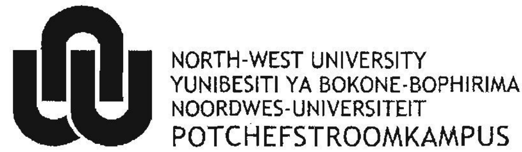

PRE-CLINICAL EVALUATION OF THE POSSIBLE ENHANCEMENT OF THE EFFICACY OF ANTIRETROVIRAL DRUGS BY PHEROID™ TECHNOLOGY.

M. M. Botha (*B.Pharm.*)

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Supervisor: Mrs. A. F. Grobler

Co-supervisor: Prof. A. F. Kotze

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// ABSTRACT

HIV/AIDS is the most threatening and challenging infectious diseases of our time, with the highest increase of newly infected cases reported. This infectious disease was discovered in the early eighties under homosexual men and was later to be discovered in heterosexuals. HIV is a systemic immunosuppressive disorder which causes a depletion of $CD4^+$ T cells and develops into the acquired immunodeficiency syndrome – AIDS.

Africa is the continent most affected by HIV/AIDS with the southern parts of Africa having the highest prevalence rates compared to the rest of Africa. Statistics indicate that AIDS is responsible for 3% of deaths in children worldwide - one in seven people dying of an HIV-related illness is a child under the age of 15 years. It was stated by the WHO that countries should develop improved antiretrovirals regimes for the prevention of mother-to-child transmission.

Difficulties in administering antiretrovirals (ARVs) to patients (especially children) are the strict dosage regimes and the severe adverse reactions. These factors complicate patient adherence. The list of problems in treating patients is endless and includes the distribution, stability as well as the low efficacy of these drugs.

Most of the above mentioned problems and obstacles related to ARVs and ARV treatment could be minimized or eliminated by the use of a stable and effective drug delivery system. Enhancing ARV treatment may be accomplished by the use of the Pheroid™ drug delivery system. Pheroids™ consists mainly of fatty acids and sterile nitrous oxide gassed water. Pharmacological active substances are entrapped into submicron and micron sized structures called Pheroids™. Research showed promising results and advantages in delivering drugs through oral and transdermal routes using Pheroid™ technology.

The focus of this study was to test the possible enhancement of the efficacy of antiretrovirals using Pheroid™ technology. The assays used to study this possible enhancement were a modified neutral red and a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. These assays confirmed and illustrated the toxic and protective properties of the tested ARVs (stavudine, lamivudine and nevirapine). An MT-2 cell line was used and infected with an HIV-1 strain, SW7-TCL.

Applying Pheroid™ technology in these assays resulted in massive cell death, due to increased ARV toxic levels within the cells. Viability tests proved that Pheroids™ had no effect on the viability of cells at the concentration typically used. This confirmed the enhancing properties of Pheroids™ in the delivery of drugs into the cells. The MTT assay was further adapted from a seven day incubation period to a three day incubation period. By using a low concentration series and a three day incubation period the loss of cells through toxicity was partially overcome.

One of the problems that arose form this study was the non-reproducibility of the results. Absorbance levels fluctuated at specific concentrations of the same ARV, which cause difficulties in comparing results. This result was repeatedly confirmed in this syncytium forming infection model.

In conclusion, Pheroid™ technology enhanced the delivery of ARVs into the cells although it resulted in cell death. Both the neutral red and MTT assays were found to be inaccurate but further development, research and assay optimization could result in improved *in vitro* studies.

The article format was used for this thesis, as described in the general academic rules in section A.13.7.3 of the North West University. Chapter 1 deals with HIV/AIDS related problems, statistics and treatment obstacles. Chapter 2 is a summary of the cell viability assays used in this study. Pheroid™ technology and its application to ARV treatment are dealt with in chapter 3. The proposed article for submission in the journal Cell Death and Differentiation has been included in chapter 4. Some of the results from the study are reported in the article and annexures, whilst other results are shown and discussed in Chapter 5. Chapter 6 gives a conclusion and final summary of this study. All other experimental methods and results are enclosed in the annexures, as is the "Guide for authors" for the article.

Keywords:

Lamivudine (3TC), stavudine (d4T), MTT, Finter's neutral red, Pheroid™, antiretroviral (ARV), HIV and AIDS, viability.

UITTREKSEL

Menslike immuniteit gebrek virus (MIV) en die geassosieerde sindroom is een van die grootste uitdagings van ons tyd, met die hoogste groeisyfer in terme van nuut geïnfekteerde gevalle. MIV is in homoseksuele mans in die vroeë tagtigs ontdek, maar die voorkoms het daarna ook uitgebrei na heteroseksuele persone. MIV veroorsaak ŉ sistemiese immuunonderdrukking sindroom wat gepaard gaan met ŉ kenmerkende afname in die $CD4^{+}$ T selle, wat op sy beurt lei tot die ontwikkeling van die verworwe immuniteitsgebrek sindroom – VIGS.

Die kontinent wat die meeste deur MIV/VIGS geraak word, is Afrika en veral die Suide van Afrika wat die hoogste prevalensie toon. VIGS is verantwoordelik vir 3% van die wêreld se sterftes onder kinders – een uit elke sewe mense wat sterf aan ŉ MIV-verwante siekte is ŉ kind onder die ouderdom van vyftien. Die WGO (Wêreld Gesondheidsorganisasie) het voorgestel dat alle ontwikkelende lande antiretrovirale regimes vir die voorkoming van moeder-tot-kind oordrag ontwikkel, omdat dit een van die grootste oorsake van MIV infeksies is.

Die antiretrovirale middels (ARVs) is streng dosis-afhanklik. Een van die struikelblokke wat die behandeling van MIV/VIGS bemoeilik, is die feit dat pasiënte die middels nie met toegewydheid neem nie weens die ernstige newe-effekte wat hulle ervaar. Verspreiding en die lae stabiliteit en effektiwiteit van die middels is kompliserende faktore. Meeste van hierdie probleme kan verminder of geëlimineer word deur gebruik te maak van ŉ stabiele en effektiewe geneesmiddel afleweringsisteem soos byvoorbeeld Pheroid™ geneesmiddel aflewering tegnologie. Pheroids™ bestaan hoofsaaklik uit essensiële vetsure en N_2O gegasde water. Farmakologies-aktiewe middels kan binne sub-mikron en mikron strukture (Pheroids™) vasgevang word. Navorsing het reeds belowende resultate en voordele van Pheroid™ tegnologie t.o.v. die aflewering van middels deur die transdermale en orale roetes uitgewys.

