridland, 1999).

The NNRTIs bind to the unique hydrophobic site of HIV-1-RT, which is distinct from the catalytic site of the polymerase. This pocket only becomes accessible upon displacement of the polypeptide chain connecting “palm and thumb” upon the interaction with an NNRTI. (Huang *et al.*, 1998; Rodgers *et al.*, 1995). This interaction locks the polymerase into an inactive form by inducing structural changes at the catalytic site. These changes lead to the alteration of the polymerase shape and thus its function (Weissbrich *et al.*, 2002).

1.4.1.3 *Protease inhibitors (PIs)*

Protease inhibitors (PIs) are peptidomimetics. There are two types of PIs: type I mimetics which are synthetic derivatives of short peptides, and type III mimetics which are chemically unrelated substances (Weissbrich *et al.*, 2002). These drugs bind to the catalytic site of HIV protease, which is essential for the cleavage of the HIV polyprotein into the different HIV proteins required for virion assembly. They act as competitive inhibitors with regards to the natural substrate i.e. viral polyproteins. Most PIs currently in use are type I mimetics and an example is ritonavir (Figure 1.9) (Srinivas and Fridland, 1999).

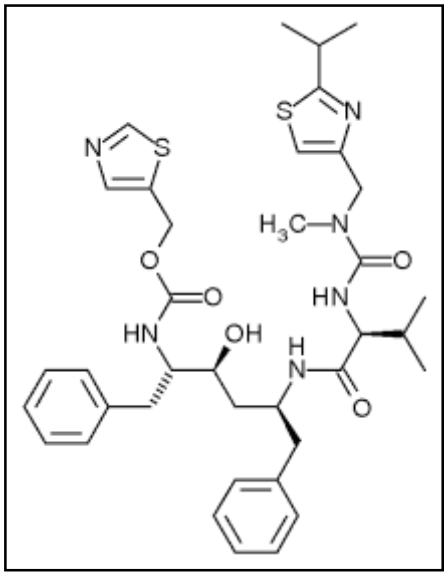


Figure 1.9: Structure of the PI ritonavir.

1.4.2 Effect of HAART on candidiasis

The ability of *Candida albicans* to cause oral candidiasis in immunocompromised hosts has been attributed to the decrease in the host's ability to prevent infection as well as the organism's virulence factors (Ramage *et al.,* 2004). Since the introduction of HAART in 1996, a remarkable decrease in oral thrush has been observed in HIV-positive patients (Ramage *et al.,* 2004). Initially, this was attributed solely to increases in CD_4+ cell counts, decreases in viral load, and immunological reconstitution of host defenses. It was later observed that HAART resulted only in late and inconsistent recovery of anti-candidal cellular immunity, and in some patients oral candidiasis was fully treated even before recovery of CD_4+ cell counts and response to *Candida* antigens (Ramage *et al.,* 2004), implying that host immune reconstitution was not the only reason for the observed decrease in oral candidiasis.

Also in 1996, an HIV-positive patient was diagnosed with oral thrush which could not be treated with fluconazole, itraconazole, amphotericin B or nystatin. Only upon the introduction of the HIV proteinase inhibitor saquinavir as part of the HAART therapy was the candidiasis cured (Zingman, 1996). In 1999, the decrease in *C. albicans* infections in HIV-positive patients was attributed to the PIs direct inhibition of *Candida* aspartyl proteases (Caudel *et al.* 1999). In addition to the SAP virulence trait of *C. albicans,* an *in vitro* study has also demonstrated that PIs inhibit the adherence of *C. albicans* to epithelial cells (Bektic *et al.*, 2001). Whilst inhibition of SAP expression in the oral cavity by PIs and not NNRTIs were observed (Cassone *et al.* 1998; Bernardis *et al.* 1999), results by

Bernardis *et al.* did not support the hypothesis that this inhibition could eliminate *Candida* or its selection in the oral cavity (Bernardis *et al.* 1999).

It therefore seems that factors other than PIs and host immunity could affect *Candida*. Increases in virulence of the organism may well be triggered by such factors, which can contribute to HIV-positive individuals eventually developing oral thrush (Sanchez-Vargas *et al.*, 2005).

CHAPTER 2: PROJECT

2.1 RATIONALE

The occurrence of *Candida* in the oral cavities of HIV/AIDS patients usually predicts the development of oral candidiasis at a later stage, since the anti-*Candida* activity of oral epithelial cells is diminished in these immunocompromised individuals (Steele *et al.,* 2000; Sanchez-Vargas *et al.,* 2005). Despite the reduction in oral thrush observed since the introduction of HAART, patients usually develop this infection when their viral loads are greater than 3000 copies/ml and CD_4+ cell counts are less than 200 (Migliorat *et al.,* 2004).

Immune reconstitution inflammatory syndrome (IRIS), a clinical deterioration of the clinical status of HAART-treated patients despite satisfactory control of viral replication and improvements in the CD_4+ lymphocyte counts, leads to inflammatory responses towards previously diagnosed or incubating pathogens, as well as unidentified antigens (Shelburne, 2003). Experimental data showed that IRIS was found to be more pronounced in patients receiving an NNRTI-HAART regime than those receiving the PI-HAART regime. This showed that immunological recovery alone upon administration of HAART did not explain the anti-SAP and anti-oral candidiasis effects of PIs (Cassone, 2002).

In the early 1960s AZT was developed as an anticancer drug, but was never patented as it proved unsuitable in a clinical setting. It was later shown to be useful against HIV. 5-fluorouracil, a nucleoside analogue used for cancer treatment was shown to increase

Candida cell virulence *in vitro* (Ueta *et al.,* 2001). Since 5-fluorouracil and NRTIs are structurally similar, we postulate that the NRTIs may have a similar effect on *Candida*.

In HIV-positive individuals a change in oral *Candida* flora, (Nguyen *et al*., 1996, Nho *et al*., 1997) and a high prevalence of antifungal resistance, particularly to the triazoles, (Law *et al.*, 1994; Sanglard and Odds, 2002), has been noted. These make the findings by Jin and co-workers (2003) and Ueta *et al.* (2001) of great importance. Virulence changes in *Candida* species upon exposure to NRTIs would be of interest in South Africa, since an estimated 5.5 million South Africans are infected with the virus (UNAIDS, 2006 Report on the global AIDS epidemic), and oral candidiasis remains the most prevalent fungal infection (Blignaut *et al.,* 2002). This study could contribute to the design of treatment of HIV patients with candidiasis.

If an increase in *Candida* spp. virulence due to NRTIs is observed, this would imply that patients should be treated for candidiasis with the appropriate antifungal drug prior to initiation of HAART therapy. It would also re-enforce the need for patients to adhere strictly to their ARV therapy.

2.2 HYPOTHESIS

Nucleoside analogues used in antiretroviral therapy for HIV/AIDS increase the virulence of *Candida albicans*.

2.3 OBJECTIVES

2.2.1 General Objective

To establish fungal biofilm, proliferation, adherence and antifungal susceptibility assays in the laboratory, and determine the effects of NRTIs on the virulence of *C. albicans.*

2.2.2 Specific Objectives

To determine the effects of NRTIs on:

i) *Candida* biofilm formation

ii) The rate of proliferation of *Candida*

iii) The adhesion of *Candida* to epithelial cells

iv) The antifungal susceptibility of *Candida* to amphotericin B

2.3 ETHICAL CLEARANCE

Establishment of techniques was done using previously identified *Candida* spp. These isolates, obtained from Dr M. Patel (Division of Oral Microbiology, Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand), were collected from HIV-positive individuals. Ethical clearance for such use was obtained from the Ethics and Biosafety Committee of the University of the Witwatersrand, reference number M010507.

Oral swabs were collected from HIV-positive individuals attending the HIV clinic of the Johannesburg Hospital, just before they started antiretroviral therapy. Patient details were not known to the investigator, and patient consent was obtained before collection of swabs. A copy of the consent form is attached as Appendix D. Ethical clearance for use of these samples was obtained from the Ethics and Biosafety Committee of the University of the Witwatersrand, reference number M040904.

CHAPTER 3: MATERIALS AND METHODS

3.1 CHEMICALS, REAGENTS AND MEDIA

All the reagents used in this study were of molecular or analytical grade. A list of all reagents, and enzymes used as well as the composition of media, buffers, solutions and stains are found in appendices A, B and C. All primers used were purchased from Inqaba Biotechnical Industries (Pty) Ltd., South Africa.

3.2 IDENTIFICATION

Identification techniques were validated using *Candida albicans* (ATCC 90028), *Candida tropicalis* (ATCC 750), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258), and a clinical isolate of *Candida parapsilosis*. Patient isolates were subsequently identified using the same techniques.

3.2.1 Microbiological Identification

Single colonies of each isolate were obtained by streaking each sample onto Sabouraud's Dextrose Agar (SDA) containing 0.005% chloramphenicol , and incubated at 36° ± 1°C for 48hrs. This selective medium was chosen for the isolation since it has a high dextrose concentration and acidic pH, which allows for the selection of fungi (Chapin & Murray, 1999 (b)).

A loopful of each of the yeast isolates was resuspended in distilled water, and this was used for further identification.

3.2.1.1 *Staining*

Staining is one of the most commonly used methods of identifying microorganisms.

3.2.1.1.1 *Gram Stain*

Gram staining, considered one of the most useful identification tests for microorganisms, stains yeasts purple (Lauderale *et al.*, 1999; CDC Appendix ML-C) (Figure 3.1). Yeasts are Gram-positive, though they stain poorly.

One hundred microlitres of each yeast suspension was heat-fixed onto a slide, and the slides were flooded with crystal violet. After one minute, the stain was rinsed off with tap water. The slides were then flooded with Gram's iodine for one minute. After rinsing off with water, the slides were flooded with Gram's decolouriser for 10s. These were then rinsed with water and flooded with safranin counterstain for 30s. The slides were rinsed, dried and observed with a light microscope at 1000x magnification.

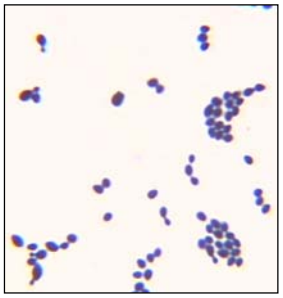


Figure 3.1: Photograph showing the characteristic phenotypic traits of *Candida* after a Gram stain at 1000x magnification

3.2.1.1.2 *Periodic Acid Schiff Base (PAS) Stain*

This stain is mainly used to detect fungi from clinical specimens. The periodic acid first hydrolyzes the cell wall aldehydes. The latter then combine with the Schiff base, colouring the cell wall carbohydrates, yielding the characteristic bright pink-magenta of fungi (Chapin and Murray, 1999 (a)) (Figure 3.2).

One hundred microlitres of each yeast suspension was heat fixed onto a slide. The slides were flooded with 0.5% periodic acid for 5mins. After rinsing the slides with tap water, the slides were completely covered with the Schiff base. These were allowed to stand for 15mins after which the slides were rinsed with running water for 10mins. The slides were flooded with 1% Light Green Solution (Merck, South Africa), a counterstain, for 1min. The slides were rinsed with water, air dried and observed at 1000x magnification.

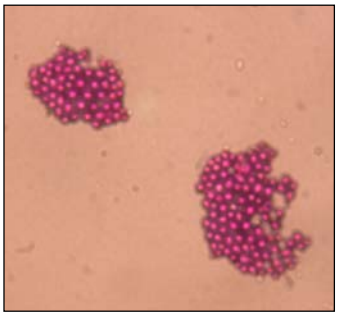


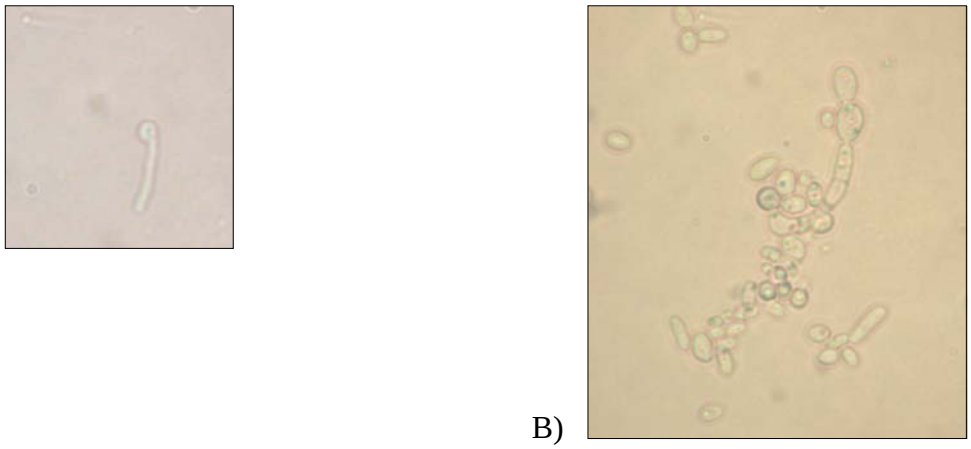
Figure 3.2: Photograph showing the characteristic phenotypic traits of *Candida* after a PAS stain at 1000x magnification

3.2.1.2 *Germ Tube Formation*

This is one of the defining tests for the preliminary identification of *Candida albicans*, since only *Candida albicans* and *Candida dubliniensis* form true hyphae (Warren and Hazen, 1999).

A single colony of each yeast isolate was inoculated in 0.5ml horse serum and incubated at 36°C± 1°C for 2.5 - 3hrs. A drop of each solution was observed under the microscope at 400x magnification for germ tube formation. *Candida albicans* (ATCC 90028) was used as the positive control while *Candida tropicalis* (ATCC 750) was used as the negative control (Figure 3.3).

A true hypha has no constriction between the blastoconidium and germ tube. *C.tropicalis* can develop hyphal initials, but the blastoconidia are larger than those of *C.albicans* and a constriction is observed between the blastoconidium and germ tube.



A) B)

Figure 3.3: Photographs showing the presence of a true hypha characteristic of *C.albicans* (A) and its absence in *C.tropicalis* (B)

3.2.2 Molecular Identification

Molecular characterisation is essential for the identification of organisms to the species level. These tests are quicker and less subjecive than biochemical tests.

3.2.2.1 *DNA Extraction*

Three methods were evaluated for the extraction of DNA from isolates.

3.2.2.1a *Phenol-Chloroform Extraction*

The extraction method is a modification from *Robert et al* (1995).

A loopful of pure yeast was inoculated in a 50ml centrifuge tube containing 3ml Sabouraud's Dextrose broth (SDB). This was incubated at 30°C in a shaker (150rpm) overnight.

One and a half millilitres of the overnight cell suspension was centrifuged at 900g for 5mins and the supernatant discarded. The pellet was washed with 500µl distilled water and centrifuged at 900g for 5mins, and the supernatant discarded. Five hundred microlitres of lysis buffer (500µl TE buffer + 100µl 10% SDS) was then added to the pellet, and incubated at 65°C for 30mins. Proteins were precipitated by the addition of 100µl 5M potassium acetate and kept on ice at 37°C for 1hr. The cell suspension was centrifuged at 12 000g for 10mins. The supernatant was treated with 100µl RNase A (10 mg/ml) at 37°C for 1hr to degrade any RNA present. Two phenol-chloroform-isoamyl alcohol extractions were performed, and the tubes were centrifuged each time at 16 000g for 5mins. The phenol denatured the proteins; the chloroform while also being a protein denaturant stabilised the boundary formed between the aqueous and phenol phase; and the isoamyl alcohol prevented the foaming of the mixture during vortexing and helped in the separation of the organic and aqueous phases. After this procedure, the DNA was present in the aqueous solution at the top of the tube, leaving the organic matter at the bottom and the

denatured proteins acting as the separating barrier between both phases.

The DNA was then precipitated with 5M NaCl adjusted to a concentration of 0.1M, completed with an equal volume of 100% ethanol and placed at –20°C for 1hr. This was centrifuged at 12 000g for 15mins. The DNA was washed twice with 1ml 70% ethanol and centrifuged at 20 800g for 3mins. The supernatant was then discarded and pellets were allowed to air dry. The DNA was resuspended in 100µl sterile water, and stored at -20°C until further use.

3.2.2.1b *Quick Extraction Method*

A loopful of pure yeast cells was resuspended in 1.5ml sterile water and boiled for 6mins. This was centrifuged at 20 800g for 4mins. The supernatant containing the DNA was transferred into a sterile microcentrifuge tube.

3.2.2.1c *Roche High Pure PCR Template Preparation Kit Method*

A loopful of pure yeast, inoculated in a 50ml centrifuge tube containing 3ml SDB, was incubated at 30°C in a shaker (150rpm) overnight.

Five hundred microlitres of cells was centrifuged at 3000g for 5mins, and the pellet resuspended in 185µl phosphate buffered saline (PBS). Ten microlitres of 10mg/ml

lyticase was then added, and this suspension was incubated for 30mins at 37°C. Binding buffer (200µl) and proteinase K (40µl) was added to the sample mixture, and was incubated at 72°C for 10mins. To this, 100µl isopropanol was added and vortexed briefly. The sample was then pipetted into the upper reservoir of a combined Filter Tube-Collection assembly, and centrifuged for 1min at 6000g.

The flowthrough and collection tube were discarded, and the filter tube was combined with a new collection tube. Inhibitor Removal Buffer (500µl) was added to the upper reservoir and the Filter Tube-Collection assembly was centrifuged at 6000g for 1min.

The flowthrough and collection tube were again discarded, and the filter tube was combined with a new collection tube. Wash Buffer (500µl) was added to the upper reservoir of this assembly, and centrifuged at 6000g for 1min. This step was repeated.

This time, only the flowthrough was discarded, and the filter tube was combined with the same collection tube. This assembly was centrifuged for 10s at 8000g to remove any residual Wash Buffer.

The collection tube was discarded and the filter tube inserted into a sterile 1.5ml microcentrifuge tube. Pre-warmed Elution Buffer (200µl at 70°C) was added to the filter tube and the centrifuged at 6000g for 1min.
The microcentrifuge tube now containing the eluted DNA was either used directly, or was stored at -20°C for further analysis.

3.2.2.2 *Multiplex Polymerase Chain Reaction*

Multiplex PCR allows for the exponential amplification of DNA with the use of more than one primer set, thereby permitting more than one DNA target to be amplified in a single reaction tube.