Die klem van hierdie studie het op die moontlike verbetering van die effektiwiteit van antiretrovirale middels deur die gebruik van Pheroid™ tegnologie geval. Die toetssisteme (assays) wat in hierdie studie vir die analisering van die moontlike

verbetering in effektiwiteit gebruik is, is die MTT- en Finter se neutrale rooi lewensvatbaarheid (viability) toetssisteme. Deur die gebruik van hierdie toetssisteme kan die toksiese effek sowel as die beskerming wat die geneesmiddel teen MIV in selkultuur studies bied, bepaal word. In dié ondersoek is die toksiese en beskermende effekte van drie antiretrovirale middels lamivudien, stavudien en nevirapien bepaal in 'n infeksie model bestaande uit 'n MT-2 sellyn wat geïnfekteer is met 'n MIV-1 virus genaamd SW7-TCL. Sodanige infeksie lei tot die vorming van sinsitiums (fusie van selle).

Die inkorporering van antiretrovirale middels in Pheroids™ het in hierdie toetssisteem tot die afsterwing van die selle gelei as gevolg van 'n verhoogde aflewering van die geneesmiddel en gevolglike toksiese vlakke van die middels in die selle. Pheroids™ het met die algemeen gebruikte verdunning van 1:1000 (verdun met N_2O-gegasde water) self geen effek op die lewensvatbaarheid van selle gehad nie; inteendeel, Pheroids™ is nie verantwoordelik vir die afsterwing van selle nie. Die blootstelling van die selle aan toksiese intrasellulêre geneesmiddel vlakke was dus vir die afsterwing van die selle verantwoordelik.

Die MTT toetssisteem wat gebruik is, is vanaf 'n 7-dag na 'n 3-dag inkubasie periode verkort en 'n laer geneesmiddel konsentrasie reeks is gebruik, ten einde die afsterwing van die selle te probeer voorkom. Dié twee veranderinge in die uitvoering van die studie protokol was gedeeltelik geslaagd.

Een van die probleme van hierdie ondersoek was dat die data nie reproduseerbaar of herhaalbaar was nie. Die absorpsie waargeneem vir spesifieke konsentrasies van dieselfde antiretrovirale geneesmiddel het verskil, wat die vergelyking van resultate bemoeilik het. 'n Ondersoek in die verband het bevestig dat die MTT toetssisteem nie produseerbaar in die sinsitium-vormende selle is nie.

Ten slotte, Pheroid™ tegnologie het wel die aflewering van geneesmiddels binne-in die selle verhoog, alhoewel die verbeterde aflewering terselfde tyd die afsterwing van selle veroorsaak het. Resultate verkry met beide die MTT en Finter se neutrale rooi lewensvatbaarheid toetssisteme was nie herhaalbaar of reproduseerbaar nie. Deur verdere ontwikkeling, navorsing en toetssisteem optimalisering kan *in vitro* studies verbeter word.

Hierdie verhandeling word voorgelê in die artikel formaat, soos vervat in die in die akademiese reëls, afdeling A.13.7.3 van die Noordwes Universiteit. Hoofstuk 1 handel oor MIV/VIGS verwante probleme, statistiek en struikelblokke met betrekking tot die behandeling van VIGS. Hoofstuk 2 beskryf die lewensvatbaarheid toetssisteme soos in die studie gebruik is, terwyl hoofstuk 3 oor die gebruik van Pheroid™ tegnologie met betrekking tot antiretrovirale middels handel. Die artikel vir die moontlike publisering in die joernaal "Cell Death and Differentiation" is vervat in hoofstuk 4. Van die resultate is opgeteken in die artikel en die addendums terwyl ander weer in hoofstuk 5 beskryf word. Hoofstuk 6 bevat 'n finale opsomming en afsluiting van die studie, terwyl alle ander resultate en metodes, asook die "Guide for Authors" vir die skryf van die artikel in die addendums saamgevat is.

Sleutel woorde:
Lamivudine (3TC), stavudine (d4T), MTT, Finter's neutrale rooi, Pheroid™, antiretrovirale middels (ARV), MIV en VIGS, lewensvatbaarheid sisteem.

PROBLEM STATEMENT, AIM AND OBJECTIVES

The treatment and management of HIV/AIDS was transformed with the introduction of highly active antiretroviral therapy (HAART). The change in treatment had a great impact on patients with increased lifespan of patients and decreased prevalence of opportunistic infections. The complexity of these regimes lies mainly in the severe side effects, patient adherence and an increase in the viral resistance to these drugs. The complexity of paediatric treatment regimes, administration and dosages adapted from that of the adult dose are problematic.

The aim of this study was to adhere to the need, stated by the WHO, of developing improved antiretroviral regimes, increasing the bioavailability and lessening adverse reactions. Using Pheroid™ technology the therapeutic effect of antiretrovirals could perhaps be enhanced on the one hand with a decrease of side effects on the other hand.

The main objectives of this study were:

- To conduct a literature overview of HIV/AIDS and the treatment thereof.
- Development of an effective *in vitro* assay for the simultaneous determination of the viability and toxicity of antiretrovirals.
- Using Pheroid™ technology in the application of these drugs in order to enhance the *in vitro* effect on the protection of cells against HIV-1 infection.
- Enhancing the efficacy of paediatric antiretrovirals and treatment using Pheroid™ technology.

CHAPTER 1

HIV and AIDS: Introduction, history, pathology and antiretroviral therapy

1.1 Introduction

The first indications of the emergence of an immunosuppressant disease were noted in 1982 when 5 previously healthy, homosexual men were diagnosed with pneumocystis carinii pneumonia. Soon reports followed that they were infected with a virus which has immunosuppressant properties. Names varied from lymphadenopathy associated virus (LAV), human T cell lymphotropic virus III (HTVL-III) and then finally human immunodeficiency virus (HIV). Two types of human immunodeficiency viruses (HIV) have been identified – HIV 1 and HIV 2 (HIV/AIDS Update, 2004).

Human immunodeficiency virus type 1 is considered a systemic immunosuppressive disorder caused by progressive depletion of $CD4^+$ T cells (Fauci, 1993). Several mechanisms have been suggested for the depletion of $CD4^+$ T cells, such as HIV-1 induced cytopathicity, syncytium formation and immune responses to the infected cells (Levy, 1993). This leads to an asymptomatic period, which may vary in length. A large portion of these patients develop the acquired immunodeficiency syndrome (AIDS) (Koot *et al.,* 1993).

1.2 Statistics on incidence

In 2003 the Centre for Disease Control (CDC) announced a 2% increase in the number of new AIDS cases since 2001, with an 18% increase since 1999 in new HIV cases in gay and bisexual men. ARV use has increased by 15% in children and young adults, with the greatest increase in the 13 to 15 year old group. An estimated 40 million people will, according to the Joint Nations Programme, be infected with HIV or will be living with AIDS. Approximately 37.5 million are adults and 2.5 million are children under the age of 15 years. There were approximately 3 million deaths from AIDS in 2003.

About 95% HIV/AIDS cases are in developing countries (HIV/AIDS Update, 2004). The WHO (World Health Organization) Africa Region stated that by the end of 2004 two thirds of the world's people diagnosed with HIV/AIDS would be living in this region. It implies that in this region 9 out of 100 people, between the ages of 15-49 will be HIV positive; thus a total of 26

million people infected with the virus. It is also estimated that 4.7 million need antiretroviral drugs (ARVs) (WHO, 2005).

The WHO has identified particular groups with higher risks for HIV infection. They include sex workers and their clients, long-distance truck drivers, the military, sexual transmitted disease (STD) patients, migrant labourers and youth (WHO, 2005).

1.2.1 *The current situation in Africa*

The following data has been generated by the WHO (2003-2004) in selected countries from the population-based HIV sero-surveys conducted in the region since 2000: HIV prevalence rates varied from <1% in Comoros, Seychelles and Madagascar, Cape Verde to 38.5% in Botswana and 42.2% in Swaziland in 2003 and 2004. Southern Africa had the highest prevalence rates compared to the rest of Africa. The median HIV prevalence rates among antenatal clinic attendees varied from 2.4% in Angola to 16.2% in Mozambique, 27.9% in South Africa and 42.2% in Swaziland. Overall national HIV prevalence rates among adults in the general population (men and woman) aged 15-49 years varied from 0.7% in Senegal to 23.6% in Lesotho (WHO, 2005).

In South Africa, a recent study of the death registration data has shown that HIV/AIDS related deaths among people 15 years of age and older increased by 62% in 1997-2002, with deaths among South Africans aged 25-44 years more than doubling. A revision of the vital registration system based on almost 2.9 million death certificates showed that more than 33% of deaths were among young adults. The proportion of total deaths occurring in the 25-44 year group increased as follows: 23.2% (1997), 25% (1998), 27.7% (1999), 29.9% (2000), 31.7% (2001), 33.6% (2002) (Dorrington *et al.*, 2001).

1.2.2 HIV in children

90% of the 1500 children that become infected with HIV daily are living in sub-Saharan Africa. Global statistics indicated that 2.3 million children that were living with HIV in 2005, acquired the virus *in-utero*. AIDS is responsible for 3% of the deaths in children under the age of 5 years worldwide, and 6% of those who live in sub-Saharan Africa. One in seven people dying of an HIV-related illness worldwide is a child under the age of 15 years. In 2005, an estimated 380 000 children died of HIV or HIV-related causes. Half of them did not live beyond their second birthday (WHO, 2006).

Statistics on the estimated number of children (0-14 years) living with HIV in 2005:

- Sub-Saharan Africa 2 000 000

- Latin America 32 000
- Caribbean 22 000
- North America and Europe 15 000
- North Africa and Middle East 31 000
- Eastern Europe and Central Asia 6 900
- Asia and Pacific 170 000

(WHO, 2006 a).

The United Nations Population Division, 2004 revision, estimated the impact of AIDS on under-five mortality rates for the period 2002-2005 in selected countries in sub-Saharan Africa. These statistics are given in table 1.1

Table 1.1: Impact of AIDS on child mortality

Country	Deaths per 1000 live births with AIDS	Deaths per 1000 live births without AIDS
Botswana	106	42
Kenya	118	98
Lesotho	123	70
Namibia	78	43
South Africa	74	43
Swaziland	143	73
Zambia	173	142
Zimbabwe	117	78

(WHO, 2006 a).

The high mortality rate of children in Zambia could be due to the following:

- The levels of child malnutrition in Zambia deteriorated from being 25% underweight in 1999 to 31% in 2002. Reasons given as the cause are drought as well as the impact of HIV/AIDS;

- Zambia showed some of the highest tendencies in children being underdeveloped in Africa, with 51.9% of children less than five years of age stunted;
- Urbanized areas in Zambia showed a greater degree of deterioration in child malnutrition.

(UNICEF, 2004)

1.3 HIV infection and the variability of strains

1.3.1 Classes of HIV

According to the literature, three categories of HIV-1 can be distinguished according to genetic variability, rapid evolution and diversification. Genetic classifications of HIV-1 are as follows: a major group (group M), an outlier group (group O) and a non-M/non-O group (group N). Most of the widespread HIV-1 strains are classified as group M viruses, which can be further subdivided into different genetic subtypes, labelled with letters form A to K. "Overall it was estimated that HIV-1 genetic subtypes and circulating recombinant forms play a different role in the regional sub-epidemics". The subtype C strains are responsible for 50% of all infections worldwide, with subtypes A, B, D and G, responsible for 12%, 10%, 3%, and 6%, respectively. The subtypes F, H, J and K together caused 0.94% of infections. The circulating recombinant forms CRF01_AE and CRF02_AG were each responsible for 5% of cases. CRF03_AB only plays a small role in Eastern Europe and Central Asia. The remaining 8% of all infections are due to other recombinant forms. Thus, 18% of infections worldwide are due to infections with recombinant HIV forms (WHO, 2005).

The variability of the strains makes it more difficult to treat the disease specifically. Investing in a method to treat this problem more efficiently could increase the life span of children.

1.3.2 Syncytium-inducing variants

Koot *et al.* (1993) studied the relation between the appearance of syncytium-inducing (SI) variants, the rate of $CD4^+$ cell depletion and the clinical progression of HIV-1 infection. This study was performed with two groups of men, with or without SI variants at the start of the study. It was found that after 30 months, the possibility of progression to AIDS for the group with SI isolates was 70.8% in contrast to the group without SI isolates, which was 15.8%. Thus it was concluded that the appearance of SI variants is prognostic for a rapid decline of $CD4^+$ cell counts. It was also found that patients with SI isolates were more severely immuno-compromised than patients without SI isolates at the time of AIDS diagnosis (Koot *et al.,* 1993).

The variability of strains therefore induces different immunological responses, which make treatment more complicated.