The reaction was set up as previously described by Li *et al.,* 2003. The reaction is based on the amplification of the internal transcribed spacer (ITS) regions 1 and 2, which are sequences of RNA in a primary transcript that lie between precursor ribosomal subunits. These are removed by splicing when the structural RNA precursor molecule is processed in a ribosome, and are coded by ribosomal DNA. ITS-1 is located between the 18S gene and the 5.8S gene, while ITS-2 is located between the 5.8S gene and the 28S gene.
The organisation of the primers' sequences (Table 2) within the ITS regions, specific for each organism is shown below, with ITS 4 acting as the universal reverse primer (Figure 3.4).

Table 2: Primer sequence and final concentration in multiplex PCR (Li *et al.*, 2003)

Primer	**Primer Sequence**	**Final Primer Concentration**
CA (*Candida albicans*)	TCAACTTGTCACACCAGATTATT	0.06μM
CP (*Candida parapsilosis*)	GGCGGAGTATAAACTAATGGATAG	0.2μM
CG (*Candida glabrata*)	CACGACTCGACACTTTCTAATT	0.06μM
CK (*Candida krusei*)	GATTTAGTACTACACTGCGTG	0.3μM
CT (*Candida tropicalis*)	AAGAATTTAACGTGGAAACTTA	0.2μM
ITS 4	TCCTCCGCTTATTGATATGC	0.12μM

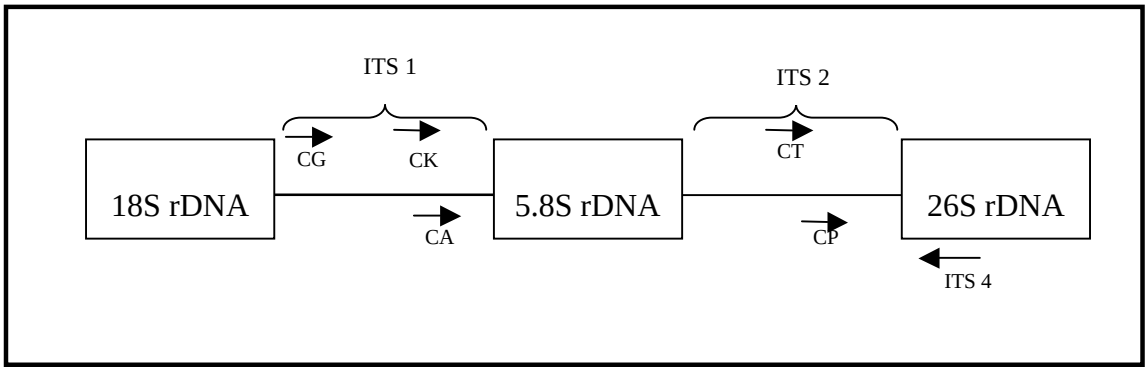


Figure 3.4: Schematic representation of the organisation of the primer sequences within the ITS regions

PCR reactions containing 1-10ng of yeast DNA, 12.5µl Roche 2x Master Mix and each primer at its final concentration (Li *et al.,* 2003) were made up to a final volume of 25µl with sterile distilled water. These reactions, all set up in a laminar flow hood using filtered tips, were performed in the iCycler PCR thermal cycler (Bio-Rad Laboratories Hercules, CA, USA). The reaction parameters used were according to Chang *et al.,* 2001 (Table 3).

Table 3: PCR reaction parameters (Chang *et al.,* 2001)

Step	**Temperature**	**Time**	**Number of Cycles**
Initial denaturation	94°C	3mins	1
Denaturation	94°C	1min	35
Annealing	60°C	1min	
Extension	72°C	1min	
Final Extension	72°C	5mins	1

In order to optimize the multiplex PCR reaction for identification of yeast isolates to species level, *C. albicans* DNA was used. The reaction yielded PCR product bands when run on a 2% agarose gel of the expected size (402bp). Increasing the $MgCl_2$ concentration from 1.5mM to 2mM and 5mM $MgCl_2$ resulted in an increase in the amount of product visualised (Lanes 3 and 5; Figure 3.5).

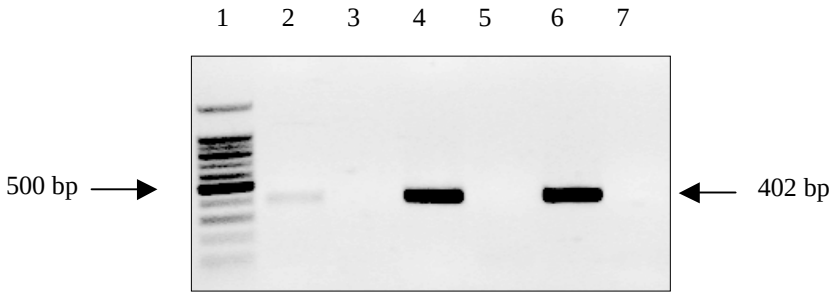


Figure 3.5: Optimization of PCR by titration of the $MgCl_2$ concentrations using *C. albicans* DNA. Lane 1: Molecular Marker; Lane 2: PCR Reaction with 1.5mM $MgCl_2$; Lane 3: Negative control; Lane 4: PCR Reaction with 2mM $MgCl_2$; Lane 5: Negative control; Lane 6: PCR Reaction with 5mM $MgCl_2$; Lane 7: Negative control

Since the difference in the amount of amplicon produced with reactions containing 2mM and 5mM $MgCl_2$ was indistinguishable visually (Figure 3.5), 2mM $MgCl_2$ was the concentration used in later multiplex PCR reactions.

3.2.2.2.1 *Detection of PCR Product*

Ten microlitres of the PCR product was loaded onto a 1% agarose gel (w/v) made with 1x TAE buffer and 3.75 x 10^{-4} μg/μl ethidium bromide. A commercially available molecular ladder was used as a marker. The fragments were separated by electrophoresis at 80V in 1x TAE buffer using a gel electrophoresis system (Amersham Pharmacia Biotech, Uppsala, Sweden). The separated fragments were then observed (BIORAD Gel Doc 1000 system,

Bio-Rad Laboratories Hercules, CA, USA). The expected PCR product size for each organism is shown in Table 4.

Table 4: Expected fragment sizes (Li *et al.*, 2003)

Organism	**Product Size**
Candida albicans	402bp
Candida parapsilosis	126bp
Candida glabrata	632bp
Candida krusei	475bp
Candida tropicalis	149bp

PCR products obtained after a multiplex PCR reaction using *C.albicans* DNA extracted by all three methods was run on a 2% agarose gel (Figure 3.6) to test the efficacy of each extraction method.

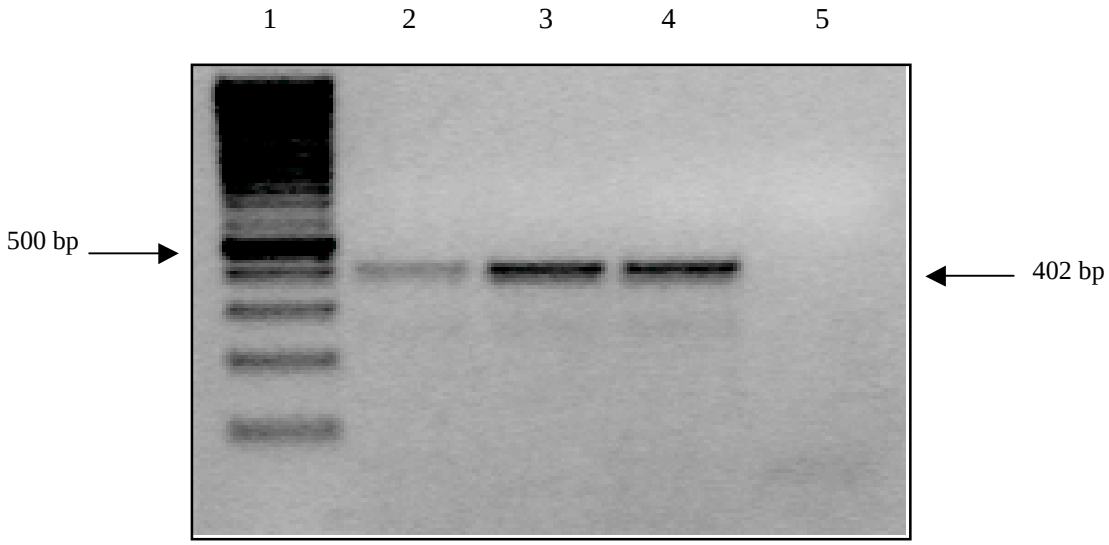


Figure 3.6: Testing the efficacy of three DNA extraction methods tested using *C.albicans*. Lane 1: Molecular Marker; Lane 2: DNA extracted using quick boiling; Lane 3: DNA extracted using phenol-chloroform; Lane 4: DNA extracted using the Roche kit; Lane 5: Negative control containing no DNA

Although all three extraction methods proved effective in extracting the DNA, as judged by the presence of the expected 402bp band, visually the quick-boiling DNA yielded the least amount of product. For subsequent PCR reactions, any of the three methods was used. The quick-boiling DNA extraction method was the method mostly used as it was quicker, cheaper, and only small quantities of DNA are required for genetic identification by PCR.

3.3 PREPARATION OF YEASTS FOR THE STUDY OF VIRULENCE TRAITS

After identification, each pure *Candida* isolate was streaked onto a SDA plate and incubated overnight at 36°C ± 1°C. A colony was then picked and inoculated into 10ml SDB supplemented with glucose at a final concentration of 8% to enhance the formation of biofilm in subsequent assays. This suspension was then incubated at 36°C ± 1°C for 18hrs.

One millilitre of each cell suspension was washed twice in PBS, centrifuged at 3400g for 2mins, and the pellets resuspended in 1ml 0.85% normal saline. The turbidity of each suspension was adjusted with SDB to the equivalent of 3 x 10^7CFU/ml, the recommended cell count (Shin *et al*., 2002) for determination of biofilm formation. This was achieved by comparative plate counts and spectrophotometric readings as follows:

Serial dilutions ranging from 10^{-1} to 10^{-6} were prepared for each sample and the absorbance at 600nm measured using a Jenway Model 6300 spectrophotometer (Jenway, Division of Barloworld Scientific, Stone, Staffordshire, England). The 10^{-2}, 10^{-4} and 10^{-6} dilutions for each sample were plated out on SDA plates and incubated overnight at 36°C ± 1°C. The colony forming units per ml were calculated as such:

CFU/ml = (number of colonies x dilution factor)/ volume (ml) spread on plate = Y

To get to the required 3 x 10^7CFU/ml cross multiplication was done i.e.

E.g. From calculation 1ml suspension = Y CFU/ml

$\therefore$ Xml suspension = 3 x 10^7CFU/ml

$\Rightarrow$ X = (3 x 10^7CFU/ml x 1ml) / Y CFU/ml

The absorbance of each *Candida* isolate was read at 600nm. As a blank, SDB with 8% final glucose concentration containing no yeast was used. The yeast concentrations were then adjusted to 3x10^7CFU/ml.

3.4 VIRULENCE ASSAYS

3.4.1 Adherence Assay

The assay used was a modification from that described previously by Samaranayake and McFarlane (1981).

$WHCO_6$ cells (established from biopsy in Prof R. Veale's Laboratory, Department of Molecular Cell Biology, University of the Witwatersrand) were cultured in a 3:1 mixture of DMEM:Ham's F_{12} medium (Sigma-Aldrich, South Africa), supplemented with 10% fetal calf serum (Highveld Biological, South Africa). The cultures were maintained in a humidified CO_2 incubator (Labotech, Forma Scientific, RSA.) at 37° C and 5% CO_2. The day prior to the assay, $WHCO_6$ cells were counted using a haemocytometer, and plated out

onto 3cm tissue culture dishes (Nalge Nunc International, Denmark) at 10^5 cells in 2ml per dish.

On the day of the assay, the cells were washed once with 1ml PBS, and inoculated with 1ml of the prepared yeast cells. These were incubated for 1 hour in a humidified CO_2 incubator (Labotec, Forma Scientific, RSA.) at 37° C and 5% CO_2, without agitation. The culture dishes were then washed with 1ml PBS for 30s and fixed with 2ml of 2% formalin in PBS for 15mins at room temperature under a laminar flow hood. The cells were then rinsed, air-dried and stained with Gram's safranin. The number of yeast cells adhering to 100 epithelial cells was counted under a light microscope.

3.4.2 Biofilm Assay

Production of biofilm, a major virulence factor, was assessed using a method described by Shin and co-workers (Shin *et al.*, 2002).

PolySorp plates were chosen for this assay as it has a hydrophobic surface that ensures better binding of lipids. Even though only 1%-7% of the yeast cell wall is lipid-based, this plate was the most suitable since cell surface hydrophobicity has been shown to play a major role in the adherence of *Candida* to host cells (Cotter and Kavanagh, 2000).

Twenty microlitres of each yeast suspension at 3 x 10^7CFU/ml was inoculated into the wells of PolySorp plates (Nalge Nunc International, Denmark). One hundred and eighty microlitres SDB was added into each well, and this was incubated at 36°C ± 1°C for 24h without agitation. Control wells with no yeast suspensions were also included.

Each well was then washed once with 200µl sterile distilled water to remove the non-adherent cells. Sterile distilled water (200µl) was then added to each well and the absorbance at 405nm was then read using an EL_x800 Universal microplate reader (BIOTEK Instruments, Inc., USA.). Percent transmittance (%T) was being recorded, and it was obtained as such:

Absorbance = $\log_{10}(1/T)$, where T= % Transmittance

The %T value of each yeast was subtracted from the %T value of the control wells to obtain the $\%T_{bloc}$ of each well i.e. the amount of light blocked when passing through the wells. This was done at each time point and for each isolate.

For each yeast isolate, this assay was performed in duplicate for each of the two time points (24hrs and 72hrs), on two different occasions.

3.4.3 Proliferation Assay

Proliferation of number of viable cells was determined since this gives an indication of the number of cells present with the potential to cause candidiasis. The greater this number, the greater the potential of the organisms to cause disease.

A standard colorimetric assay, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega), was used as per manufacturer's instructions. The CellTiter 96® AQ_{ueous} One Solution reagent contains a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-2(4-sulfophenyl)2-H-tetrazolium, inner salt; MTS, a tetrazolium compound and an electron coupling reagent, phenazine ethosulfate, PES. This solution is bioreduced by the cells into a coloured formazan product by metabolically active cells. This conversion is made possible by Nicotinamide Adenine Dinucleotide Phosphate (NADPH) or Nicotinamide Adenine Dinucleotide (NADH) produced by the dehydrogenase enzymes in the active cells.

Twenty microlitres of the CellTiter 96® AQ_{ueous} One Solution was pipetted into the wells of a 96-well microtitre plate. One hundred microlitres of each yeast suspension at 3 x 10^7CFU/ml was added into the wells. The plate was incubated for 1-4 hours at 36°C ± 1°C. The absorbance was then recorded using an EL_x800 Universal microplate reader (BIOTEK Instruments, Inc., USA) at 490nm to measure the amount of soluble formazan produced by cellular reduction of the MTS. Control wells were also included on the plate where only

SDB was added to the CellTiter 96® AQ_{ueous} One Solution. This was used to correct any background absorbance as well as to check for contamination. For each yeast isolate, this assay was performed in duplicate for each of the two time points (24hrs and 72hrs) on two different occasions.

3.5 ANTIFUNGAL SUSCEPTIBILITY TESTING

Antifungal susceptibility of each yeast isolate was performed for amphotericin B (Davies Diagnostics, South Africa) according to the M27-A method of June 1997 (guidelines of the Clinical and Laboratory Standards Institute (CLSI) formerly known as the National Committee for Clinical Laboratory Standards (NCCLS). The minimum inhibitory concentration (MIC) for each isolate was obtained with the broth microdilution method using the microtitre plate method.

Amphotericin B was diluted in dimethyl sulphoxide (DMSO) to a concentration of 3200µg/ml. This was serially diluted (1/10) to a final concentration of 0.03µg/ml. A 100µl volume of each dilution was inoculated in wells 1-11 of a flat bottom microtitre plate. To each of these dilutions, 100µl of 0.5-2.5 x 10^3 CFU/ml of each yeast isolate was added. A positive control i.e. “yeast with no amphotericin B” was added into well 11 of the microtitre plate, and a negative control i.e. “RPMI with no yeast” was added into well 12.

The plates were then incubated at 36°C ± 1°C for 48hrs, and evaluated visually, by eye, observing the presence or absence of visible growth. The MIC was defined as the lowest concentration of amphotericin B that prevented all visible growth, when compared to positive controls. As a technical control, *C.albicans* ATCC 90028 was used.

3.6 EXPOSURE OF *CANDIDA ALBICANS* TO NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

3.6.1 Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

The NRTIs currently recommended for use in South Africa (Simelela, 2004) are didanosine (ddI), lamivudine (3TC), stavudine (d4T) and zidovudine (AZT). The ARVs used in this project were kindly donated by Ranbaxy Laboratories Ltd. (Pharma Manufacturing, Dewas, India).

3.6.2 Collection and Sample Identification of Patient Isolates

Oral swabs were collected from antiretroviral-naïve patients attending the HIV clinic of the Johannesburg Hospital, South Africa, prior to initiation of antiretroviral therapy. Patient attendance at this clinic is by referral, and patients attending this clinic are World Health Organisation (WHO) Stage IV HIV/AIDS individuals, or have CD_4+ counts <200cells/mm^3.

Forty-five oral samples collected using sterile cotton swabs from the tongue and/or palate of patients, were plated out on SDA containing 0.005% chloramphenicol within 3hrs of collection, and incubated at 36°C ± 1°C for 48 hours. All yeast isolates were identified using the microbiological and molecular identification techniques described in Section **3.2.**

3.6.3 Virulence Assays and Antifungal Susceptibility Testing

All isolates were prepared as described in Section **3.3**.

Prior to virulence and antifungal susceptibility assays, all isolates were exposed to the anticipated *in vivo* peak concentration (Goodman & Gilman's, 2001), double the peak concentration and half the peak concentration of each NRTI in Sabouraud's Dextrose broth for 24 and 72hrs (Table 5).

Table 5: Concentrations of NRTIs used

Concentration/ARV	ddI	3TC	Stavudine	AZT
_ Peak Concentration	1.05µg/ml	0.5µg/ml	0.6µg/ml	0.8µg/ml
Peak Concentration	2.1µg/ml	1.0µg/ml	1.2µg/ml	1.6µg/ml
2x Peak Concentration	4.2µg/ml	2.0µg/ml	2.4µg/ml	3.2µg/ml

In the case of the biofilm assay, all NRTIs (at the abovementioned concentrations) were added to the yeast cells before inoculation in PolySorp microtitre plates (Nalge Nunc International). Unless otherwise stated, adherence, biofilm, proliferation and antifungal susceptibility assays were performed as described in Sections **3.4.1**, **3.4.2, 3.4.3** and **3.5** respectively. In all assays *Candida* isolates grown in the absence of NRTIs were used as controls.