1.4 Antiretroviral therapy

The treatment as well as the management of HIV was transformed in the late 1990's with the introduction of highly active antiretroviral therapy (HAART). The change in treatment had a great impact on patients; patients were not only living longer, but opportunistic infections were decreasing. Antiretroviral regimens are of the more complex treatment regimes, have severe side effects, and adherence to the number of medications that must be taken on a daily basis is problematic. There is also the risk of viral resistance because of non-compliance or suboptimal levels of antiretroviral drugs (HIV/AIDS Update, 2004).

Advantages of early treatment with ARVs are the following:

- Viral load suppression;
- Immune system protection and the avoidance of opportunistic infections;
- Lengthened survival;
- Decrease in viral spread.

Disadvantages include:

- Inhibition on the quality of life, due to severe side-effects;
- Decreased obedience in treatment;
- Drug resistance development;
- Long term use toxicity;
- Transmission of HIV resistance to current drugs.

(HIV/AIDS Update, 2004).

The primary goal of antiretroviral therapy is to decrease HIV related morbidity and mortality. The patient should experience fewer HIV related illnesses, $CD4^{+}$ count should rise and remain above the baseline (initial $CD4^{+}$) count and the viral load should become undetectable (<400 copies/ml) and remain undetectable on ARV therapy. The overall quality of life should be improved. The secondary goal is to decrease the incidence of HIV through the increased uptake of voluntary testing and counselling and practicing safer sex, the reduction of transmission in discordant couples and reducing the risk of transmission from mother to child (DHHS, 2004).

Antiretroviral therapy will be discussed in general with regards to adult treatment, but the specific treatment regimes will be discussed according to paediatric recommended regimes, which are the focus of this study.

1.4.1 Initiation of HAART therapy: patient selection criteria.

The latest guidelines by the WHO (March 23, 2004) recommend the initiation of antiretroviral therapy in asymptomatic patients that have a $CD4^{+}T$ count (cells/mm^3) less than or equivalent to 350/mm^3 or plasma HIV RNA levels greater than 55,000 copies/ml (HIV/AIDS Update, 2004).

The clinical staging of the WHO divides patients in 4 stages. Some of the symptoms will be highlighted.

- Clinical stage 1: Asymptomatic, persistent generalized lymphadenopathy.
- Clinical stage 2: Persistent hepatosplenomegaly, papular pruritic eruptions, wart virus infection, recurrent oral ulcerations, herpes zoster and fungal nail infections.
- Clinical stage 3: Unexplained moderate malnutrition, unexplained persistent diarrhoea (14 days or more), unexplained persistent fever (above 37.5 °C), oral candidiases, oral hairy leukoplakia, acute necrotizing ulcerative gingivitis, pulmonary tuberculosis (TB) and unexplained anaemia.
- Clinical stage 4: Unexplained severe wasting, stunting or severe malnutrition, *pneumocystis pneumonia*, recurrent severe bacterial infections, chronic herpes simplex infection, extrapulmonary TB, Kaposi sarcoma, oesophageal candidiasis, CNS toxoplasmosis, cytomegalovirus infection, chronic cryptosporidiosis, cerebral or B cell non-Hodgkin lymphoma and HIV-associated cardiomyopathy (WHO, 2006 b).

The WHO recommendations for ARV treatment initiation in infants and children can be summarized as follows:

1. Infants and children with established HIV infection should be started on antiretroviral therapy if they have:
    - WHO defined paediatric clinical stage 4 disease (irrespective of $CD4^{+}$ count);
    - WHO defined paediatric clinical stage 3 disease (irrespective of $CD4^{+}$ count, but it may be used as guidance); children aged over 12 months with tuberculosis, lymphocytic interstitial pneumonia, oral hairy leukoplakia or thrombocytopaenia.

ARV initiation may be delayed if the $CD4^+$ count is available and above threshold values for initiating ART;

- WHO defined paediatric clinical stage 2 disease and $CD4^+$ count at or below threshold;
- WHO defined paediatric clinical stage 1 disease and $CD4^+$ count at or below threshold (WHO, 2006 b).

2. If virological testing is not available to confirm HIV infection, HIV antibody-positive infants and children under the age of 18 months should be considered for ARV treatment if they have clinically diagnosed presumed severe HIV disease (WHO, 2006 b).

South African patient selection criteria differ from the international (WHO) criteria. According to the National Antiretroviral Therapy Guideline of South-Africa (2004), the following factors must be met before a child is considered for therapy:

Medical criteria:

- Recurrent hospitalizations (>2 admissions per year) for HIV-related disease, or prolonged hospitalization (>4 weeks), OR
- Modified WHO defined stage 2 or 3 disease, OR
- $CD4^+$ percentage <20% in a child under 18 months old, irrespective of disease stage, OR
- $CD4^+$ percentage <15% in a child over 18 months old, irrespective of disease stage.

Psychosocial criteria:

- An identifiable adult who is able to administrate medication;
- Demonstrated reliability in the adult caregiver;
- Supportive social environment;
- Another adult living in the same household is encouraged so that there is someone else who can assist with the child's ARV dosing.

1.4.2 Classification of ARVs and regimes

ARV drugs are classified based on the specific step in the viral cycle that it inhibits. The four groups include NRTIs (nucleoside reverse trancriptase inhibitors), NNRTIs (non-nucleoside reverse transcriptase inhibitors), PIs (protease inhibitors) and FIs (fusion inhibitors).

1.4.2.1 NRTIs (nucleoside reverse transcriptase inhibitors)

NRTIs target the reverse transcriptase of HIV (after the drug has been phosphorylated to a triphosphate) and proceeds as a chain terminator in the reverse transcriptase reaction (De Clercq, 2004; Ho and Hitchcock, 1989).

- Zidovudine (AZT, ZDV): well tolerated in children, but associated with metabolic complications of therapy, although to a lesser extent than stavudine (d4T). This drug can cause severe drug-related side effects (anaemia and neutropenia). d4T may be a substitute for AZT in intolerance, and *vice versa*, except in cases of lactic acidosis. Do not administer AZT in combination with d4T.

- Didanosine (ddI): This drug is usually reserved for second-line regimes. It has weak acid stability and is easily damaged by stomach acid. It also has a low, but rapid absorption.

- Lamivudine (3TC): a potent NRTI with good efficacy, safety and tolerability in HIV-infected children, and the core of NRTI therapy.

- Stavudine (d4T): better tolerated than AZT, with no laboratory requirements as to the monitoring of hemoglobin.

- Abacavir (ABC): Reports have shown that ABC-containing dual NRTI regimes are more effective than regimes containing AZT + 3TC in children with HIV-1 who have not been treated previously. This drug has very little hematological toxicity. It may serve as a good backbone for NRTI therapy with NNRTIs as part of a triple nucleoside regimen.

- Tenofovir (TDF): The use of tenofovir in the therapy of children should not be encouraged due to the lack of safety data.

- Emtricitabine (FTC): a newer NRTI, which has been included for the treatment of adults and children. Its resistance is similar to 3TC, due to its similarity in structure. It can be given to children over 3 months of age, as a substitute to 3TC.

(WHO, 2006 b).

1.4.2.2 NNRTIs (non-nucleoside reverse transcriptase inhibitors)

This group targets an allosteric, non-substrate binding site of the HIV-1 reverse transcriptase (De Clercq, 2004) and interrupts the enzyme's catalytic site (Veldkamp *et al.,* 2001).

- Nevirapine (NVP): This drug should be used in combination with other ARVs, except when used for perinatal HIV prophylaxis. A high occurrence of rash is commonly associated with NVP which may be severe and/or life threatening. It is not a recommended choice for treatment in children.

- Efavirenz (EFV): This drug is not recommended for use in children under the age of three years due to no established dosing regimes. It induces rash, teratogenicity and toxicities related to the central nervous system. NVP is the better choice of drug in this class.

(WHO, 2006 b).

1.4.2.3 PIs (*protease inhibitors*)

This group inhibits viral multiplication by means of a transition-state, hydroxyethylene-based, peptidomimetic inhibitor of the HIV protease (De Clercq, 2004).

- Ritonavir (RTV): RTV is one of the only ARV drugs that inhibit a liver enzyme that usually metabolizes PIs. RTV requires the use of a cold chain, and has poor tolerability.

- Lopinavir (LPV): LPV is one of the drugs preferred for second-line regimes only.

- Indinavir (IDV): Indinavir is not often used, due to the lack of suitable paediatric formulations.

- Nelfinavir (NFV): This drug is an alternative for the PI component if an RTV-enhanced PI is not available. It has poor tolerability.

- Saquinavir (SQV): As with LPV, it is one of the drugs preferred only for second-line regimens.

1.4.2.4 FI (fusion inhibitor)

FI inhibit virus-cell fusion (De Clercq, 2004).

- Enfuvirtide (T-20): T-20 inhibits virus-cell fusion and is being used in combination treatment with other anti-HIV agents in the treatment of HIV-1 infection (De Clercq, 2004).

(WHO, 2006 b).

1.4.3 International treatment regimes

The ideal option when choosing a first-line regimen is to combine two NRTIs and one NNRTI. This combination prevents HIV replication by inhibition of reverse transcriptase. Such NRTI/NNRTI regimens are effective and less expensive, generic formulations are often available and a cold chain is not required. Protease inhibitors (PIs) are also included in a second line regimen (WHO 2006).

1.4.3.1 First-line regimen

The following combinations are recommended as a first-line regimen for children (2 NRTIs + NNRTI):

AZT + 3TC + NVP/EFV or

d4T + 3TC + NVP/EFV or

ABC + 3TC + NVP/EFV

A triple NRTI regimen (AZT/d4T + 3TC + ABC) can be considered as an alternative for simplifying initial therapy in special circumstances (WHO, 2006 b).

1.4.3.2 Second-line regimen

In the case of treatment failure, the complete first-line regimen should be changed to a second-line regimen, which should include at least three new drugs. Using a second-line drug in children is difficult, due to a lack of information and research. It is recommended by the WHO that a new regimen should be based on a protease inhibitor (PI), with ritonavir (RTV) as booster and given in combination with two new NRTIs, with ddI as one of the two.

Due to cross resistance, AZT + ddI would be the alternative regimen.

In the event of treatment failure with 2 NRTIs and 1 NNRTI, the following can be given as an alternative second-line regimen:

- In the case of treatment failure with a regime containing AZT or d4T, substitute the regime with the following:

  ddI + ABC + LPV or SQC or NFV

- In the case of treatment failure with a regime containing ABC, substitute the regime with:

  ddI + AZT + LPV or SQC or NFV

In the case of treatment failure with three NRTIs the following regime is proposed:

ddI + EFV/NVP + LPV or SQC or NFV

(WHO, 2006 b).

1.4.4 South-African treatment regimens

In 2003, 20 antiretrovirals were approved by the Department of Health of South-Africa. These antiretrovirals can be divided into 4 classes, with combination therapy of 3 drugs in each regime, as described for HAART therapy (DOH, 2004).

The following regimes have been categorized into first- and second-line regimes, fridge (cold storage) availability and age or weight.

1.4.4.1 First-line regimen

1.4.4.1.1 Cold storage availability

For children under the age of 3 years the following regimes have been proposed by the Department of Health (South-Africa):

- Stavudine (d4T) with lamivudine (3TC) and ritonavir (RTV) or,
- Stavudine (d4T) with lamivudine (3TC) and nevirapine (NVP) for those not previously exposed to NVP.

The proposed regime for children reaching the age of 3 years or older:

Stavudine (d4T) with lamivudine (3TC) and efavirenz (T-20) (GOV, 2004).

1.4.4.1.2 Cold storage not available

The following regimes have been proposed for children under the age of 3 years:

- Zidovudine (AZT) with lamivudine (3TC) and ritonavir (RTV) or,
- Zidovudine (AZT) with lamivudine (3TC) and nevirapine (NVP) for those not previously exposed to NVP (DOH, 2004).

1.4.4.2 Second-line regimen

There are three reasons to move to second-line therapy in children: virological (rebound of the viral load baseline), clinical (if there is a persistent oral thrush not responding to

treatment) and immunological (a persistent decline in the $CD4^+$ count over a period of two months without the presence of TB).

The following regimes have been categorized into first- and second-line regimes, fridge (cold storage) availability and age or weight.

1.4.4.2.1 Cold storage availability

These second-line regimens have been proposed for children under the age of 3 years or below a body weight of 10 kg:

- Didanosine (ddI) with zidovudine (AZT) and lopinavir (LPV) or,
- Didanosine (ddI) with zidovudine (AZT) and ritonavir (RTV) or,
- Didanosine (ddI) with zidovudine (AZT) and nevirapine (NVP) for those not previously exposed to NVP.

The following regimens have been proposed for children over the age of 3 years and with a body weight above 10 kg:

- Didanosine (ddI) with zidovudine (AZT) and lopinavir (LPV) or,
- Didanosine (ddI) with zidovudine (AZT) and ritonavir (RTV) (DOH, 2004).