3.7 STATISCAL ANALYSES

3.7.1 Determination of Sample Size

In consultation with a biostatistician, Dr P. Becker (Biostatistics Unit, Medical Research Council, Pretoria, South Africa), a minimum sample size of 20 patients was decided upon as there would be adequate degrees of freedom for testing the hypotheses of interest using the appropriate analysis of variance (ANOVA).

3.7.2 Analyses of the Results

An analysis of variance was performed using the software Statistix 8.0. A cross-sectional time-series regression model with random effects was employed.

CHAPTER 4: RESULTS

4.1 IDENTIFICATION

Techniques were established and validated using the control yeasts *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258), and a clinical isolate of *Candida parapsilosis* (Figure 4.1) as described in Chapter 3, Section 3.2.

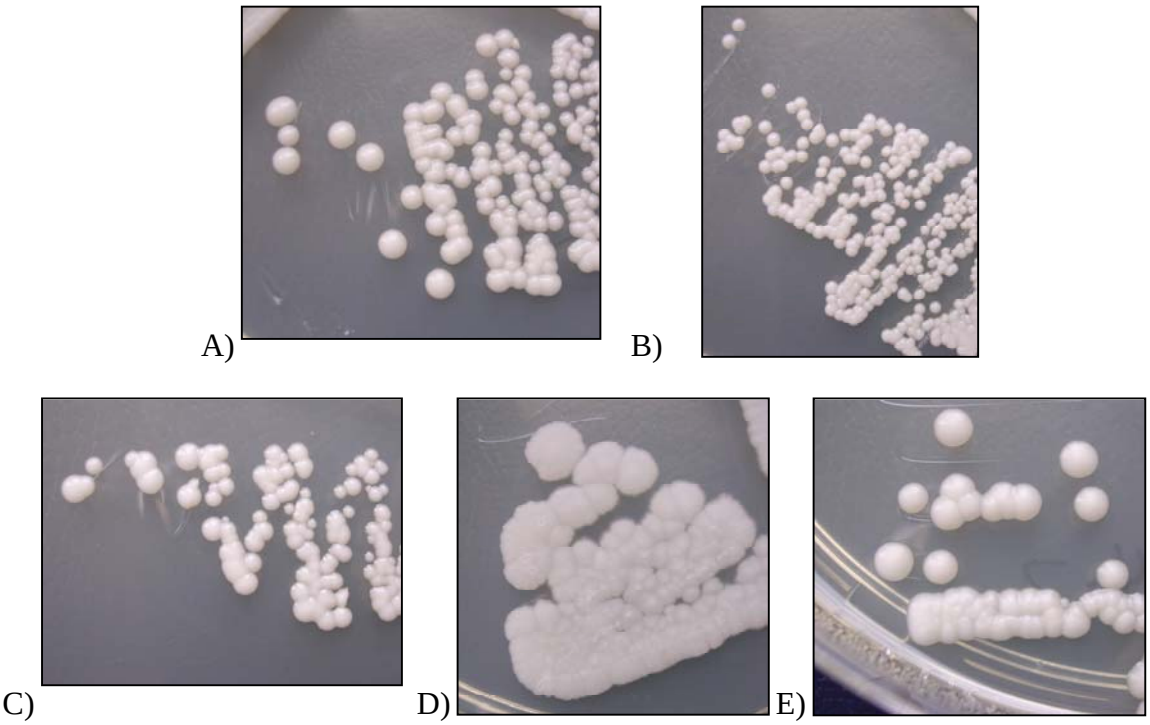


Figure 4.1: SDA plate showing the characteristic colonial morphologies of *C. albicans* (A), *C. parapsilosis* (B), *C. glabrata* (C), *C. krusei* (D), and *C. tropicalis* (E).

Gram-stains and PAS stains revealed the characteristic phenotypes of yeast (Figures 3.1&3.2).

Multiplex PCR reactions set up to confirm the species of each control strain showed the expected band size for each control strain (Table 4) (Figure 4.2).

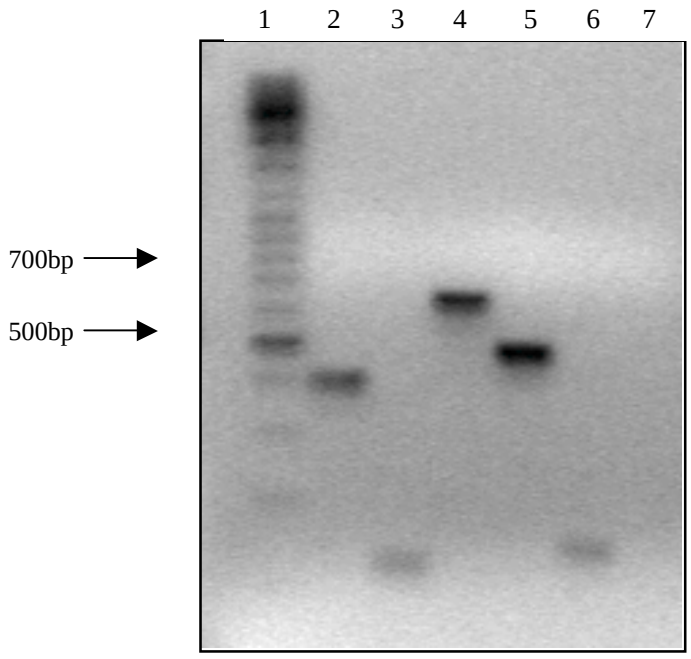


Figure 4.2: A 2% agarose gel showing products of a multiplex PCR of each control yeast; Lane 1: Molecular Marker; Lane 2: *C. albicans* - 402bp, Lane 3: *C. parapsilosis* - 126bp, Lane 4: *C. glabrata*- 632bp, Lane 5: *C. krusei* - 475bp, Lane 6: *C. tropicalis* - 149bp, Lane 7: No DNA control.

Of the forty-five oral swabs collected from HIV-positive patients attending the antiretroviral (ARV) clinic of the Johannesburg Hospital, South Africa, prior to initiation of antiretroviral therapy, thirty nine of the isolates were identified as *C. albicans*, while six of the isolates remained unidentified. It is possible that these were either *C. albicans* isolates that did not have the IS1 genotype (Blignaut *et al.* 2002(b)), or *Candida dubliniesis* since the isolates produced true hyphae but were not identified *C. albicans* using the multiplex PCR. Since *C. dubliniensis* primers were not used in the multiplex PCR reaction, this was not conclusively proven.

4.2 PREPARATION OF YEASTS FOR THE STUDY OF VIRULENCE TRAITS

The absorbance of each control broth (Table 6) was read at 600nm.

Table 6: Absorbance of each control strain at 600nm

Control Strain	Absorbance at 600nm
Candida albicans (ATCC 90028)	1.370
Candida parapsilosis (JHB Hospital outbreak)	0.741
Candida glabrata (ATCC 90030)	1.113
Candida krusei (ATCC 6258)	0.868

The number of colonies produced after plating 10^{-2}, 10^{-4}, and 10^{-6} serial dilutions of each yeast onto SDA were counted (Table 7).

Table 7: Number of colonies obtained with each serial dilution

	Number of colonies		
Control Strain	**10^{-2} dilution**	**10^{-4} dilution**	**10^{-6} dilution**
C. albicans (ATCC 90028)	Too many to count	12	0
C. parapsilosis (JHB Hospital outbreak)	Too many to count	11	0
C. glabrata (ATCC 90030)	Too many to count	19	0
C. krusei (ATCC 6258)	Too many to count	12	0

Using the 10^{-4} dilutions, the colony forming unit per ml (CFU/ml) for each yeast was obtained (Table 8).

Table 8: CFU/ml of each control strain

Control Strain	**CFU/ml**
C. albicans (ATCC 90028)	1.2×10^8
C. parapsilosis (JHB Hospital outbreak)	1.1×10^8
C. glabrata (ATCC 90030)	1.9×10^8
C. krusei (ATCC 6258)	1.2×10^8

For subsequent assays, each cell suspension was adjusted to 3×10^7 CFU/ml. This was achieved using the values in Table 8 as described in Section 3.3.

After a 3 x 10^7CFU/ml cell suspension of each patient isolate was obtained, the cells were grown in the anticipated *in vivo* peak concentration, double the peak concentration and half the peak concentration of ddI, 3TC, stavudine and AZT for 24 and 72 hours, in duplicate and on two different occasions.

After each time period, the virulence traits of biofilm formation, proliferation, and adherence to epithelial cells were tested, to assess the effects of each NRTI.

4.3 VIRULENCE FACTORS ASSAYS

The assays were validated using control yeasts. Comparisons of growth at different concentrations of NRTIs and for different time intervals were with respect to growth in the absence of NRTIs, which was used as the baseline.

The results obtained for all assays performed in the presence of NRTIs are tabulated in Appendix F. The summary of the total effects of each NRTI at each concentration are represented as figures.

4.3.1 Adherence Assay

This assay was only performed on clinical strains as described in Section 3.4.1. The number of adherent yeast cells to 100 fixed oesophageal cells was counted microscopically for 29 of the 39 *C. albicans* isolates (Figure 4.3) due to delays in obtaining reagents from the respective manufacturers.

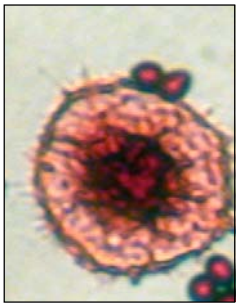


Figure 4.3: Example of two yeast cells adhering to an epithelial cell at 400x magnification.

The effect of each NRTI on the adherence of the yeast is represented diagrammatically below (Figure 4.4). The values of the isolates exposed at each time point was compared to the value of the controls at that time point. At all three concentrations of ddI, 3TC, d4T and AZT, the mean number of yeast cells adhering to 100 epithelial cells did not differ significantly from that grown in NRTI-free medium ($p>0.05$).

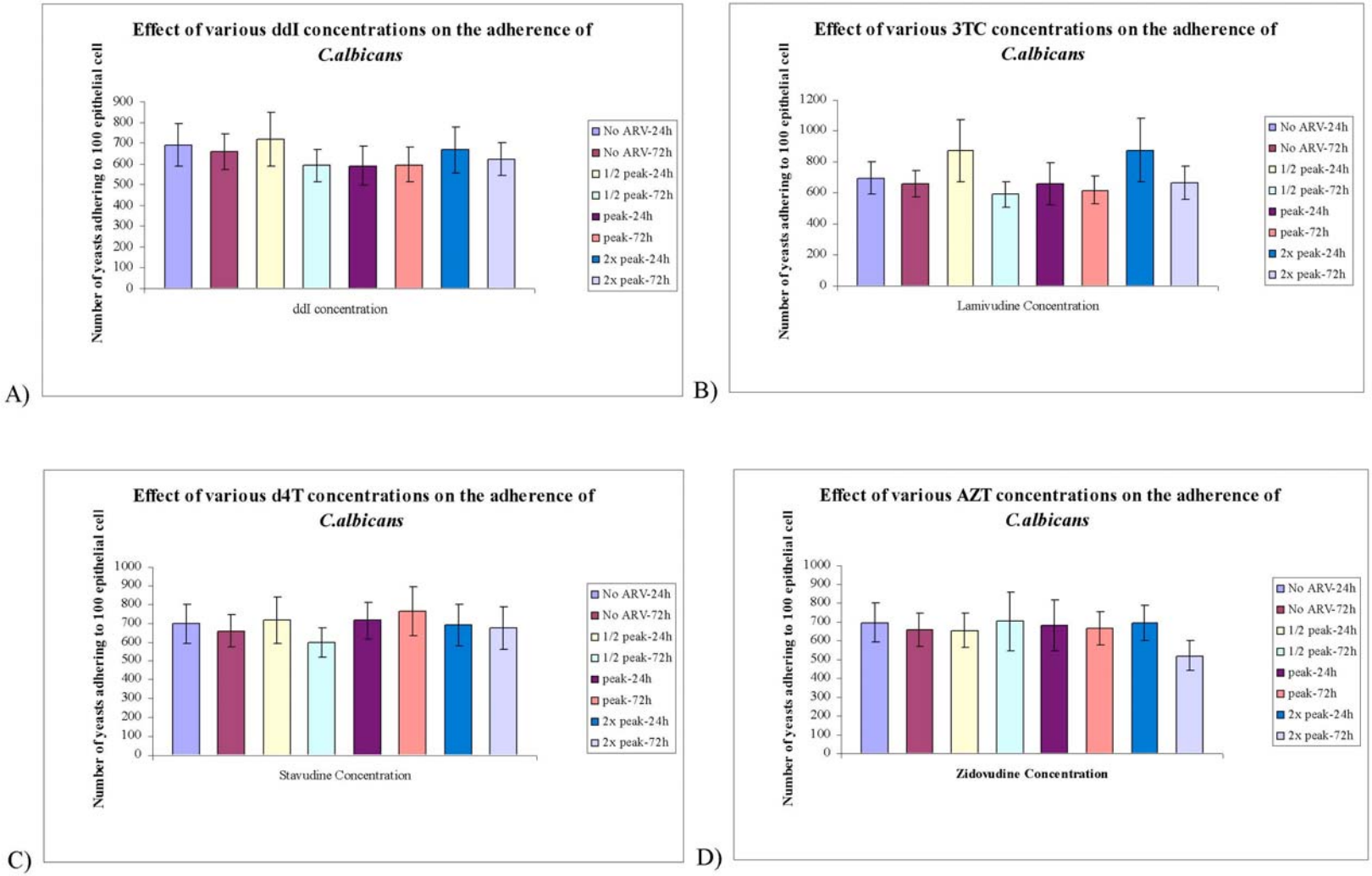


Figure 4.4: Effects of different NRTI concentrations on the adherence of *C.albicans* isolates collected from HIV-positive patients prior to initiation of HAART to oesophageal cells. The error bars represent the standard error of the mean (SEM). The number of yeast cells adhering to epithelial cells was obtained once at each time point (please refer to page **53**)

4.3.2 Biofilm Assay

This assay was performed as described in Section 3.4.2.

To establish this assay, control yeasts were used. The $\%T_{bloc}$ for each yeast was reproducible on both occasions, as shown by the error bars which represented standard errors of the means (Figure 4.5). The amount of biofilm formed differed between *Candida* species. *C. albicans* produced the least amount of biofilm, followed by *C. krusei,* and *C. glabrata,* while *C. parapsilosis* produced the greatest amount of biofilm.

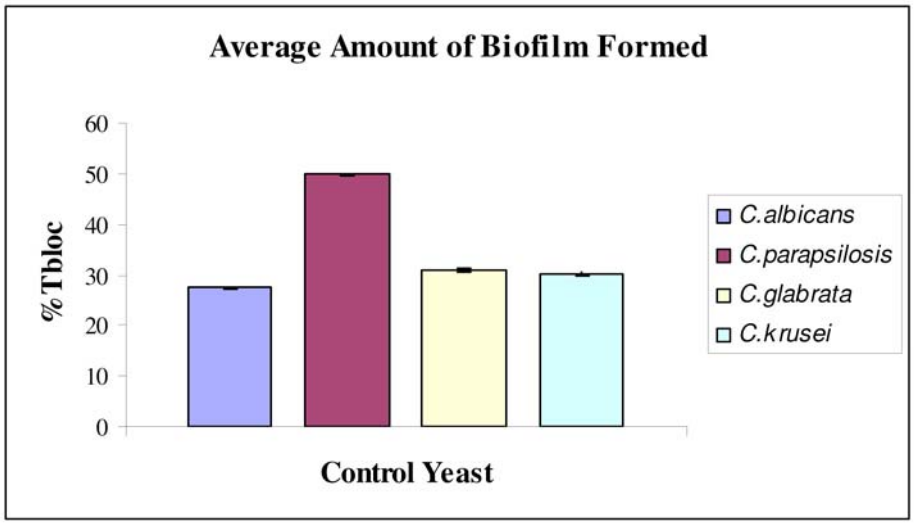


Figure 4.5: Biofilm formation of control strains as assessed by calculating $\%T_{bloc}$ using the absorbances obtained for each stain at 405nm. Error bars represent the standard error of the means.

The extent of biofilm formation was then measured spectrophotometrically for only 29 of the 39 clinical isolates (Figure 4.6) due to delays in obtaining reagents from the respective manufacturers. All values obtained were differences from zero i.e. the baseline, since the $\%T_{bloc}$ calculation took into account the yeasts grown in the absence of NRTIs. This calculation gives an indication of the amount of biofilm formed upon treatment with NRTIs, such that only the net effect of exposure to the drug would be determined. The "No ARV" baseline was obtained separately for each isolate exposed to each NRTI at each time points. Hence, negative values indicate that the isolates produced less biofilm when exposed to NRTIs, while positive values indicate that isolates produced more biofilm upon exposure to NRTIs than the control.

The results (Figure 4.6) showed no significant difference in the extent of biofilm formation of the yeasts upon exposure to each NRTI concentration ($p>0.05$). This large variation observed (error bars) could be as a result of the fact that the analysis took into account all *C. albicans* isolates. However, each patient isolate may have produced different amounts of biofilm. As a result, it was concluded that the ARVs had no effect on this virulence factor.