1.4.4.2.2 Cold storage not available

The following regimes have been proposed by the Department of Health where cold storage is not available for children under the age of 3 years or under a body mass of 10 kg:

- Didanosine (ddI) with abacavir (ABC) and lopinavir (LPV) or,
- Didanosine (ddI) with abacavir (ABC) and ritonavir (RTV) or,
- Didanosine (ddI) with abacavir (ABC) and nevirapine (NVP) for those not previously exposed to NVP.

The following second-line regime has been proposed for children above the age of 3 years or with a body weight above 10 kg:

- Didanosine (ddI) with zidovudine (AZT) and lopinavir (LPV) or
- Didanosine (ddI) with zidovudine (AZT) and ritonavir (RTV).

(DOH, 2004).

1.4.5 Adverse reactions

The following reflects a summary of the above mentioned antiretrovirals' adverse reactions. The selected safety monitoring will also be discussed.

- Abacavir (ABC): within 6 weeks of treatment initiation a potentially fatal hypersensitivity reaction may develop in 3-5% of patients. Other symptoms (2 or more present) that could suggest a hypersensitivity reaction are fever, a maculopapular pruritic rash, gastrointestinal (GI) symptoms, pharyngitis, dyspnoea, coughing, musculoskeletal disorders, malaise, fatigue, lymphadenopathy and paraesthesia. Never administer abacavir to a child who has previously developed an abacavir hypersensitivity reaction. Fasting cholesterol and triglycerides at baseline should be determined with a 6 month follow-up after therapy initiation, and every 12 months thereafter.
- Didanosine (ddI): pancreatitis, peripheral neuropathy, GI effects (bloating, flatulence, nausea and diarrhoea) and lactic acidosis. Safety should be monitored clinically.
- Efavirenz (EFV): CNS disturbances (dysphoria, vivid dreams, distractedness and dizziness), GI symptoms and skin rash. Safety monitoring as for didanosine.
- Lamivudine (3TC): diarrhoea, pancreatitis and lactic acidosis. Safety monitoring as for didanosine.
- Lopinavir (LPV): GI symptoms, lipid abnormalities (in 5% of patients) and lypodystrophy changes. Safety monitoring as for ABC.
- Ritonavir (RTV): GI symptoms, bad taste, raised liver enzyme activity, raised cholesterol and triglycerides, lipid abnormalities (in 5% of patients) and lypodystrophy changes. Safety monitoring as for ABC.
- Nevirapine (NVP): Skin rash (occurs in 16% of patients), nausea, vomiting and hepatitis which can be fatal. Safety monitoring as for ABC but including a $CD4^+$ count.
- Stavudine (d4T): Peripheral neuropathy, hepatic steatosis, lactic acidosis and pancreatitis. Safety monitoring as for didanosine.
- Zidovudine (AZT): Bone marrow suppression (anaemia and neutropenia), GI symptoms, myopathy, lactic acidosis and cardio-myopathy in children. Safety monitoring includes a full blood count (FBC) before the initiation of therapy, repeated every month for the first 3 months, then a FBC every 6th month, including $CD4^+$ and viral load counts (NOH, 2004).

1.4.6 Mitochondrial toxicity and HIV therapy (NRTIs)

HIV requires integration of its genetic material in the host cell genome after entering the cell. To enable the virus to do this, it needs to convert its single stranded viral RNA into double stranded DNA by means of the enzyme reverse transcriptase. NRTIs bear a resemblance to the natural nucleosides without the free 3' hydroxyl group. When the NRTI is incorporated into the growing DNA, termination occurs (White, 2001). This may also occur in the mitochondria.

Adverse effects associated with ARVs have been highlighted in the previous discussion. Some of these reactions are thought to occur due to their effect on mitochondria. This major energy generator of the cell (the mitochondria) has its own double stranded circular DNA of about 16 000 bases. The mitochondrial DNA replicates with the assistance of an enzyme called polymerase gamma. NRTIs affect the function of this enzyme, which may lead to the depletion of mitochondrial DNA or changes in qualitative properties (White, 2001) in different cell lines (Cossarizza & Moyle, 2004). The depletion leads to morphological changes in the mitochondria (White, 2001).

Both mitochondria and its DNA depends on the cell type and the metabolic activity of the cell (Cossarizza & Moyle, 2004). Studies suggested a ranking for effects on mitochondrial polymerase gamma: ddC > ddI > d4T > 3TC > ZDV > ABC. Termination of treatment or dose reduction may result in the remission of the toxicity (White, 2001).

The cell viability or cytotoxicity *in vitro* assay to be used in this study is based on the MTT assay developed by Mosmann (1983). This assay can be used for determining the number of viable cells in culture after HIV-1 infection. The mechanism is based on the reduction of MTT by active mitochondria which results in the formation of formazan crystals (Mosmann, 1983). This principle was applied for determining the toxic and protective properties of the ARVs tested *in vitro*.

1.5 Conclusion on antiretroviral therapy

To conclude the preceding discussion on the South-African antiretroviral regimen, the following regimen has been decided on to investigate in this study:

Lamivudine (3TC) + stavudine (d4T) + nevirapine (NVP).

This regimen falls under the first-line regimens for the treatment of HIV/AIDS and the two NRTIs target both HIV-1 and HIV-2. It has the least number of adverse effects when used in the treatment of paediatric patients. One of the concerns with this regimen is the necessity

for cold-storage, which complicates adherence. Inadequacies of this regimen are availability and the lack of age appropriate antiretroviral formulations for children (Clayden, 2006).

Using Pheroid™ technology in the formulation of a combined dosage form, these obstacles could perhaps be defeated. Pheroid™ technology will be discussed in chapter 3 as a novel delivery system with its associated advantages.

A summary of the classification, dosages and mechanisms of action of the three selected drugs are listed in table 1.2

Table 1.2: Summary of the classification, dosages and mechanisms of action of d4T, 3TC and NVP

	Stavudine (d4T)	**Lamivudine (3TC)**	**Nevirapine (NVP)**
Mechanism of action	Acts as an alternate substrate for kinases which activates it to triphosphate. This competes for *binding to the HIV reverse* transcriptase.[1]	As for d4T. [1]	NVP is a non-nucleoside reverse transcriptase inhibitor that binds to the viral enzyme and interrupts the enzyme's catalytic site. [2]
Antiretroviral drug classification	NRTI	NRTI	NNRTI
WHO recommended paediatric target dose	1 mg/kg/dose twice daily. [3]	4 mg/kg/dose twice daily to a maximum of 150 mg twice daily.[3]	160 – 200 mg/m^2 to a maximum dose of 200 mg twice daily. [3]
Activity spectrum	HIV-1 and -2. [4]	HIV-1 and -2; HBV (hepatitis B virus). [4]	HIV-1. [4]
Principle indication	Advanced HIV disease and/or HIV infection; use in combination with other ARVs. [4]	HIV and HBV infections. Used in conjunction with other antiretrovirals. [4]	For HIV-1 infection. Used primarily with NRTIs. [4]

1) Ho and Hitchcock, 1989. 2) Veldkamp *et al.*, 2001. 3) WHO, 2006. 4) De Clercq, 2004.