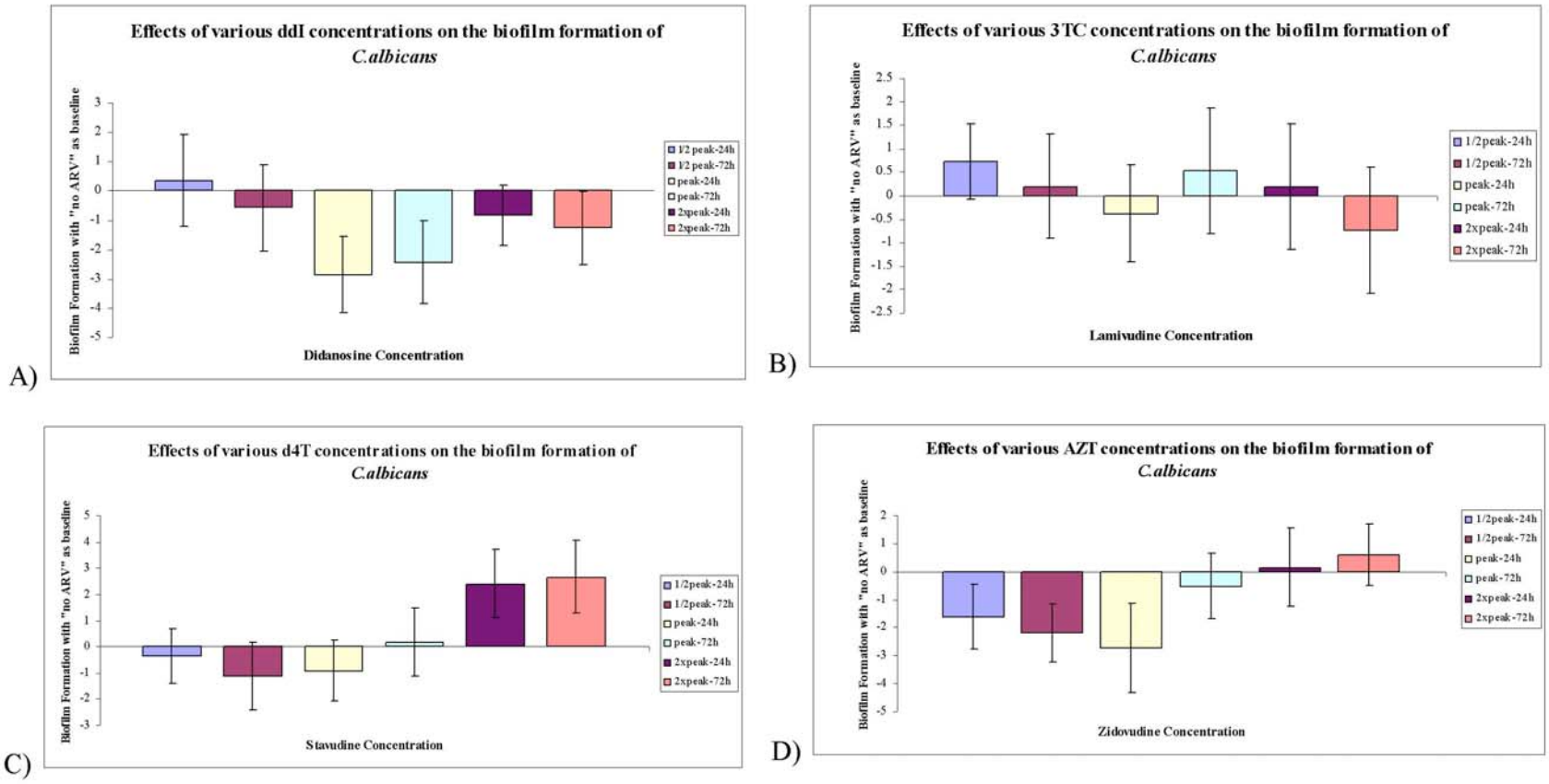


Figure 4.6: The effects of different NRTI concentrations on the extent of biofilm formation of *C.albicans* clinical strains collected from HIV-positive patients prior to initiation of HAART. The error bars represent the standard error of the mean (SEM).
The amount biofilm formed by each isolate was obtained in duplicate, on two different occasions, for each time point (please refer to page **63**)

4.3.3 Proliferation Assay

This assay was performed as described in Section 3.4.3.

To establish this assay, the coloured formazan product formed by metabolically active cells was detected for each control yeast strain. *C. parapsilosis* produced more viable cells than *C. krusei*, which in turn had more viable cells than *C. glabrata*. *C. albicans* was the control yeast that produced the least number of viable cells. This assay, performed in duplicate on six different occasions, was reproducible as observed by the small error bars (Figure 4.7).

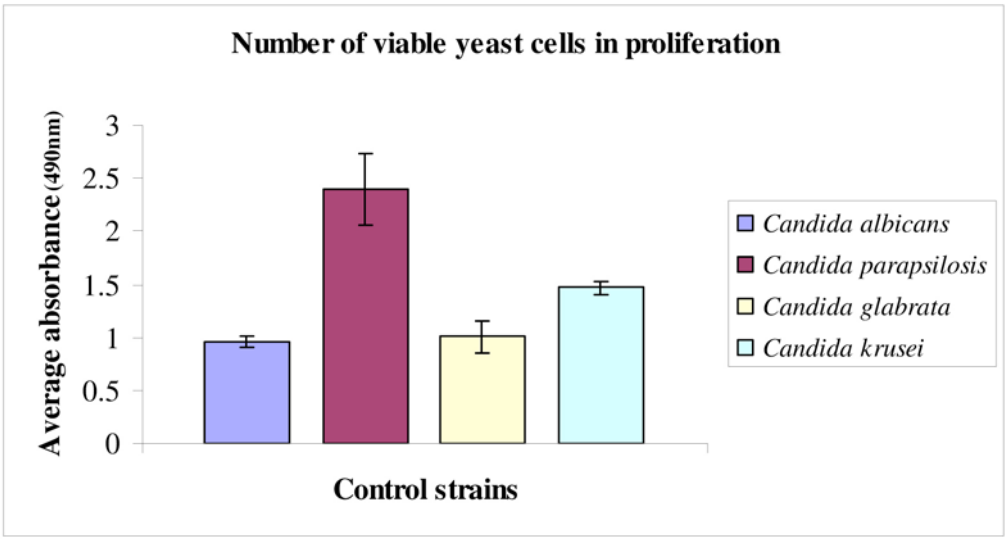


Figure 4.7: Number of viable control yeast cells as assessed spectrophotometrically at 490nm using a colorimetric assay. Error bars represent the standard error of the means (SEM).

This colorimetric assay was then used to determine the number of viable cells of 23 clinical isolates when exposed to NRTIs (Figure 4.8). Of the 39 *C. albicans* isolates, 23 were used in this assay due to delays in obtaining reagents from the respective manufacturers.

At all three concentrations of the NRTIs ddI, d4T and AZT, the rate of proliferation of *C. albicans* did not differ significantly from that yeast grown in medium devoid of ARV (Figure 4.8 A, C, D). A significant increase in the rate of proliferation was however observed when *C. albicans* isolates were grown in double the peak concentration of 3TC recommended for an adult HIV-positive individual ($p<0.001$) (Figure 4.8 B), at both time points.

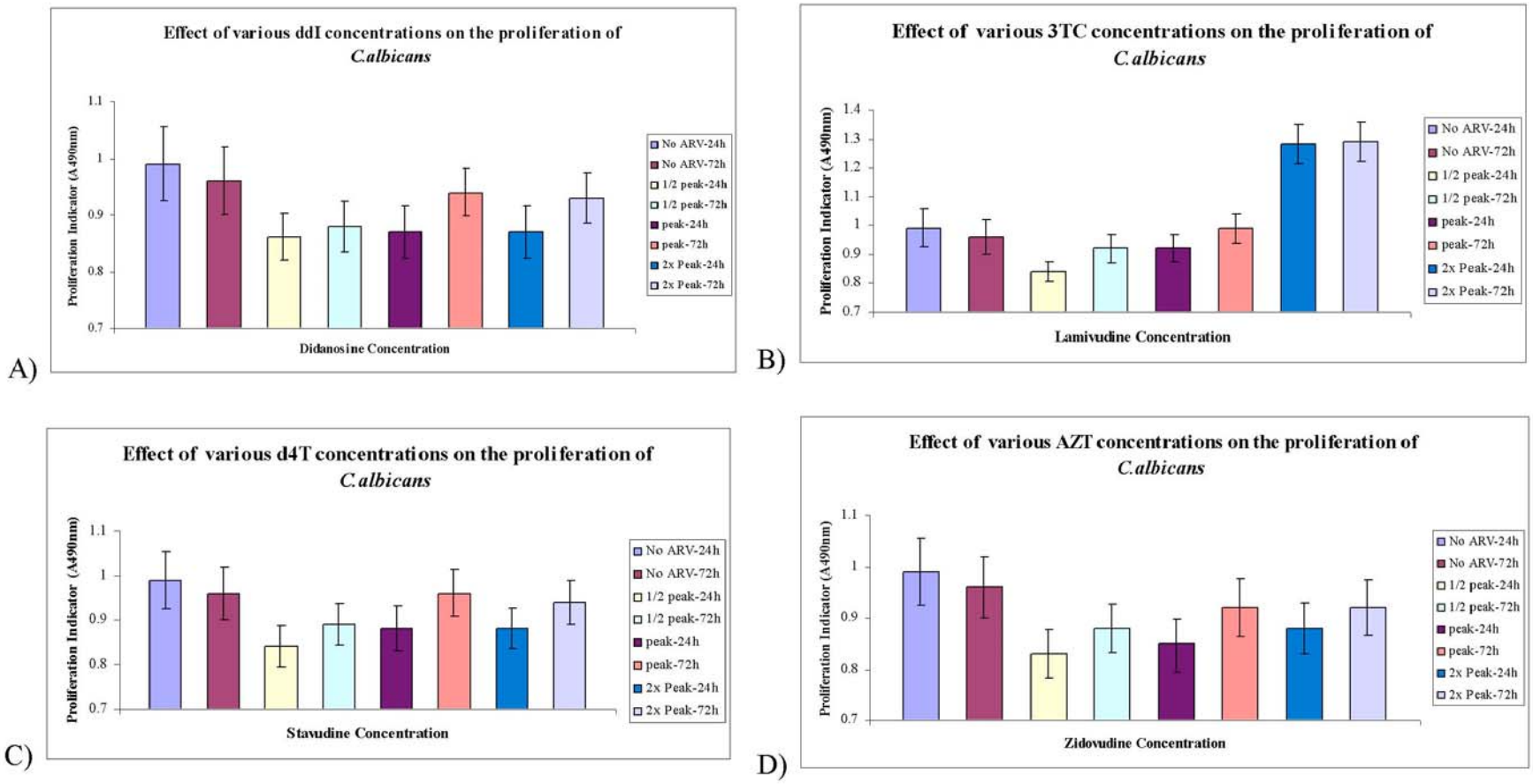


Figure 4.8: The effects of different NRTI concentrations on the rate of proliferation of *C.albicans* isolates collected from HIV-positive patients prior to initiation of HAART. The error bars represent the standard error of the mean (SEM).
For each isolate, the number of viable cells was obtained in duplicate and on two different occasions, for each time point.
(please refer to page **66**)

4.5 ANTIFUNGAL SUSCEPTIBILITY ASSAY

C. albicans ATCC 90028 was used to establish the antifungal susceptibility assay in the laboratory. This test performed in duplicate on two separate occasions yielded an MIC of 1µg/ml of amphotericin B. This value indicates that the yeast was susceptible to amphotericin B. The MIC guidelines established by the CLSI indicate that MIC range of sensitive *Candida* to amphotericin B is 0.5-1µg/ml.

Thirty-one isolates were chosen for this assay because those were the isolates used in the virulence assays. The MICs obtained for these patient isolates were the same for each of two occasions tested. Growth of isolates in media containing each NRTI at all three concentrations for 24hrs and 72hrs yielded identical MICs of 1µg/ml of amphotericin B, when compared to the yeasts grown in media devoid of ARVs. Thus, in our clinical isolates, NRTIs had no effect on the susceptibility of *C. albicans* to amphotericin B.

CHAPTER 5: DISCUSSION

The opportunistic organism *Candida albicans* is known to cause both localised and systemic infections in humans (Haynes, 2001). Globally, *C. albicans* remains the principal causative agent of oral thrush (Spencer, 2005). The worldwide trend shows that approximately 90% of all HIV-positive individuals develop oral candidiasis at least once during the course of their disease, progression from HIV-positive to full blown AIDS (Repitigny *et al.*, 2004). A large study performed using *Candida* isolates obtained from the oral cavities of 339 HIV-positive individuals attending three comprehensive care AIDS clinics in Pretoria and GaRankuwa, Gauteng Province, South Africa showed that approximately 90% of these isolates were identified as *C. albicans* (Blignaut *et al.*, 2002). These results were similar to those obtained in Thailand where *C. albicans* was the most recovered *Candida* sp. in HIV-positive individuals (96.67%) (Teanpaisan & Nittayananta, 1998). Another study performed in South Africa found the overall *Candida* carrier rate (81.3%) in HIV-positive individuals in South Africa to be higher than in Italy (61.9%) and Thailand (66.67%) (Campisi *et al.*, 2002; Teanpaisan & Nittayananta, 1998). The *C. albicans* carriage was however lower at 78.6% in South Africa when compared to 96.67% in Thailand (Patel *et al.*, 2003; Teanpaisan & Nittayananta, 1998).

To answer the question of whether HIV nucleoside analogue drugs have a similar effect as 5-FU on the virulence of *Candida albicans*, we collected *Candida* isolates from the oral cavities of ARV-naïve HIV-positive individuals attending the HIV Clinic of the Johannesburg Hospital, Gauteng Province, South Africa. Identification of these isolates

showed that out of 45 *Candida* isolates collected, 39 were *C. albicans* (87%), while 6 isolates (13%) were considered to be either *C. albicans* isolates that did not have the IS1 genotype (Blignaut *et al.* 2002(b)) or *C. dubliniensis* by a process of elimination. Although the sample size is small, these results are similar to Patel and co-workers' (2003) where out of 173 isolates obtained from HIV-positive individuals attending two ARV clinics in the same province, 78.6% were identified as *C. albicans*, and whilst 6.3% were *C. dubliniensis*.

It is generally accepted that various molecules may have an effect on the virulence of pathogenic organisms. Some of these molecules increase the virulence of these organisms, whilst others, such as non-steroidal anti-inflammatory drugs decrease their pathogenicity (Alem and Douglas, 2004). This is no exception with *C. albicans*. In 2001, Ueta and colleagues demonstrated that 5-fluorouracil, a cancer nucleoside analogue, increased the virulence of two *C. albicans* isolates *in vitro*. To assess the effects of HIV/AIDS nucleoside analogue drugs on the virulence of the *C. albicans*, isolates collected were grown in media containing didanosine (ddI), lamivudine (3TC), stavudine (d4T) and zidovudine (AZT). Biofilm, adherence and proliferation assays were then performed to determine the effects of these drugs on the virulence factors adherence, biofilm formation and proliferation. In addition, an antifungal susceptibility assay was also used to determine what effect the above drugs would have on the susceptibility of these yeasts to an antifungal agent. All isolates were assumed to be genetically different since they were obtained from different patients. Due to cost and time limitations, fingerprinting was not performed to confirm this.

Adherence Assay

Adhesion of microorganisms to host cells is the first step of pathogenesis (Sundstrom, 2002).

To determine the effect of HIV nucleoside analogues on the adherence of *C. albicans* to epithelial cells, 29 isolates grown in the presence of each NRTI were allowed to adhere to oesophageal cells *in vitro*. The mean number of adherent yeasts to 100 oesophageal cells was comparable for the isolates grown in NRTI-free media and those grown in the presence of NRTIs at all three concentrations (half the peak, the peak and double the peak concentrations recommended for an AIDS patient) ($p>0.05$). Previous experimental data has attributed increased adhesion of *C. albicans* to epithelial cells to a 4-fold increase in the binding activity of concanavalin A (a mannose residue) (Ueta *et al.*, 2001). It is possible that in this case, the NRTIs did not promote the expression of molecules that bind to mannose residues or other molecules involved in adherence. Since the epithelial cells used in this study were obtained from a biopsy, cells should express adherence receptors similar to the *in vivo* situation. However, immortalisation of the cell line and passaging may have had an effect on this expression. The most likely explanation for the results obtained in this study is that the NRTIs had no effect on mechanisms that trigger the virulence trait of adherence in the system used.

Contradictory data have been reported with regard to differences in adherence properties of *Candida* isolates, from both HIV-positive and HIV-negative individuals, to buccal epithelial cells (BECs). Enhanced adherence of HIV-positive *C. albicans* to BECs (Sweet

et al., 1995), less adherence of *C. albicans* from patients in the initial AIDS stages to BECs Pereiro *et al.*, 1997), as well as similar adherence of *C. albicans* from HIV-positive and HIV-negative to BECs (Tsang *et al.*, 1999) have been reported. In HIV-positive individuals an increase in the rate of *C. albicans* carriage and frequency of oral candidiasis has also been observed (Jin *et al.,* 2003). Jin and co-workers (2003) suggested that this was as a result of the compromised immune systems and, possible alterations in the oral environment and mucosal cells. It seems that the quality and quantity of saliva in HIV-positive individuals during the course of their disease affects adherence and colonization of the yeast to the BECs. Saliva is made up of components such as lysozyme and histatins which have anti-candidal properties (Samaranayake *et al.,* 2002). HIV infection has been noted to have an effect on these components (Samaranayake *et al.* 2002). It seems that factors such as the inability of some of these proteins to interact with *C.albicans* may play a role in the reduced anti-candidal effect of saliva in HIV-positive individuals (Repitigny *et al.*, 2004). In this study, adhesion was assessed in the absence of the normal oral environment (i.e. no saliva), and it is likely that alterations in the oral environment of immunocompromised individuals plays a significant role in the increased carriage and oral thrush observed which was not seen in the *in vitro* system**.** It would be interesting to conduct the adherence experiments in an environment similar to that of the oral cavity in order to assess whether or not NRTIs affect components such as histatins to increase their antifungal activity.

Biofilm Assay

Biofilms are responsible for a large number of infections that afflict humans (Douglas, 2003). The ability of *Candida* to produce biofilms is believed to be an important virulence trait. These *Candida* biofilms show increased resistance to antifungal therapy and the cells within the polymeric matrix can resist the host's immune system (Shin *et al.*, 2002).

In this study the average amount of biofilm produced by each control *Candida* strain was in keeping with observations made by previous workers, in that *C. parapsilosis* formed the most biofilm followed by *C. glabrata* and then by *C.albicans* in SDB medium containing 8% glucose (Shin *et al.,* 2001). Based on these observations, and the small standard deviations obtained for repeat experiments, it was concluded that the experimental system was adequate and could be used for clinical isolates. The results also showed that *C. albicans* produced biofilm in the presence of glucose-rich media, as seen previously (Jin *et al.,* 2003).

To determine the effect of the HIV nucleoside analogues on biofilm formation, 29 *C. albicans* isolates were grown in the presence of the NRTIs at various concentrations. When the amount of biofilm produced was compared to that produced when the isolates were grown in media devoid of the NRTIs, no observable significant difference was noted ($p>0.05$). The difference between the two time points was still not statistically different, as *C. albicans* grown for longer periods of time, i.e. 72hrs as opposed to 24hrs, did not make any difference in the amount of biofilm produced ($p>0.05$).

These results differ from others (Tsang *et al.,* 1999; Jin *et al.*, 2003), where statistical models correlated increases in biofilm formation with exposure to AZT. The difference could be attributed to the fact that another *in vitro* system was used. However, because regression studies linked a higher degree of biofilm formation of *C. albicans* with exposure to AZT (Jin *et al.*, 2003), it would be interesting to study the effect of each NRTI on the biofilm producing ability of *C. albicans* in an *in vitro* environment that more closely mimics the *in vivo* situation. It may also be interesting to study the effect of NRTIs on *C. albicans* quorum sensing in a more physiological biofilm model.

Proliferation Assay

The ability of an organism to proliferate is of importance to the disease process. The greater the number of cells produced, the higher the chance the organism may cause disease (in the presence of other virulence factors). In immunocompromised individuals, *Candida* cells proliferate easily because, amongst others, the *Candida*-killing activity of phagocytes is suppressed (Ueta *et al.*, 2001).

Experiments with *Candida* control strains showed that the assay was able to detect proliferation over time, with little variation seen between replicates and repeat experiments.

Previous experimental data have revealed increases in *C. albicans* proliferation upon exposure to 5-FU (Ueta *et al.*, 2001). The authors suggested that it was likely that the

multiplying cells were less sensitive to 5-FU, with the more sensitive ones being killed by the drug. Upon treatment of *C. albicans* with ddI, d4T, and AZT, no significant difference in the number of proliferating viable cells was observed when compared to the yeasts grown in the absence of these drugs ($p>0.05$). However, when the isolates were grown in double the anticipated peak concentration of 3TC in an adult HIV/AIDS patient, at both 24hrs and 72hrs, an increase in the number of viable cells was noticed ($p<0.001$). The observations made by Ueta and co-workers (2001) indicated that 5-FU either potentiates the virulence of *Candida* cells or they eliminate the cells of low virulence thereby increasing the risk of oral and systemic candidiasis. In contrast, results in this study seem to suggest that only potentiation of *Candida* cell virulence is responsible for increases in proliferation since 3TC has no anti-fungal activity *per se*, as it as a nucleoside reverse transcriptase inhibitor.

3TC differs from the other NRTIs used in this study in that it is a cytidine analogue. This agent seems to act similarly to 5-FU which also increases proliferation of *C. albicans* in a dose-dependent manner (Ueta *et al.*, 2001). The mechanism of action by which 5-FU prevents DNA strand elongation is comparable to that of other anti-cancer cytidine nucleoside analogue drugs, where the phosphorylated drugs get incorporated into the DNA and subsequently prevent DNA strand elongation (Damaraju *et al.*, 2003). Since the main difference between the two kinases responsible for this (thymidine kinase for 5-FU and cytidine kinase for 3TC) is the presence of a 2'–OH group on the pentose of the cytidine residue which is absent on pentose of the thymine residue, it is suggested that anti-cancer cytidine nucleoside analogue drugs increase the virulence of *Candida* cells in a similar manner to 5-FU.

5-FU has been reported to stimulate the proliferation of eukaryotic cells by activating a signal-transduction pathway (Wu *et al*, 1998). Vital processes such as DNA replication and mitosis are triggered by a cell-cycle control system. This system is made up of two phases (S and M) and two checkpoints (G_1 and G_2). In the G_1 phase the cells grow to an appropriate size where they are large enough to enter the cell cycle. At this checkpoint the cell checks whether the environment is favourable for cell proliferation. If it is, the cell proceeds to the S phase. Otherwise, the cells go into a stationary phase known as START or G_0 (Alberts *et al.*, 1998).

In the S-phase, the DNA replication machinery is set into motion, and the DNA is replicated. After this, the cell proceeds to the G_2 checkpoint where the cell ensures that DNA replication is complete. If this is complete, the cells proceed into the M phase where the cell undergoes mitosis. The G_1 and G_2 checkpoints allow the cell system to be regulated by signals from other cells, e.g. growth factors, as well as other extracellular signal molecules which promote or inhibit cell proliferation (Alberts *et al.*, 1998). Extracellular signals that stimulate cell proliferation lead to the activation of G_1 cyclin-dependent protein kinases complexes. These then phosphorylate the *Retinoblastoma* (Rb) protein, thus changing its conformation. This change results in the release of bound gene regulatory proteins which then activate the genes responsible for cell proliferation. These extracellular signals override the signals that stop cell proliferation (Alberts *et al.*, 1998).

Wu and colleagues (1998) have demonstrated that addition of 5-FU to 5-FU-resistant human cells results in an increase in the amount of mitogen-activating protein (MAP) kinases produced. Filamentation and *C .albicans* virulence are regulated by two-parallel

signalling cascades, one of which is through the MAP-kinase pathway (Lengler *et al.*, 2000). Given that 3TC is a cytidine analogue, it is not implausible that this drug may act in a similar manner to 5-FU, and activates the MAP-kinase signalling pathway, thereby acting as an extracellular signal stimulating cell proliferation. Because cytidine has been found to induce the production of uridine phosphorylase and thymidine phosphorylase, two key enzymes in DNA replication (Vita *et al.*, 1983), it is also possible that 3TC induces production of these enzymes, which leads to more DNA replication.

On the other hand, because resistance of eukaryotic cells to 5-FU has mainly been attributed to over-expression of thymidylate synthase (Marsh, 2005), this over-expression might also be responsible for the increases in the amount of MAP-kinases produced when 5-FU is added to resistant cells. As such, if 3TC induces over-expression of thymidylate synthase, the MAP-signaling pathway may be upregulated, and therefore increase proliferation.

Antifungal Susceptibility

Since the 1980s an increasing trend of fungal infections has been observed. This has been attributed to the increase in the number of immunosuppressed individuals, especially those infected with HIV (Espinel-Ingroff *et al.*, 1999). As a result of this, a wide variety of antifungal drugs have been developed to combat these infections by targeting various components of the fungal cell. Amphotericin B is an established antifungal agent, with broad spectrum activity, that binds to ergosterol resulting in membrane damage. It is used

for the treatment of invasive fungal infections when conventional therapy (with azoles) has failed (Espinel-Ingroff *et al.*, 1999).

A study carried out in Venezuela showed that most *Candida albicans* isolates obtained from HIV-positive individuals (66 out of 67) were susceptible to amphotericin B (Magaldi *et al.*, 2000). The situation in South Africa, another developing country, appears to be different. Susceptibility testing of 446 *C.albicans* isolates obtained from HIV-positive and HIV-negative individuals showed an 8.4% overall resistance to the drug (Blignaut *et al.*, 2002). The susceptibility profiles of the two groups (HIV-positive and HIV-negative) were similar. In this study, all 31 isolates of *C. albicans* grown in all NRTI concentrations, and subjected to different concentrations of amphotericin B, were susceptible to the drug (MIC <1μg/ml). No effect of NRTIs on the susceptibility of *C. albicans* to amphotericin B has been observed in our study. However our sample size was small and no conclusions can be drawn with this finding.

CHAPTER 6: CONCLUSION

This study addressed the research question of whether HIV nucleoside analogue drugs increase selected virulence traits of *Candida albicans.* Of the three virulence traits of *C. albicans* studied, only the rate of proliferation was increased upon exposure to 3TC. It may be that 3TC acted in a similar manner to 5-FU by increasing the rate of proliferation of *C. albicans* in a dose-dependent manner and potentially enhancing the virulence of *C. albicans.*

It would be worthwhile performing similar experiments with ddC, another HIV cytidine analogue drug, to see if this too acts in a similar manner to 3TC. If this occurs, signalling pathway-studies would be required to determine the mechanism of action of HIV cytidine analogue drugs on the rate of proliferation of these yeasts. However, since proliferation was the only virulence trait to be significantly affected by one of the four NRTIs used, this data suggests that NRTIs may have little effect on the virulence of *C. albicans.*

Although *in vitro* simulations form the basis for subsequent *in vivo* studies, the findings of this study cannot be extrapolated to predict the effect of NRTIs on *C. albicans* in a clinical setting since there are significant differences between *in vitro* models and human disease. Nonetheless, *in vitro* testing of NRTI effects on *C. albicans* infection may adequately model some aspects of the drugs on human candidal disease, and give insight to underlying mechanisms. With the increasing number of immunocompromised individuals globally, the development of new cytidine analogue drugs against HIV or cancer would necessitate

the testing of their effects on opportunistic yeasts such as *Candida*. The effects of these drugs on virulence traits such as enolase activity and secreted aspartyl protease activities should also be studied in addition to the characteristics examined here. Increases in the proliferation of *Candida* or other yeasts as a result of application of these drugs, may result in a greater risk of oral disease. This could mean persistent localized and even systemic disease, which in turn would decrease the quality of life of these individuals.

Future clinical studies may be warranted to specifically evaluate whether NRTIs and/or other cytidine analogue drugs affect the *Candida* infection outcome in HIV patients on ARV therapy using an *in vivo* model.

CHAPTER 7: REFERENCES

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CHAPTER 8: APPENDICES

Appendix A: Table of Chemicals used and their Suppliers

All chemicals and reagents used in this study were of analytical or molecular quality.

Chemicals and Reagents	Source
Agarose	Whitehead Scientific-Brakenfell, South Africa
Amphotericin B	Davies Diagnostics, South Africa
Boric acid	Sigma-Aldrich
Bovine serum albumin	Amersham Biosciences- Uppsala, Sweden
Crystal violet	NHLS Diagnostic Media Products, South Africa
DNA 100bp molecular weight marker	Promega
DMEM	Sigma-Aldrich
EDTA	Sigma-Aldrich
Ethanol (99.7-100%)	Analar- Midrand, South Africa
Fetal calf serum	Highveld Biological
Glacial acetic acid	Merck
D-Glucose monohydrate	Merck
Gram decolourizer	NHLS Diagnostic Media Products, South Africa
Ham's F_{12} medium	Sigma-Aldrich
Hydrochloric acid	Merck
Horse Serum	South African Vaccine Producers (Pty) Ltd
Isoamyl alcohol	Sigma-Aldrich
Isopropyl alcohol	Sigma-Aldrich
Iodine	NHLS Diagnostic Media Products South Africa
6x Blue/Orange Loading Dye G190A	Promega
Light Green Solution	Merk
Magnesium chloride	Roche Applied Science
Mineral oil	Sigma-Aldrich
O' Gene Ruler DNA Mix #SM1178	Fermentas
O' Gene 1kb DNA Ladder #SM1168	Fermentas
PCR grade water	Roche Applied Science
PCR Master Mix	Roche Applied Science
Phenol-chloroform-isoamyl alcohol	Sigma-Aldrich
Potassium acetate	Analar- Midrand, South Africa
Periodic Acid	Saarchem, Merk
Pulsed field gel electrophoresis agarose	Bio-Rad Laboratories- Hercules, CA,USA
Pulsed field gel electrophoresis marker I	Bio-Rad Laboratories
Safranin	NHLS Diagnostic Media Products, South Africa
Schiff Base	BDH, VWR International Ltd, Poole, England
Sodium chloride	Merck
Sodium dodecyl sulphate (SDS)	Merck
Sodium hydroxide	Merck
Tris	Roche Applied Science

Appendix B: Media, Buffers and Stains

All solutions and media were obtained from the Diagnostics Media Products Division of the National Health Laboratory Services (NHLS), Sandringham, South Africa.

1. 2% Agarose (40ml)
   0.4g Agarose
   40ml 1x TAE buffer
   1.5µl Ethidium bromide (10µg/ml)

Media

1. Sabouraud's Dextrose Agar with Chloramphenicol
   10g Mycological Peptone Oxoid L40
   40g (D)-Glucose ACE G0979NN00.500
   d.H_2O
   0.05 g chloramphenicol
Dissolve in 1L d.H_2O.

2. Semi solid agar vials
   Nutrient broth N_2 (Oxoid CM67) 25g
   Bitek agar (Difco 214530) 9g
   Deionized water make up to 1L

Buffers

Otherwise stated, all buffers were autoclaved.

1. Phosphate buffered saline (PBS) (1L)
   10.7g Na_2HPO_4
   2.72g KH_2PO_4
   8.5g NaCl
   d.H_2O
Dissolve and make up to 1L with d.H_2O

2. Normal Saline (1L)
   8.5g Sodium chloride
   d.H_2O
Dissolve all in 1L d.H_2O

3. TE Buffer
   10mM Tris-Cl
   1mM EDTA, pH 8.0
   d.H_2O

4. 1M Tris pH 8.0 (200ml)
   24.2g Tris base
   d.H_2O
Dissolve powder in 150ml d.H_2O. Add approximately 8.4 ml concentrated HCl for a solution with a pH of 8.0, and check with a pH meter.

5. 10% SDS (100ml)
   10g SDS
   d.H_2O
Add SDS powder to d.H2O, and make up to 100mL. Heat at 68°C to dissolve the powder. Adjust pH to 7.2 with 1M HCl. Do not autoclave.

6. 50x TAE buffer (100ml)
   24.2g Tris base
   5.7ml glacial acetic acid
   10ml 0.5M EDTA (pH 8)
   d.H_2O
Dissolve Tris and EDTA.2H_2O in d.H_2O. Add the acetic acid and make up to 100ml with d.H_2O.

7. 5M Sodium Chloride (100ml)
   29.2g NaCl
   d.H_2O
Dissolve powder in 100ml d.H_2O and autoclave.

8. 0.5M EDTA (1L)
   186.1g Na_2EDTA.2H_2O
   d.H_2O
Dissolve the Na_2EDTA.2H_2O powder in d.H2O, and adjust pH to 8.0 with 10M NaOH (~50mL). Make up to 1L with d.H_2O.

9. 10x TBE (400ml)
   43.2g Tris base
   22g Borate
   16ml 0.5M EDTA
   d.H_2O
Dissolve in 400ml d.H_2O.

10. 1M HCl (1L)
   86.2mL concentrated HCl
   d.H_2O
Add 86.2ml concentrated HCl to 913.8ml d.H_2O. Do not autoclave.

11. Sabouraud's Dextrose Broth
   10g Mycological Peptone Oxoid L40
   40g (D)-Glucose ACE G0979NN00.500
   d.H_2O
Dissolve and make up to 1L with d.H_2O.

12. 5M Potassium Acetate (80ml)
   39.3g Potassium Acetate
   d.H_2O
Dissolve and make up to 80ml with d.H_2O.

Stains

1. Ethidium Bromide (10mg/ml) (10ml)
   100mg Ethidium bromide
   d.H_2O

Make up to 10ml with d.H_2O. Do not autoclave. Store in the dark

2. Gram's Crystal Violet Stain
   10g 90% crystal violet dye
   500ml absolute Ethanol

3. Gram's Iodine
   6g Iodine
   12g KI
   1800ml distilled water
4. Gram's Decolourizer
   400ml acetone
   1200ml 95% Ethanol

5. Gram's Safranin
   10g safranin dye
   1000ml distilled water

6. Light Green Solution
   1g Light Green dye
   0.25ml Acetic acid
   100ml 80% Ethanol

Appendix C: List of Enzymes and Manufacturers

Enzyme	Manufacturer
Lyticase	Roche Applied Science
Proteinase K	Roche Applied Science
RNase A	Roche Applied Science

Appendix D: Subject Information Form and Consent Form

Subject Information Sheet

Good day.
My name is Bintou Ahmadou Ahidjo. I am a student in the Department of Clinical Microbiology and Infectious Diseases at the University of the Witwatersrand, where I am currently doing research for my Master's degree. For my research, I have chosen to study the fungus called *Candida*. This causes candidiasis, which is also known as thrush, and is a common infection.
Most people who are infected with HIV/AIDS have thrush. This is usually seen as a white/cream mucous in the mouth and throat of individuals. Recently, a drug called 5-fluorouracil, a drug used for the treatment of cancer, has been found to make thrush difficult to treat. I want to know if antiretrovirals also make thrush difficult to treat. If it does, then better treatment for people infected with HIV/AIDS will be needed. I would be very grateful if you could help me with this research.
For this research, a bit of the white mucous needs to be taken from your mouth before you begin antiretroviral treatment. This will be done by a qualified doctor, with a sterile cotton bud, so that no other infection occurs.
This process will however cause slight discomfort and a small amount of bleeding. This is because the mucous is attached to the mouth.
This is a once-only procedure, and none of this will be of an additional cost to you.
Participation is completely voluntary and not taking part will in no way affect the treatment you will be receiving. Also, you can withdraw from this procedure at anytime without any explanation, and this too will not affect the treatment you will be receiving.
Unfortunately, none of the results obtained from the research with your sample will be reported back to you because to maintain confidentiality none of your details for example name or hospital number will be recorded.

Thank you very much for your cooperation,
Yours Sincerely,
B. Ahmadou Ahidjo

Informed Consent Form

I ....., hereby agree to have mouth/oral swabs taken from me by a qualified doctor at no additional cost to me. I understand that none of the results obtained will be reported back to me.
I also understand that participation is completely voluntary, and that I can withdraw at anytime without any explanation and this will not affect the treatment I will be receiving.

…… …….
(Signature) (Date)

Appendix F: Data Points

The data points obtained for each of virulence assay for each clinical isolate exposed to each NRTI at 3 different concentrations at two time points and for two different occasions are tabulated below.

Legend: Time 1: 24h exposure; Time 2: 72h exposure;
NRTI 1: ddI, NRTI 2: 3TC; NRTI 3: d4T; NRTI 4: AZT
Concentration 0: No NRTI i.e. control; Concentration 1: Peak concentration of NRTI; concentration 2: 2x Peak concentration of NRTI; Concentration 3: _ Peak concentration of NRTI.

The values obtained for each replicate was the average of the duplicates.

The blanks in the table indicate where the assay was not performed. This could have been due to delays in obtaining reagents from their respective manufacturers.