1.6 Physical-chemical properties

1.6.1 Stavudine

1.6.1.1 Chemical properties

The chemical properties of stavudine are listed in table 1.5.

1.6.1.2 Stability

The stability of stavudine in aqueous medium was studied by Kawaguchi *et al.* (1989). The chemical stability of stavudine at 37 °C at various pH conditions are given in table 6 below.

Table 1.3: The chemical stability of stavudine at different pH values (Kawaguchi *et al.*, 1989)

pH	1.0	3.0	5.0	7.0	9.0
$t_{½}$	26	92	105	102	97

The table shows that stavudine has the highest stability in the pH range of 5.0 – 7.0.

The degradation of stavudine takes place through hydrolysis of the glycosyl-thymine bond under all pH conditions (Kawaguchi *et al.*, 1989).

No information or evidence on the oxidative or other degradation pathways could be found in a comprehensive literature study (Dekker & Lötter, 2002).

1.6.1.3 Absorption, distribution and elimination

- Absorbed rapidly after oral administration;
- Reach peak plasma concentrations after 1 hour;
- Bioavailability of 86%;
- Administration with food delays absorption, but does not reduce it;
- Crosses the blood-brain barrier;
- After 6 -24 hours, 40% of the drug is excreted in the urine (Kaul *et al.*, 1998; Grasela *et al.,* 2000).

1.6.2 Lamivudine

1.6.2.1 Chemical properties

The chemical properties of lamivudine are listed in table 1.5.

1.6.2.2 Stability

Inadequate information is available on the degradation and stability of lamivudine (Dekker & Lötter, 2002). In a study by Nguyen *et al.* (1995) five factors that affected the stability were investigated. These factors were: pH, sucrose, propylene glycol, glycerine and EDTA, each at a concentration of 1 mg/ml. The formulations were analysed after 3 months at 40 °C. The formulations were sealed in ampoules, thus humidity had no impact. The pH was found to be the main factor affecting the stability of lamivudine. Lamivudine was least stable at a pH of 4.5.

No degradation was found in urine samples after 5 hours at 58 °C, and a degradation of 4.5% was obtained in samples stored at room temperature for 4 days (Morris & Selinger, 1994). Similar findings have also been reported by Hoetelmans *et al.*, (1998) and Harker *et al.* (1994).

Degradation of lamivudine takes place through a process of hydrolysis of the C-N bond (see table 1.5), resulting in the formation of cytosine, which is similar to the degradation of stavudine. (Kawaguchi *et al.*, 1989).

1.6.2.3 Absorption, distribution and elimination

- Lamivudine is rapidly absorbed after oral administration (Mueller *et al.,* 1998);
- It reaches maximum serum concentrations after 0.5 to 1.5 hours;
- Absolute bioavailability is approximately 82% in adults and 68% in children;
- Absorption is delayed but not reduced by food intake;
- Diffuses freely across the placenta in pregnant woman;
- Excreted in breast milk;
- Lamivudine concentration in cerebrospinal fluid is low to modest;
- Approximately 70% of an oral dose is eliminated renally as unchanged drug;
- Hepatic impairment does not affect the pharmacokinetic properties of lamivudine (Johnson *et al.,* 1999).

1.6.3 Nevirapine

1.6.3.1 Chemical properties

The chemical properties of nevirapine are listed in table 1.5.

1.6.3.2 Stability

Stress studies were carried out by Chan *et al.* (2000) as part of the validation of an HPLC method for nevirapine. The results obtained are listed in table 1.4.

Table 1.4: Remaining nevirapine under certain stress conditions

Stress conditions	Nevirapine remaining (%)
1 N HCl	30
1 N NaOH	82
3% H_2O_2	72
Light (600 foot candles)	100

Nevirapine was found to be stable towards exposure to light and in solution. (Chan *et al.*, 2000).

1.6.3.3 Absorption, distribution and elimination

- Nevirapine is readily absorbed after an oral dose;
- Absorption is not affected by food intake;
- Bioavailability is more than 90%;
- Nevirapine reaches peak plasma concentrations after 4 hours;
- 60% of the active compound binds to plasma proteins;
- 45% of the concentration of nevirapine in the plasma is found in the cerebrospinal fluid;
- NVP is metabolized by cytochrome P450 isoenzymes;
- NVP is mainly excreted through the kidneys as a glucuronide conjugate of the hydroxylated metabolites (Cheeseman *et al.*, 1993; Zhou *et al.,* 1999).

Table 1.5: Summary of the chemical properties of stavudine, lamivudine and nevirapine

Chemical properties	Stavudine (d4T)	Lamivudine (3TC)	Nevirapine (NVP)
Chemical IUPAC Name	1-[5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methyl-1H-pyrimidine-2,4-dione	4-amino-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1H-pyrimidin-2-one	1-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido [3,2-b:2',3'-e][1,4] diazepin-6-one
Chemical Formula	$C_{10}H_{12}N_2O_4$	$C_8H_{11}N_3O_3S$	$C_{15}H_{14}N_4O$
Chemical Structure	CH3 O N NH O HO O	NH2 N O N O S HO	N N N NH CH3 O
Molecular Weight	224.213 g/mol	229.257 g/mol	266.298 g/mol
Melting point	159 – 160 °C	160 – 162 °C	196.06 °C
H_20 solubility	5 – 10 g/100ml at 21 °C	7 g/100ml	7.046 x 10^{-5} g/100ml

1.7 Paediatric pharmacotherapy

There are a number of factors to take into consideration when applying pharmacotherapy. Development and growth of an infant can affect drug absorption and disposition. The undeveloped renal functions, distorted hepatic enzyme activity and differences in the drug absorption may lead to a deviation in the systemic distribution and absorption of antiretrovirals in infants. Paediatric dosages are based on a few principles, which make it similar on the one hand and extremely different from the adult therapy and kinetics on the other hand. Paediatric dosage is based on: age, weight and/or body surface area, the pharmacokinetic and -dynamic properties of drugs and physiological changes which, as a consequence of aging, causes differences in absorption, distribution, metabolism and excretion of drugs (King *et al.*, 2002).