Table: Experimental Data Obtained for the virulence traits of each isolate

Pat_Id	Time	NRTI	Concentration	Replicate	Proliferation Assay	Biofilm Assay	Adherence Assay
1	1	1	0	1	1.632	0	
1	1	1	0	2	2.927	0	
1	1	1	1	1	1.3625	-19.65995802	
1	1	1	1	2	1.573	-16.83078866	
1	1	1	2	1	1.4745	1.361997208	
1	1	1	2	2	1.682	-3.902678641	
1	1	1	3	1			
1	1	1	3	2			
1	1	2	0	1	1.632	0	
1	1	2	0	2	2.927	0	
1	1	2	1	1	1.376	-13.79791841	
1	1	2	1	2	1.738	-10.52478909	
1	1	2	2	1	1.462	2.345762613	
1	1	2	2	2	1.715	-13.95949199	
1	1	2	3	1			
1	1	2	3	2			
1	1	3	0	1	1.632	0	
1	1	3	0	2	2.927	0	
1	1	3	1	1	1.3985	-6.02387212	
1	1	3	1	2	1.6935	-16.94236116	
1	1	3	2	1	1.44	10.8622474	
1	1	3	2	2	1.6165	-11.49834351	
1	1	3	3	1			
1	1	3	3	2			
1	1	4	0	1		0	
1	1	4	0	2	2.927	0	
1	1	4	1	1			

1	1	4	1	2	1.5895	-54.48666915	
1	1	4	2	1			
1	1	4	2	2	1.769	-17.8872717	
1	1	4	3	1			
1	1	4	3	2			
1	2	1	0	1	2.25	0	
1	2	1	0	2	1.8465	0	
1	2	1	1	1	1.308	18.62137967	
1	2	1	1	2	1.462	-0.899293086	
1	2	1	2	1	1.3325	2.914316942	
1	2	1	2	2	1.3135	3.570229792	
1	2	1	3	1			
1	2	1	3	2			
1	2	2	0	1	2.25	0	
1	2	2	0	2	1.8465	0	
1	2	2	1	1	1.269	20.61283355	
1	2	2	1	2	1.243	2.605582474	
1	2	2	2	1	1.471	12.97937968	
1	2	2	2	2	1.2735	3.907499465	
1	2	2	3	1			
1	2	2	3	2			
1	2	3	0	1	2.25	0	
1	2	3	0	2	1.8465	0	
1	2	3	1	1	1.264	15.08816864	
1	2	3	1	2	1.2665	1.37189937	
1	2	3	2	1	1.282	-0.923358588	
1	2	3	2	2	1.279	2.164525924	
1	2	3	3	1			
1	2	3	3	2			
1	2	4	0	1		0	

1	2	4	0	2	1.8465	0	
1	2	4	1	1			
1	2	4	1	2	1.283	1.489478781	
1	2	4	2	1			
1	2	4	2	2	1.225	-2.697006302	
1	2	4	3	1			
1	2	4	3	2			
2	1	1	0	1	1.7245	0	
2	1	1	0	2	2.927	0	
2	1	1	1	1	1.514	18.07016936	
2	1	1	1	2	1.493	16.01953233	
2	1	1	2	1	1.377	4.523579123	
2	1	1	2	2	1.4635	15.63402526	
2	1	1	3	1			
2	1	1	3	2			
2	1	2	0	1	1.7245	0	
2	1	2	0	2	2.927	0	
2	1	2	1	1	1.4395	16.84184854	
2	1	2	1	2	1.559	15.39950512	
2	1	2	2	1	1.4885	9.494638324	
2	1	2	2	2	1.522	16.04499686	
2	1	2	3	1			
2	1	2	3	2			
2	1	3	0	1	1.7245	0	
2	1	3	0	2	2.927	0	
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2	1	3	1	2	1.54	12.94470763	
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2	1	3	2	2	1.513	20.91195852	
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2	1	3	3	2			
2	1	4	0	1	2.927	0	
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2	1	4	2	1			
2	1	4	2	2	1.561	9.966650115	
2	1	4	3	1			
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2	2	1	0	1	1.632	0	
2	2	1	0	2	1.5305	0	
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2	2	1	3	1			
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2	2	2	0	2	1.5305	0	
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2	2	2	1	2	2.165	23.50653813	
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2	2	2	2	2	2.2635	10.72409062	
2	2	2	3	1			
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2	2	3	0	1	1.632	0	
2	2	3	0	2	1.5305	0	
2	2	3	1	1	1.3985	-36.19053876	
2	2	3	1	2	2.147	19.06761516	
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2	2	4	0	1		0	
2	2	4	0	2	1.5305	0	
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2	2	4	2	2	2.0395	15.57653846	
2	2	4	3	1			
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3	1	1	0	2	0.87	0	
3	1	1	1	1	0.789	-13.83156304	84
3	1	1	1	2	0.7805	-10.80454161	
3	1	1	2	1	0.7495	-10.47064773	52
3	1	1	2	2	0.8635	-14.02687337	
3	1	1	3	1	0.7985	-4.311599637	39
3	1	1	3	2	0.857	50.47679705	
3	1	2	0	1	0.9435	0	259
3	1	2	0	2	0.87	0	
3	1	2	1	1	0.856	-23.31595225	95
3	1	2	1	2	0.8635	-3.362766992	
3	1	2	2	1	1.389	-19.66621985	74
3	1	2	2	2	1.492	-0.805993019	
3	1	2	3	1	0.878	-23.02469469	69
3	1	2	3	2	0.864	-1.69697802	
3	1	3	0	1	0.9435	0	259
3	1	3	0	2	0.87	0	
3	1	3	1	1	0.827	-29.43506492	350

3	1	3	1	2	0.8255	-8.29075427	
3	1	3	2	1	0.8315	-24.47025688	180
3	1	3	2	2	0.833	-1.398614262	
3	1	3	3	1	0.7425	-32.99486995	90
3	1	3	3	2	0.8155	-1.473076437	
3	1	4	0	1	0.9435	0	259
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3	1	4	1	1	0.9415	-28.87222733	185
3	1	4	1	2	0.859	4.694811506	
3	1	4	2	1	0.859	-26.62239894	361
3	1	4	2	2	0.8805	-0.365095346	
3	1	4	3	1	0.9465	-25.12097297	280
3	1	4	3	2	0.8925	6.812491003	
3	2	1	0	1	1.2855	0	265
3	2	1	0	2	1.2635	0	
3	2	1	1	1	1.003	-14.07745595	31
3	2	1	1	2	1.042	-12.63147633	
3	2	1	2	1	1.222	-14.15856273	77
3	2	1	2	2	1.2105	-10.97763334	
3	2	1	3	1	1.197	-3.399555889	33
3	2	1	3	2	0.9355	-1.606567479	
3	2	2	0	1	1.2855	0	265
3	2	2	0	2	1.2635	0	
3	2	2	1	1	1.402	-21.50256426	70
3	2	2	1	2	1.1145	-3.876514235	
3	2	2	2	1	1.71	-21.21957027	76
3	2	2	2	2	1.9445	-0.845610277	
3	2	2	3	1	1.342	-7.774740876	54
3	2	2	3	2	1.1655	0.573027223	
3	2	3	0	1	1.2855	0	265

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3	2	3	1	2	1.1365	6.065261753	
3	2	3	2	1	1.325	-27.02667141	350
3	2	3	2	2	1.1945	-19.74297079	
3	2	3	3	1	1.267	-31.46710643	135
3	2	3	3	2	1.1805	-2.220881875	
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3	2	4	0	2	1.2635	0	
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3	2	4	1	2	1.1775	1.463206117	
3	2	4	2	1	1.423	-1.688672495	145
3	2	4	2	2	1.128	3.081885407	
3	2	4	3	1	1.4955	-9.320464574	365
3	2	4	3	2	1.174	3.250416542	
4	1	1	0	1	1.0435	0	160
4	1	1	0	2	1.013	0	
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4	1	1	1	2	0.8325	10.76972329	
4	1	1	2	1	1.0115	-46.72848151	368
4	1	1	2	2	0.911	20.78867439	
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4	1	1	3	2	1.003	13.1647863	
4	1	2	0	1	1.0435	0	160
4	1	2	0	2	1.013	0	
4	1	2	1	1	0.9675	-35.73451708	90
4	1	2	1	2	0.953	15.23212079	
4	1	2	2	1	1.6345	-32.89693458	195
4	1	2	2	2	1.5115	21.18033175	
4	1	2	3	1	1.059	-25.29146203	175

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4	1	3	0	2	1.013	0	
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4	1	3	1	2	0.874	3.318759278	
4	1	3	2	1	0.9525	-43.58953396	40
4	1	3	2	2	0.957	34.32094751	
4	1	3	3	1	1.0345	-41.84902518	156
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4	1	4	1	2	0.9265	0.9176565	
4	1	4	2	1	0.949	-32.51204354	60
4	1	4	2	2	0.859	57.68968656	
4	1	4	3	1	0.989	-36.62041381	220
4	1	4	3	2	0.941	8.20605089	
4	2	1	0	1	1.2925	0	170
4	2	1	0	2	1.2855	0	
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4	2	1	1	2	1.172	1.613098214	
4	2	1	2	1	1.268	-52.64425531	85
4	2	1	2	2	1.136	-15.47499198	
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4	2	1	3	2	1.2085	5.141670452	
4	2	2	0	1	1.2925	0	170
4	2	2	0	2	1.2855	0	
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4	2	2	1	2	1.1405	-4.818737812	
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4	2	2	2	2	2.2785	-6.896700837	
4	2	2	3	1	1.35	-45.55618693	120
4	2	2	3	2	1.212	-1.258552087	
4	2	3	0	1	1.2925	0	170
4	2	3	0	2	1.2855	0	
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4	2	3	1	2	1.1235	9.394678158	
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4	2	3	2	2	1.354	3.484905025	
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4	2	4	0	1	1.2925	0	170
4	2	4	0	2	1.2855	0	
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4	2	4	1	2	1.1715	-8.97831989	
4	2	4	2	1	1.4025	-49.80975993	100
4	2	4	2	2	1.319	0.805905389	
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4	2	4	3	2	1.214	0.266000896	
5	1	1	0	1	0.6505	0	186
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5	1	1	1	1	0.5605	6.166338301	83
5	1	1	1	2	0.682	-17.95749139	
5	1	1	2	1	0.5055	1.872097806	40
5	1	1	2	2	0.6375	-10.28429855	
5	1	1	3	1	0.5045	42.37983215	250
5	1	1	3	2	0.6635	26.95476686	
5	1	2	0	1	0.6505	0	186
5	1	2	0	2	0.706	0	
5	1	2	1	1	0.598	20.02862779	55

5	1	2	1	2	0.657	-15.67959945	
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5	1	2	3	1	0.7095	10.83439879	79
5	1	2	3	2	0.737	1.126913787	
5	1	3	0	1	0.6505	0	186
5	1	3	0	2	0.706	0	
5	1	3	1	1	0.6945	0.693954954	67
5	1	3	1	2	0.7415	0.454661407	
5	1	3	2	1	0.594	29.46983816	99
5	1	3	2	2	0.6985	-1.395817164	
5	1	3	3	1	0.662	-2.201366416	75
5	1	3	3	2	0.715	3.159675786	
5	1	4	0	1	0.6505	0	186
5	1	4	0	2	0.706	0	
5	1	4	1	1	0.504	-3.807977468	160
5	1	4	1	2	0.6875	-16.19219781	
5	1	4	2	1	0.658	-3.267500471	50
5	1	4	2	2	0.6385	-15.61585597	
5	1	4	3	1	0.5855	4.561754038	95
5	1	4	3	2	0.7265	-18.55822253	
5	2	1	0	1	0.674	0	115
5	2	1	0	2	1.0955	0	
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5	2	1	1	2	1.065	-23.17635547	
5	2	1	2	1	0.4445	-13.25200114	78
5	2	1	2	2	1.097	-14.61398444	
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5	2	1	3	2	1.175	37.93468274	
5	2	2	0	1	0.674	0	115

5	2	2	0	2	1.0955	0	
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5	2	2	2	1	1.185	28.95306064	245
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5	2	2	3	1	0.7055	25.14562185	125
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5	2	3	0	1	0.674	0	115
5	2	3	0	2	1.0955	0	
5	2	3	1	1	0.586	19.06733118	150
5	2	3	1	2	1.1195	-10.32483484	
5	2	3	2	1	0.431	20.86566865	40
5	2	3	2	2	0.9605	-15.98160609	
5	2	3	3	1	0.6845	25.93990573	170
5	2	3	3	2	1.0495	-15.06671291	
5	2	4	0	1	0.674	0	115
5	2	4	0	2	1.0955	0	
5	2	4	1	1	0.468	-0.214410509	170
5	2	4	1	2	0.8775	-3.376361467	
5	2	4	2	1	0.548	6.040490071	90
5	2	4	2	2	1.2315	-18.00777317	
5	2	4	3	1	0.4895	-6.453872199	15
5	2	4	3	2	1.0195	-15.75169646	
6	1	1	0	1	0.6625	0	160
6	1	1	0	2	0.5185	0	
6	1	1	1	1	0.618	1.332525992	180
6	1	1	1	2	0.7275	-12.23245107	
6	1	1	2	1	0.6705	6.118968829	160
6	1	1	2	2	0.705	-1.96197907	
6	1	1	3	1	0.7085	0.632378786	378

6	1	1	3	2	0.782	15.64749376	
6	1	2	0	1	0.6625	0	160
6	1	2	0	2	0.5185	0	
6	1	2	1	1	0.624	4.391429247	275
6	1	2	1	2	0.711	-1.441944339	
6	1	2	2	1	1.5395	5.598465769	212
6	1	2	2	2	1.5375	0.260481698	
6	1	2	3	1	0.7345	5.361505652	360
6	1	2	3	2	0.748	1.153504461	
6	1	3	0	1	0.6625	0	160
6	1	3	0	2	0.5185	0	
6	1	3	1	1	0.5495	-4.4270208	70
6	1	3	1	2	0.535	-3.761743477	
6	1	3	2	1	0.641	3.769566745	165
6	1	3	2	2	0.707	-10.22481586	
6	1	3	3	1	0.661	6.682478345	455
6	1	3	3	2	0.78	0.582728111	
6	1	4	0	1	0.6625	0	160
6	1	4	0	2	0.5185	0	
6	1	4	1	1	0.554	4.992496803	350
6	1	4	1	2	0.648	2.169696416	
6	1	4	2	1	0.597	1.192828667	585
6	1	4	2	2	0.718	20.55047994	
6	1	4	3	1	0.652	0.617674779	150
6	1	4	3	2	0.5395	1.247489161	
6	2	1	0	1	0.386	0	157
6	2	1	0	2	0.7715	0	
6	2	1	1	1	1.297	7.979219089	395
6	2	1	1	2	0.853	-5.452920604	
6	2	1	2	1	1.22	10.80514085	375

6	2	1	2	2	0.789	2.786341354	
6	2	1	3	1	1.0875	10.54334306	228
6	2	1	3	2	0.899	7.280332186	
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6	2	2	0	2	0.7715	0	
6	2	2	1	1	1.2105	-13.54757303	258
6	2	2	1	2	0.8595	5.355457497	
6	2	2	2	1	1.241	6.500351896	275
6	2	2	2	2	0.9075	7.280332186	
6	2	2	3	1	1.207	2.70143202	720
6	2	2	3	2	0.9695	10.18192542	
6	2	3	0	1	0.386	0	157
6	2	3	0	2	0.7715	0	
6	2	3	1	1	1.335	-4.811277151	225
6	2	3	1	2	0.847	4.538712406	
6	2	3	2	1	1.229	0.713373451	130
6	2	3	2	2	0.9225	9.512983402	
6	2	3	3	1	1.279	2.725142247	210
6	2	3	3	2	0.953	9.609538946	
6	2	4	0	1	0.386	0	157
6	2	4	0	2	0.7715	0	
6	2	4	1	1	1.0925	4.579360323	160
6	2	4	1	2	0.8095	9.079938312	
6	2	4	2	1	1.1555	8.4996086	147
6	2	4	2	2	0.8545	-4.165570194	
6	2	4	3	1	1.1935	-9.881714602	192
6	2	4	3	2	0.8985	0.16089217	
7	1	1	0	1	0.609	0	198
7	1	1	0	2	0.848	0	
7	1	1	1	1	0.4825	8.107906557	12

7	1	1	1	2	0.8265	-3.47202861	
7	1	1	2	1	0.512	4.949989044	150
7	1	1	2	2	0.7535	-2.332863872	
7	1	1	3	1	0.5375	3.290904334	240
7	1	1	3	2	0.8105	-6.36368791	
7	1	2	0	1	0.609	0	198
7	1	2	0	2	0.848	0	
7	1	2	1	1	0.599	-5.097201318	210
7	1	2	1	2	0.6015	4.239618679	
7	1	2	2	1	0.595	-0.708815358	295
7	1	2	2	2	0.7725	6.831152995	
7	1	2	3	1	0.6255	-9.033894354	310
7	1	2	3	2	0.936	4.773804476	
7	1	3	0	1	0.609	0	198
7	1	3	0	2	0.848	0	
7	1	3	1	1	0.4645	0.431141653	525
7	1	3	1	2	0.76	5.623744878	
7	1	3	2	1	0.5555	1.892351082	125
7	1	3	2	2	0.82	1.786991413	
7	1	3	3	1	0.5395	-4.588778208	84
7	1	3	3	2	0.8035	3.192800067	
7	1	4	0	1	0.609	0	198
7	1	4	0	2	0.848	0	
7	1	4	1	1	0.454	-2.463011162	325
7	1	4	1	2	0.783	-1.433290107	
7	1	4	2	1	0.5465	1.571010209	308
7	1	4	2	2	0.774	0.916635717	
7	1	4	3	1	0.53	2.498435501	360
7	1	4	3	2	0.819	8.051001622	
7	2	1	0	1	0.5075	0	105

7	2	1	0	2	0.616	0	
7	2	1	1	1	1.1785	-3.919745989	144
7	2	1	1	2	0.534	-5.440532233	
7	2	1	2	1	1.1305	-17.6302107	85
7	2	1	2	2	0.5695	-1.833416026	
7	2	1	3	1	1.144	-5.886163302	330
7	2	1	3	2	0.6245	-2.720748822	
7	2	2	0	1	0.5075	0	105
7	2	2	0	2	0.616	0	
7	2	2	1	1	1.082	0.80677771	342
7	2	2	1	2	0.727	-5.485636309	
7	2	2	2	1	1.2485	1.665536498	302
7	2	2	2	2	0.7155	-7.452068645	
7	2	2	3	1	1.337	1.819292217	162
7	2	2	3	2	0.7655	-7.526723347	
7	2	3	0	1	0.5075	0	105
7	2	3	0	2	0.616	0	
7	2	3	1	1	1.128	-1.374550849	330
7	2	3	1	2	0.602	-3.925571925	
7	2	3	2	1	1.339	2.727751586	215
7	2	3	2	2	0.653	1.198308438	
7	2	3	3	1	1.3375	-5.547683819	100
7	2	3	3	2	0.625	-11.19674091	
7	2	4	0	1	0.5075	0	105
7	2	4	0	2	0.616	0	
7	2	4	1	1	1.157	-8.555286139	335
7	2	4	1	2	0.5415	-2.318341786	
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8	1	2	1	2	0.76	1.031451793	
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8	1	4	2	1	0.3405	-14.30309728	315

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8	2	1	0	1	0.815	0	430
8	2	1	0	2	0.516	0	
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8	2	2	0	1	0.815	0	430
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20	2	1	1	2	0.811	-7.766697965	
20	2	1	2	1	1.61	3.418389898	743
20	2	1	2	2	0.8155	0.600379994	
20	2	1	3	1	1.438	-3.696628442	1342
20	2	1	3	2	0.9115	-5.77402072	
20	2	2	0	1	1.463	0	346

20	2	2	0	2	0.826	0	
20	2	2	1	1	1.886	1.864982133	403
20	2	2	1	2	0.707	2.729480532	
20	2	2	2	1	2.384	1.42940181	106
20	2	2	2	2	1.4205	2.126295311	
20	2	2	3	1	1.8305	4.336247056	546
20	2	2	3	2	0.671	0.637058049	
20	2	3	0	1	1.463	0	346
20	2	3	0	2	0.826	0	
20	2	3	1	1	1.7195	3.165748844	1943
20	2	3	1	2	0.7325	-0.577961583	
20	2	3	2	1	1.2405	4.636777501	1461
20	2	3	2	2	0.931	1.507597718	
20	2	3	3	1	1.735	1.287394135	247
20	2	3	3	2	0.7365	-1.28905427	
20	2	4	0	1	1.463	0	346
20	2	4	0	2	0.826	0	
20	2	4	1	1	2.007	-1.439754903	1360
20	2	4	1	2	0.7785	0.299229167	
20	2	4	2	1	1.7615	-1.709292891	246
20	2	4	2	2	0.8415	0.329972155	
20	2	4	3	1	1.4505	2.109510099	288
20	2	4	3	2	0.804	2.528671122	
21	1	1	0	1	0.961	0	1042
21	1	1	0	2	0.323	0	
21	1	1	1	1	1.0065	1.621050621	660
21	1	1	1	2	0.8035	0.089369246	
21	1	1	2	1	1.2725	0.813243145	988
21	1	1	2	2	0.8825	2.510649962	
21	1	1	3	1	1.235	-2.136152147	342

21	1	1	3	2	1.064	2.909496208	
21	1	2	0	1	0.961	0	1042
21	1	2	0	2	0.323	0	
21	1	2	1	1	1.006	-0.803321104	706
21	1	2	1	2	0.8365	-0.358539312	
21	1	2	2	1	1.0375	1.24788532	360
21	1	2	2	2	1.04	3.775474937	
21	1	2	3	1	1.012	-7.437804983	581
21	1	2	3	2	0.7075	1.802464312	
21	1	3	0	1	0.961	0	1042
21	1	3	0	2	0.323	0	
21	1	3	1	1	1.4705	-1.597422106	944
21	1	3	1	2	1.2255	-1.633324361	
21	1	3	2	1	1.03	5.05345191	682
21	1	3	2	2	0.8195	6.793373016	
21	1	3	3	1	0.994	-0.448920645	524
21	1	3	3	2	0.889	-1.482379466	
21	1	4	0	1	0.961	0	1042
21	1	4	0	2	0.323	0	
21	1	4	1	1	1.031	-11.47144172	460
21	1	4	1	2	0.722	1.123845226	
21	1	4	2	1	1.0455	-5.374585502	349
21	1	4	2	2	0.956	-2.712334337	
21	1	4	3	1	1.091	-7.11520409	704
21	1	4	3	2	0.7965	-7.914433523	
21	2	1	0	1	1.071	0	346
21	2	1	0	2	0.891	0	
21	2	1	1	1	1.213	-13.10305073	1480
21	2	1	1	2	0.823	-9.794160849	
21	2	1	2	1	1.0325	-3.313618536	1124

21	2	1	2	2	0.7675	4.695137876	
21	2	1	3	1	1.085	-2.603003806	622
21	2	1	3	2	0.5735	6.312856327	
21	2	2	0	1	1.071	0	346
21	2	2	0	2	0.891	0	
21	2	2	1	1	1.213	0.197826314	480
21	2	2	1	2	0.9325	4.477922436	
21	2	2	2	1	1.024	-0.536321885	563
21	2	2	2	2	0.873	7.213493148	
21	2	2	3	1	1.212	-2.181162219	831
21	2	2	3	2	0.695	3.034755778	
21	2	3	0	1	1.071	0	346
21	2	3	0	2	0.891	0	
21	2	3	1	1	1.3245	-0.750941916	249
21	2	3	1	2	0.7065	-0.0139777	
21	2	3	2	1	1.229	3.381504769	423
21	2	3	2	2	0.7025	11.11997712	
21	2	3	3	1	1.1755	-0.852658448	905
21	2	3	3	2	0.56	3.583221376	
21	2	4	0	1	1.071	0	346
21	2	4	0	2	0.891	0	
21	2	4	1	1	1.204	-12.56747987	722
21	2	4	1	2	1.018	-2.320509203	
21	2	4	2	1	1.1155	-5.012642146	321
21	2	4	2	2	0.9505	7.902333407	
21	2	4	3	1	1.1695	-1.651027359	1347
21	2	4	3	2	0.646	4.805643173	
22	1	1	0	1	1.121	0	1520
22	1	1	0	2	1.114	0	
22	1	1	1	1	0.836	-2.582351261	900

22	1	1	1	2	1.1885	2.976027278	
22	1	1	2	1	1.085	0.146515341	1300
22	1	1	2	2	1.096	3.296421595	
22	1	1	3	1	1.0915	-2.901593232	800
22	1	1	3	2	1.088	-3.302016474	
22	1	2	0	1	1.121	0	1520
22	1	2	0	2	1.114	0	
22	1	2	1	1	0.8605	-3.319772529	440
22	1	2	1	2	1.193	4.345553258	
22	1	2	2	1	1.681	-42.67840665	720
22	1	2	2	2	1.7055	4.865101728	
22	1	2	3	1	0.832	4.948061054	780
22	1	2	3	2	0.9965	4.659510811	
22	1	3	0	1	1.121	0	1520
22	1	3	0	2	1.114	0	
22	1	3	1	1	0.937	1.021823164	840
22	1	3	1	2	0.956	5.259946337	
22	1	3	2	1	0.8935	3.339004446	1060
22	1	3	2	2	1.082	4.268394879	
22	1	3	3	1	0.9925	4.740082154	1100
22	1	3	3	2	1.06	5.186742752	
22	1	4	0	1	1.121	0	1520
22	1	4	0	2	1.114	0	
22	1	4	1	1	0.8015	-1.513478002	500
22	1	4	1	2	1.201	-0.630157247	
22	1	4	2	1	0.94	-3.657930776	1860
22	1	4	2	2	1.118	4.790402102	
22	1	4	3	1	0.996	-2.799137819	1220
22	1	4	3	2	1.1155	-26.80365701	
22	2	1	0	1	1.1515	0	1160

22	2	1	0	2	0.861	0	
22	2	1	1	1	1.0535	0.290447908	480
22	2	1	1	2	0.837	0.290447908	
22	2	1	2	1	1.1395	1.171377247	520
22	2	1	2	2	0.5815	1.171377247	
22	2	1	3	1	1.139	0.290447908	780
22	2	1	3	2	0.449	-7.671079378	
22	2	2	0	1	1.1515	0	1160
22	2	2	0	2	0.861	0	
22	2	2	1	1	1.091	2.225831335	860
22	2	2	1	2	0.841	2.225831335	
22	2	2	2	1	1.7145	0.229236654	1160
22	2	2	2	2	1.487	-0.582137344	
22	2	2	3	1	1.2325	-0.543067848	820
22	2	2	3	2	0.6725	-0.543067848	
22	2	3	0	1	1.1515	0	1160
22	2	3	0	2	0.861	0	
22	2	3	1	1	0.9305	-0.345452607	680
22	2	3	1	2	0.7505	-0.345452607	
22	2	3	2	1	1.182	-0.582137344	620
22	2	3	2	2	0.646	0.229236654	
22	2	3	3	1	1.1255	-1.122533767	500
22	2	3	3	2	0.6085	-1.122533767	
22	2	4	0	1	1.1515	0	1160
22	2	4	0	2	0.861	0	
22	2	4	1	1	0.838	1.693143653	1140
22	2	4	1	2	0.778	1.693143653	
22	2	4	2	1	1.0275	0.788042606	1100
22	2	4	2	2	0.7525	0.788042606	
22	2	4	3	1	1.148	1.343578688	900

22	2	4	3	2	0.6455	1.343578688	
23	1	1	0	1	1.3145	0	880
23	1	1	0	2	1.2705	0	
23	1	1	1	1	1.249	2.968532717	360
23	1	1	1	2	1.177	2.830370063	
23	1	1	2	1	1.249	5.440333624	320
23	1	1	2	2	1.1885	3.873461203	
23	1	1	3	1	1.323	6.594801788	840
23	1	1	3	2	1.3225	6.846937883	
23	1	2	0	1	1.3145	0	880
23	1	2	0	2	1.2705	0	
23	1	2	1	1	1.2025	6.129071796	1140
23	1	2	1	2	1.2835	4.63871579	
23	1	2	2	1	0.924	5.786392021	900
23	1	2	2	2	1.2535	5.600908572	
23	1	2	3	1	0.9005	3.673917705	980
23	1	2	3	2	1.187	6.057017546	
23	1	3	0	1	1.3145	0	880
23	1	3	0	2	1.2705	0	
23	1	3	1	1	1.1255	6.158072351	360
23	1	3	1	2	1.244	4.461128962	
23	1	3	2	1	1.33	10.16758686	660
23	1	3	2	2	1.292	9.963501517	
23	1	3	3	1	1.049	6.750516101	1160
23	1	3	3	2	1.251	6.99175518	
23	1	4	0	1	1.3145	0	880
23	1	4	0	2	1.2705	0	
23	1	4	1	1	1.2385	4.490360045	880
23	1	4	1	2	1.3065	6.073982675	
23	1	4	2	1	1.2685	4.582165903	520

23	1	4	2	2	1.3	6.326909141	
23	1	4	3	1	1.0845	4.987087495	440
23	1	4	3	2	1.3085	6.691927699	
23	2	1	0	1	1.366	0	840
23	2	1	0	2	0.795	0	
23	2	1	1	1	1.2725	3.248226166	920
23	2	1	1	2	0.71	0.728355635	
23	2	1	2	1	1.284	3.921367635	640
23	2	1	2	2	0.656	1.505353257	
23	2	1	3	1	1.418	0.728355635	880
23	2	1	3	2	0.6725	1.74948928	
23	2	2	0	1	1.366	0	840
23	2	2	0	2	0.795	0	
23	2	2	1	1	1.379	3.716636071	1800
23	2	2	1	2	1.0435	1.384005119	
23	2	2	2	1	1.349	4.591761443	1340
23	2	2	2	2	0.841	1.31605379	
23	2	2	3	1	1.2825	5.895779931	1120
23	2	2	3	2	0.5505	1.279688413	
23	2	3	0	1	1.366	0	840
23	2	3	0	2	0.795	0	
23	2	3	1	1	1.3395	4.977370408	460
23	2	3	1	2	0.5635	1.912662691	
23	2	3	2	1	1.3875	6.847490526	540
23	2	3	2	2	0.5905	3.442651098	
23	2	3	3	1	1.3465	6.180663986	480
23	2	3	3	2	0.6945	2.515676606	
23	2	4	0	1	1.366	0	840
23	2	4	0	2	0.795	0	
23	2	4	1	1	1.402	3.903181263	1040

23	2	4	1	2	0.588	-0.882930252	
23	2	4	2	1	1.3955	3.834231568	1100
23	2	4	2	2	0.651	0.46631346	
23	2	4	3	1	1.404	1.404859621	640
23	2	4	3	2	0.6335	0.447895308	
24	1	1	0	1	0.878	0	440
24	1	1	0	2	0.4535	0	
24	1	1	1	1	1.038	-8.918214248	900
24	1	1	1	2	0.348	1.235557017	
24	1	1	2	1	0.854	-0.9175263	340
24	1	1	2	2	0.5035	0.525322754	
24	1	1	3	1	0.879	-7.12801153	640
24	1	1	3	2	0.452	-6.647930059	
24	1	2	0	1	0.878	0	440
24	1	2	0	2	0.4535	0	
24	1	2	1	1	1.016	-4.479417984	700
24	1	2	1	2	0.351	0.180374548	
24	1	2	2	1	1.5345	1.179814074	860
24	1	2	2	2	0.679	1.346659888	
24	1	2	3	1	0.832	-1.768196116	760
24	1	2	3	2	0.3515	1.826036137	
24	1	3	0	1	0.878	0	440
24	1	3	0	2	0.4535	0	
24	1	3	1	1	0.79	-1.418671882	1340
24	1	3	1	2	0.299	0.677696712	
24	1	3	2	1	0.801	-1.574698234	1080
24	1	3	2	2	0.218	2.808956909	
24	1	3	3	1	0.8595	-3.013302629	920
24	1	3	3	2	0.2675	3.477089707	
24	1	4	0	1	0.878	0	440

24	1	4	0	2	0.4535	0	
24	1	4	1	1	0.8785	-8.199287231	880
24	1	4	1	2	0.2805	0.627372473	
24	1	4	2	1	0.8195	0.537200583	760
24	1	4	2	2	0.054	1.830972826	
24	1	4	3	1	0.8725	-4.294783926	600
24	1	4	3	2	0.31	3.029718384	
24	2	1	0	1	1.0895	0	1480
24	2	1	0	2	0.2335	0	
24	2	1	1	1	1.0985	-6.542361529	360
24	2	1	1	2	0.311	-0.399329395	
24	2	1	2	1	0.9445	-1.254391438	480
24	2	1	2	2	0.241	7.094677598	
24	2	1	3	1	0.989	-0.399329395	340
24	2	1	3	2	0.1025	18.56846916	
24	2	2	0	1	1.0895	0	1480
24	2	2	0	2	0.2335	0	
24	2	2	1	1	1.1415	0.967562454	740
24	2	2	1	2	0.157	17.92065789	
24	2	2	2	1	1.6685	-2.339911403	520
24	2	2	2	2	0.8805	9.780240807	
24	2	2	3	1	1.023	-3.475932412	420
24	2	2	3	2	0.271	13.79405222	
24	2	3	0	1	1.0895	0	1480
24	2	3	0	2	0.2335	0	
24	2	3	1	1	1.114	2.270540089	1160
24	2	3	1	2	0.1925	16.99448008	
24	2	3	2	1	1.1285	4.667188004	600
24	2	3	2	2	0.1295	16.79501339	
24	2	3	3	1	0.9845	-4.225303229	700

24	2	3	3	2	0.303	17.82768896	
24	2	4	0	1	1.0895	0	1480
24	2	4	0	2	0.2335	0	
24	2	4	1	1	0.978	2.076127098	1000
24	2	4	1	2	0.164	13.7536211	
24	2	4	2	1	0.9745	-11.53948539	380
24	2	4	2	2	0.134	18.8446528	
24	2	4	3	1	1.0165	-7.475603531	300
24	2	4	3	2	0.436	13.79405222	
25	1	1	0	1	0.9435	0	2020
25	1	1	0	2	0.6315	0	
25	1	1	1	1	0.9795	-5.429470811	1460
25	1	1	1	2	0.878	0.878179501	
25	1	1	2	1	0.9675	-3.695136044	1400
25	1	1	2	2	0.6135	1.677009779	
25	1	1	3	1	0.922	-0.196352656	1540
25	1	1	3	2	0.778	0.353341274	
25	1	2	0	1	0.9435	0	2020
25	1	2	0	2	0.6315	0	
25	1	2	1	1	1.0215	-2.01218968	620
25	1	2	1	2	0.5005	-0.337484546	
25	1	2	2	1	0.996	-0.348181352	1120
25	1	2	2	2	0.454	1.610935513	
25	1	2	3	1	0.971	-0.039561907	1420
25	1	2	3	2	0.534	0.216039478	
25	1	3	0	1	0.9435	0	2020
25	1	3	0	2	0.6315	0	
25	1	3	1	1	0.986	-1.961719493	840
25	1	3	1	2	0.733	0.018860672	
25	1	3	2	1	0.851	3.261717476	1820

25	1	3	2	2	0.631	4.456248243	
25	1	3	3	1	0.86	0.499906952	1120
25	1	3	3	2	0.7585	0.515657721	
25	1	4	0	1	0.9435	0	2020
25	1	4	0	2	0.6315	0	
25	1	4	1	1	0.872	-2.151248312	520
25	1	4	1	2	0.59	1.818418356	
25	1	4	2	1	0.8745	-0.736243475	1040
25	1	4	2	2	0.577	2.501912885	
25	1	4	3	1	0.8205	-1.538705462	1680
25	1	4	3	2	0.613	1.525586608	
25	2	1	0	1	1.017	0	640
25	2	1	0	2	0.4405	0	
25	2	1	1	1	1.148	-2.355697168	500
25	2	1	1	2	0.457	-0.51205951	
25	2	1	2	1	1.109	0.814880721	520
25	2	1	2	2	0.5045	0.849472632	
25	2	1	3	1	1.167	-0.51205951	740
25	2	1	3	2	0.295	2.125482671	
25	2	2	0	1	1.017	0	640
25	2	2	0	2	0.4405	0	
25	2	2	1	1	1.1755	-8.19226813	680
25	2	2	1	2	0.283	-5.722382274	
25	2	2	2	1	1.169	-16.11655866	380
25	2	2	2	2	0.2815	-1.112587313	
25	2	2	3	1	1.21	0.454144825	520
25	2	2	3	2	0.3725	1.590297682	
25	2	3	0	1	1.017	0	640
25	2	3	0	2	0.4405	0	
25	2	3	1	1	1.122	-10.049754	600

25	2	3	1	2	0.1945	-2.237631495	
25	2	3	2	1	0.959	6.016811813	1380
25	2	3	2	2	0.412	13.0789766	
25	2	3	3	1	0.9755	-8.404041034	700
25	2	3	3	2	0.278	1.225215454	
25	2	4	0	1	1.017	0	640
25	2	4	0	2	0.4405	0	
25	2	4	1	1	0.999	-8.636787502	660
25	2	4	1	2	0.234	-11.55195238	
25	2	4	2	1	0.993	-1.851056564	720
25	2	4	2	2	0.303	-2.162599573	
25	2	4	3	1	1.012	-1.789914823	700
25	2	4	3	2	0.261	-16.14141772	
26	1	1	0	1	0.9055	0	540
26	1	1	0	2	1.111	0	
26	1	1	1	1	0.823	2.124350558	500
26	1	1	1	2	0.8385	1.74767498	
26	1	1	2	1	0.6675	2.566822832	660
26	1	1	2	2	0.812	1.515989417	
26	1	1	3	1	0.3735	-8.419285846	700
26	1	1	3	2	1.0045	-8.214507487	
26	1	2	0	1	0.9055	0	540
26	1	2	0	2	1.111	0	
26	1	2	1	1	0.8595	1.962782772	800
26	1	2	1	2	1.651	1.30274818	
26	1	2	2	1	1.634	1.186463193	1200
26	1	2	2	2	0.848	1.480858613	
26	1	2	3	1	0.7715	1.226212658	820
26	1	2	3	2	0.764	-0.644096134	
26	1	3	0	1	0.9055	0	540

26	1	3	0	2	1.111	0	
26	1	3	1	1	-0.168	0.441190417	860
26	1	3	1	2	0.9045	0.395560089	
26	1	3	2	1	0.3945	2.172401063	520
26	1	3	2	2	0.917	1.737979771	
26	1	3	3	1	0.8105	0.128137207	280
26	1	3	3	2	0.8165	0.919100054	
26	1	4	0	1	0.9055	0	540
26	1	4	0	2	1.111	0	
26	1	4	1	1	0.437	2.428641179	1020
26	1	4	1	2	0.7585	-0.021001295	
26	1	4	2	1	1.219	2.780153922	320
26	1	4	2	2	0.8205	1.639163483	
26	1	4	3	1	0.565	0.676505297	360
26	1	4	3	2	0.657	1.009907191	
26	2	1	0	1	0.903	0	960
26	2	1	0	2	0.5595	0	
26	2	1	1	1	0.746	10.11075877	540
26	2	1	1	2	0.6005	0.439973189	
26	2	1	2	1	1.0755	3.397976814	680
26	2	1	2	2	0.554	2.099282132	
26	2	1	3	1	0.899	12.58689822	420
26	2	1	3	2	0.807	-7.641675385	
26	2	2	0	1	0.903	0	960
26	2	2	0	2	0.5595	0	
26	2	2	1	1	0.9245	10.00511648	400
26	2	2	1	2	0.5975	2.085326611	
26	2	2	2	1	1.699	-4.19480258	660
26	2	2	2	2	1.4805	1.871839729	
26	2	2	3	1	0.943	-16.43175036	640

26	2	2	3	2	0.585	-0.868296683	
26	2	3	0	1	0.903	0	960
26	2	3	0	2	0.5595	0	
26	2	3	1	1	0.9405	16.62157262	720
26	2	3	1	2	0.7705	0.023536727	
26	2	3	2	1	0.905	13.47993929	300
26	2	3	2	2	0.7055	1.155367413	
26	2	3	3	1	0.674	-2.509925381	1000
26	2	3	3	2	0.685	0.861825867	
26	2	4	0	1	0.903	0	960
26	2	4	0	2	0.5595	0	
26	2	4	1	1	0.8445	19.8495471	280
26	2	4	1	2	0.606	0.551830806	
26	2	4	2	1	0.789	14.85586202	960
26	2	4	2	2	0.5235	2.712807274	
26	2	4	3	1	0.7855	-13.37447819	780
26	2	4	3	2	0.552	-0.43632987	
27	1	1	0	1	1.7365	0	1120
27	1	1	0	2	1.0005	0	
27	1	1	1	1	1.563	-1.178011087	680
27	1	1	1	2	0.6745	0.937122957	
27	1	1	2	1	1.439	0.446742185	600
27	1	1	2	2	0.568	-2.49730135	
27	1	1	3	1	1.422	-0.741675775	480
27	1	1	3	2	0.6345	-0.123261808	
27	1	2	0	1	1.7365	0	1120
27	1	2	0	2	1.0005	0	
27	1	2	1	1	1.2595	0.538888356	300
27	1	2	1	2	0.7365	-0.333973852	
27	1	2	2	1	1.205	0.102684271	920

27	1	2	2	2	0.5985	1.741480919	
27	1	2	3	1	0.8405	-2.437319724	960
27	1	2	3	2	0.643	0.734198635	
27	1	3	0	1	1.7365	0	1120
27	1	3	0	2	1.0005	0	
27	1	3	1	1	1.522	0.419585898	440
27	1	3	1	2	0.7215	1.62386965	
27	1	3	2	1	1.0945	1.384921533	400
27	1	3	2	2	0.6095	2.493607354	
27	1	3	3	1	1.402	-0.593431718	980
27	1	3	3	2	0.636	0.940080531	
27	1	4	0	1	1.7365	0	1120
27	1	4	0	2	1.0005	0	
27	1	4	1	1	1.393	0.823036896	1080
27	1	4	1	2	0.5975	-0.637247101	
27	1	4	2	1	1.1625	-0.475254675	860
27	1	4	2	2	0.583	-0.768110122	
27	1	4	3	1	1.257	-0.396927809	740
27	1	4	3	2	0.657	-2.115894512	
27	2	1	0	1	0.7795	0	1040
27	2	1	0	2	0.4805	0	
27	2	1	1	1	0.5785	11.77281649	520
27	2	1	1	2	0.5635	-2.462988478	
27	2	1	2	1	0.754	3.294361104	880
27	2	1	2	2	0.446	-2.288328928	
27	2	1	3	1	0.5665	-0.220384043	680
27	2	1	3	2	0.3795	-11.03510687	
27	2	2	0	1	0.7795	0	1040
27	2	2	0	2	0.4805	0	
27	2	2	1	1	0.7745	14.76924948	640

27	2	2	1	2	0.559	-1.380476381	
27	2	2	2	1	0.773	-10.82971424	720
27	2	2	2	2	0.4925	-2.253210513	
27	2	2	3	1	0.6	6.72147081	420
27	2	2	3	2	0.366	-2.04834102	
27	2	3	0	1	0.7795	0	1040
27	2	3	0	2	0.4805	0	
27	2	3	1	1	0.787	15.26401708	840
27	2	3	1	2	0.5115	-3.828724218	
27	2	3	2	1	0.7345	20.14532547	1140
27	2	3	2	2	0.466	1.072980738	
27	2	3	3	1	0.5595	-4.872311461	1040
27	2	3	3	2	0.43	-7.70800773	
27	2	4	0	1	0.7795	0	1040
27	2	4	0	2	0.4805	0	
27	2	4	1	1	0.6525	14.60533399	880
27	2	4	1	2	0.566	-2.959720852	
27	2	4	2	1	0.745	2.452415449	580
27	2	4	2	2	0.4415	-3.364406833	
27	2	4	3	1	0.557	1.114516579	660
27	2	4	3	2	0.544	-8.372344781	
28	1	1	0	1		0	400
28	1	1	0	2		0	
28	1	1	1	1		-3.523779469	700
28	1	1	1	2		5.847486212	
28	1	1	2	1		-5.688794304	320
28	1	1	2	2		1.245622267	
28	1	1	3	1		-6.527039733	260
28	1	1	3	2		2.688156307	
28	1	2	0	1		0	400

28	1	2	0	2		0	
28	1	2	1	1		-2.412330833	280
28	1	2	1	2		7.627262345	
28	1	2	2	1		-2.137944466	520
28	1	2	2	2		6.011935633	
28	1	2	3	1		-2.412330833	960
28	1	2	3	2		8.738255866	
28	1	3	0	1		0	400
28	1	3	0	2		0	
28	1	3	1	1		-3.257761637	820
28	1	3	1	2		8.141153561	
28	1	3	2	1		-1.24434665	720
28	1	3	2	2		8.494294848	
28	1	3	3	1		-5.743842877	760
28	1	3	3	2		8.973663443	
28	1	4	0	1		0	400
28	1	4	0	2		0	
28	1	4	1	1		-1.015356426	400
28	1	4	1	2		2.540351659	
28	1	4	2	1		-1.909694485	680
28	1	4	2	2		5.172381414	
28	1	4	3	1		-2.004032795	940
28	1	4	3	2		6.076924326	
28	2	1	0	1		0	340
28	2	1	0	2		0	
28	2	1	1	1		4.572629864	720
28	2	1	1	2		5.340097818	
28	2	1	2	1		4.9126723	700
28	2	1	2	2		12.70519257	
28	2	1	3	1		6.476004519	580

28	2	1	3	2		8.932895471	
28	2	2	0	1		0	340
28	2	2	0	2		0	
28	2	2	1	1		9.642593975	660
28	2	2	1	2		4.035001628	
28	2	2	2	1		8.239460516	560
28	2	2	2	2		-8.366231138	
28	2	2	3	1		7.136123872	460
28	2	2	3	2		-2.415088674	
28	2	3	0	1		0	340
28	2	3	0	2		0	
28	2	3	1	1		7.30767386	540
28	2	3	1	2		-2.179486038	
28	2	3	2	1		12.168656	400
28	2	3	2	2		19.42698797	
28	2	3	3	1		8.059854683	680
28	2	3	3	2		-2.283896993	
28	2	4	0	1		0	340
28	2	4	0	2		0	
28	2	4	1	1		0.737421268	420
28	2	4	1	2		-2.049646698	
28	2	4	2	1		6.583235043	320
28	2	4	2	2		6.396602524	
28	2	4	3	1		5.570545996	620
28	2	4	3	2		-3.54742983	
29	1	1	0	1		0	380
29	1	1	0	2		0	
29	1	1	1	1		2.357367294	560
29	1	1	1	2		0.476506268	
29	1	1	2	1		3.08000318	240

29	1	1	2	2		1.09838334	
29	1	1	3	1		-0.297511614	520
29	1	1	3	2		1.061820922	
29	1	2	0	1		0	380
29	1	2	0	2		0	
29	1	2	1	1		1.71531238	340
29	1	2	1	2		-2.145528383	
29	1	2	2	1		2.191035316	760
29	1	2	2	2		1.81504882	
29	1	2	3	1		1.36219159	720
29	1	2	3	2		1.087966985	
29	1	3	0	1		0	380
29	1	3	0	2		0	
29	1	3	1	1		1.112856123	660
29	1	3	1	2		0.506011993	
29	1	3	2	1		5.638820176	420
29	1	3	2	2		4.603031398	
29	1	3	3	1		2.789061791	500
29	1	3	3	2		0.819608297	
29	1	4	0	1		0	380
29	1	4	0	2		0	
29	1	4	1	1		-0.069291529	760
29	1	4	1	2		-0.974796169	
29	1	4	2	1		-2.435931429	740
29	1	4	2	2		0.051496441	
29	1	4	3	1		1.524117722	200
29	1	4	3	2		0.301982788	
29	2	1	0	1		0	520
29	2	1	0	2		0	
29	2	1	1	1		-0.014176113	660

29	2	1	1	2		2.599308826	
29	2	1	2	1		0.653821791	520
29	2	1	2	2		3.487744939	
29	2	1	3	1		-8.66706699	460
29	2	1	3	2		-3.633773735	
29	2	2	0	1		0	520
29	2	2	0	2		0	
29	2	2	1	1		0.088653922	440
29	2	2	1	2		3.894609987	
29	2	2	2	1		0.716672736	580
29	2	2	2	2		3.077205605	
29	2	2	3	1		0.508101752	300
29	2	2	3	2		3.435532112	
29	2	3	0	1		0	520
29	2	3	0	2		0	
29	2	3	1	1		0.394779044	540
29	2	3	1	2		5.402407504	
29	2	3	2	1		0.014127235	680
29	2	3	2	2		4.795505569	
29	2	3	3	1		-0.722156878	600
29	2	3	3	2		3.924664265	
29	2	4	0	1		0	520
29	2	4	0	2		0	
29	2	4	1	1		1.500153357	720
29	2	4	1	2		2.857257393	
29	2	4	2	1		-0.129365045	620
29	2	4	2	2		3.815654358	
29	2	4	3	1		-1.617465269	260
29	2	4	3	2		3.235394582	
30	1	1	0	1	1.1785	0	800

30	1	1	0	2	1.1605	0	
30	1	1	1	1	0.778	3.005786546	500
30	1	1	1	2	0.979	1.580629177	
30	1	1	2	1	0.534	3.247752292	580
30	1	1	2	2	1.06	-1.266351164	
30	1	1	3	1	1.094	1.805545041	540
30	1	1	3	2	0.9495	-2.125969178	
30	1	2	0	1	1.1785	0	800
30	1	2	0	2	1.1605	0	
30	1	2	1	1	1.1325	2.799496965	360
30	1	2	1	2	1.249	3.116610949	
30	1	2	2	1	2.704	2.524531004	340
30	1	2	2	2	1.7485	4.312293018	
30	1	2	3	1	1.465	0.547518719	420
30	1	2	3	2	1.226	3.229281863	
30	1	3	0	1	1.1785	0	800
30	1	3	0	2	1.1605	0	
30	1	3	1	1	1.25	2.619803154	380
30	1	3	1	2	1.255	4.408762855	
30	1	3	2	1	1.027	6.15577237	220
30	1	3	2	2	1.3175	8.275752482	
30	1	3	3	1	1.748	1.10232046	540
30	1	3	3	2	1.2085	-0.140082247	
30	1	4	0	1	1.1785	0	800
30	1	4	0	2	1.1605	0	
30	1	4	1	1	1.1615	-0.967984852	700
30	1	4	1	2	1.198	4.934545131	
30	1	4	2	1	1.334	-0.124891347	860
30	1	4	2	2	1.254	1.703888118	
30	1	4	3	1	1.4175	-1.470042851	560

30	1	4	3	2	1.916	3.775241844	
30	2	1	0	1	0.761	0	1080
30	2	1	0	2	0.8395	0	
30	2	1	1	1	0.826	2.719390597	120
30	2	1	1	2	0.722	-1.406854144	
30	2	1	2	1	0.6965	1.687912497	280
30	2	1	2	2	0.723	-5.951886193	
30	2	1	3	1	0.734	-0.08479751	180
30	2	1	3	2	0.757	-1.079245937	
30	2	2	0	1	0.761	0	1080
30	2	2	0	2	0.8395	0	
30	2	2	1	1	0.689	1.435477601	320
30	2	2	1	2	0.7345	-2.742216142	
30	2	2	2	1	1.258	1.687912497	440
30	2	2	2	2	1.156	-0.812695784	
30	2	2	3	1	0.882	-0.265023302	660
30	2	2	3	2	0.878	-0.283073342	
30	2	3	0	1	0.761	0	1080
30	2	3	0	2	0.8395	0	
30	2	3	1	1	0.8325	1.549610474	220
30	2	3	1	2	0.815	-0.991970597	
30	2	3	2	1	0.74	4.609501866	80
30	2	3	2	2	0.5415	2.409822491	
30	2	3	3	1	0.7755	-0.204324496	160
30	2	3	3	2	0.772	-1.496661481	
30	2	4	0	1	0.761	0	1080
30	2	4	0	2	0.8395	0	
30	2	4	1	1	0.7615	-1.884167289	380
30	2	4	1	2	0.742	0.312017539	
30	2	4	2	1	0.727	0.453300672	340

30	2	4	2	2	0.734	-0.124400016	
30	2	4	3	1	0.7275	-2.561234629	520
30	2	4	3	2	0.7135	0.319174288	
31	1	1	0	1	0.665	0	240
31	1	1	0	2	1.092	0	
31	1	1	1	1	0.2175	6.685558547	600
31	1	1	1	2	0.585	2.080532274	
31	1	1	2	1	0.371	3.246815186	320
31	1	1	2	2	0.6045	0.807092454	
31	1	1	3	1	0.724	8.763429259	620
31	1	1	3	2	0.8525	-0.672336259	
31	1	2	0	1	0.665	0	240
31	1	2	0	2	1.092	0	
31	1	2	1	1	0.526	9.574182091	340
31	1	2	1	2	0.578	2.553114401	
31	1	2	2	1	0.572	8.674950748	580
31	1	2	2	2	0.676	3.432408596	
31	1	2	3	1	0.5865	10.61341398	440
31	1	2	3	2	0.8565	3.590464898	
31	1	3	0	1	0.665	0	240
31	1	3	0	2	1.092	0	
31	1	3	1	1	0.3635	8.895006309	500
31	1	3	1	2	0.791	3.803092667	
31	1	3	2	1	0.453	10.37203583	740
31	1	3	2	2	0.9025	5.583265721	
31	1	3	3	1	0.1275	9.7653075	840
31	1	3	3	2	0.881	1.487312446	
31	1	4	0	1	0.665	0	240
31	1	4	0	2	1.092	0	
31	1	4	1	1	0.3205	12.34449138	420

31	1	4	1	2	0.8605	0.741943673	
31	1	4	2	1	0.5885	16.07651839	780
31	1	4	2	2	0.8405	3.402447537	
31	1	4	3	1	0.3885	12.73759218	260
31	1	4	3	2	0.541	2.961437477	
31	2	1	0	1	0.746	0	500
31	2	1	0	2	0.8975	0	
31	2	1	1	1	0.6425	-2.768780745	260
31	2	1	1	2	0.7955	-3.335539196	
31	2	1	2	1	0.7085	0.239260162	660
31	2	1	2	2	0.8485	-3.8468639	
31	2	1	3	1	0.77	4.673745487	400
31	2	1	3	2	0.88	-6.4838045	
31	2	2	0	1	0.746	0	500
31	2	2	0	2	0.8975	0	
31	2	2	1	1	0.6775	3.979530034	540
31	2	2	1	2	0.802	1.163765233	
31	2	2	2	1	0.744	0.49475557	600
31	2	2	2	2	0.836	2.556666526	
31	2	2	3	1	0.7505	7.461132936	320
31	2	2	3	2	0.832	1.699164721	
31	2	3	0	1	0.746	0	500
31	2	3	0	2	0.8975	0	
31	2	3	1	1	0.6585	2.44128522	500
31	2	3	1	2	0.777	0.296467638	
31	2	3	2	1	0.7135	-0.779837156	540
31	2	3	2	2	0.7015	1.603232175	
31	2	3	3	1	0.6945	5.387774171	580
31	2	3	3	2	0.753	2.000029392	
31	2	4	0	1	0.746	0	500

31	2	4	0	2	0.8975	0	
31	2	4	1	1	0.658	3.32901121	700
31	2	4	1	2	0.7665	-1.279037015	
31	2	4	2	1	0.7095	-0.779837156	520
31	2	4	2	2	0.76	-0.807029476	
31	2	4	3	1	0.664	3.745089691	840
31	2	4	3	2	0.7745	-0.333961787	