1.7.1 Identification of age ranges

Selection of the appropriate drug dosage is based upon the stages of growth and development of the infant. Identification according to the age ranges allows consistency among these patients (King *et al.*, 2002).

The following table gives a classification of the terms used in pharmacotherapy and –kinetics of early infant development.

Table 1.6: Categorization based on children's age

Age	Class
Birth before 38 weeks of gestation	Neonate
<1 month	Term or preterm
1-24 months	Infant
2-12 years	Child
13-17 years	Adolescents

(King *et al.*, 2002).

1.7.2 Dosage selection according to weight and body surface area

Dose selection based on age only is inaccurate and not recommended, because of variability of weight. HIV-infected children tend to weigh less than healthy children. The most common method for determining dosages in children is based on the body surface area (BSA). This calculates for a higher dose than required. In cases where the dosage of an antiretroviral is not established, extrapolation of the adult dosage (based on weight) will be necessary (King *et al.*, 2002).

1.7.3 Effect of age on drug absorption

Age-related factors in paediatric patients influence the absorption of drugs. These factors are:

- gastric acid secretion;
- gastric emptying time;
- intestinal transit time and
- gastrointestinal motility.

Intestinal transit time in infants is much longer than in children and body composition varies with age and plays a role in the distribution of drugs (King *et al.*, 2002).

1.7.4 Body composition

The total body water (TBW) as a percentage of bodyweight changes with age, as shown by table 1.7 (King *et al.*, 2002).

Table 1.7: Total body water as a percentage of bodyweight

Age	TBW as a % of Bodyweight
Preterm	90%
Birth	78%
First few months	55-60%

1.7.5 Tissue binding characteristics

Drug distribution is effected by plasma protein binding. This characteristic differs between adults and infants. The total plasma protein concentration is lower in neonates. α_1- acid glycoprotein and lipoprotein are lower at birth and reach adult values at the 8th to 12th month of age respectively (King *et al.*, 2002).

1.7.6 Central nervous system

HIV-related encephalopathy is problematic in HIV-infected children. Drugs should be selected with caution because of the increased permeability of the central nervous system membranes of the infant (King *et al.*, 2002).

1.7.7 Altered hepatic enzymes

Drug metabolism is decreased during the postnatal period due to altered hepatic enzyme activity. Metabolisms of drugs are mostly via hepatic enzymes. These enzymes are divided into two classes: phase I and phase II.

Phase I reactions are: hydroxylation, reduction and oxidation.

Phase II reactions include: glucoronidation, sulfation and methylation.

All of these reactions are depressed at birth and reach adult values later in the infant's life (King *et al.*, 2002).

1.8 Pharmacokinetics

The pharmacokinetics of the neonate differ completely from that of adults due to the fact that the neonate is a developing organism. Functions such as rate of drug metabolism and renal elimination develop during the first few weeks of life (Pacifici, 2005). A pharmacokinetic summary of the three ARVs (stavudine, lamivudine and nevirapine) is given in table 1.8. The difference between adults and infants in the time required for the drug to reach maximum plasma levels (T_{max}), is especially prounounced.

Table 1.8: Pharmacokinetic properties of stavudine, lamivudine and nevirapine

		Stavudine (d4T)	Lamivudine (3TC)	Nevirapine (NVP)
C_{max} (mg/L)	Adult	0.9 ± 0.3, obtained from a dose of 40 mg (single dose).[1]	1.4 ± 1.1, obtained form a dose of 150 mg (single dose).[1]	8.3 ± 2.8, obtained from a dose of 200 mg (single dose).[1]
	Paediatric	0.44 obtained from a dose of 1 mg/kg/dose. [2]	1.32 obtained form a dose of 4 mg/kg/dose. [2]	9.9 obtained from a dose of 160-200 mg/m^2. [2]
C_{min} (mg/L)	Adult	0.09 [2]	0.09 [2]	3.7 [2]
	Paediatric	The same as C_{min} for adults [3]	0.07 [3]	6.0 [2]
T_{max} (h)	Adults	0.5 – 0.7 [4]	0.5 – 1.5 (single 100 mg dose). [4]	2.0 – 4.0 [4]
	Paediatric	2.5 [4]	3.0 [4]	14.0 [4]
AUC (mg • h/L)	Adult	Not available [3]	Not available [3]	Not available [3]
	Paediatric	1.63 [3]	7.14 [3]	54.5 [3]
$t_{½}$ (h)	Adult	1.2 – 2.0 (40 mg single dose).[1]	6.0 – 9.0 (150 mg single dose).[1]	24.0 – 76.0 (200 mg single dose).[1]
	Paediatric	1.2 (similar to that of adults) [4]	6.0 [4]	37.3 [4]

1) Hosseinipour *et al.*, 2007. 2) Clayden, 2007. 3) King *et al.*, 2002. 4) Pacifici, 2005.

1.9 Conclusion

To conclude this literature overview, paediatric antiretrovirals do contribute to the treatment of HIV/AIDS infants, but there is room left for improvement. Strict dosage regimes and severe adverse reactions make the use of these drugs difficult to administer. A list of problems in the greater parts of Africa is the following: distribution, stability, adherence to the taking of drugs, adverse reactions as well as the low efficacy of ARVs.

In the American Journal of Obstetrics and Gynaecology (Dao *et al.,* 2007) it was stated by the WHO that countries should develop better antiretroviral regimes to increase the effectiveness in the prevention of mother-to-child transmission. Combination therapy as well as better delivery may contribute to the effectiveness of this treatment. In chapter 3, Pheroid™ technology will be discussed as a drug delivery system with benefits in this regard. In the next chapter specific cell viability assays will be discussed. These assays are used for determining the efficacy of ARVs and the effect of Pheroid™ technology on it. In the future, a combined 3-in-1 ARV dose for the treatment of HIV/AIDS, with Pheroid ™ technology as drug delivery system, may be of great value.

CHAPTER 2

Cell viability assays and methods

2 HIV-1-specific $CD4^+$ T cell responses

Various models and mechanisms exist for detecting $CD4^+$ T cell depletion, HIV-1 infection and ARV treatment efficacy and these will be discussed in the following section.

2.1 T cell response and depletion of $CD4^+$ cells

Maintenance of an effective immunity during chronic viral infections is important with virus-specific $CD4^+$ T helper lymphocytes critical in this defense system. These lymphocytes are characteristically undetectable in HIV-1 infections (Rosenberg *et al.,* 1997). Mechanisms have been proposed to explain the depletion of $CD4^+$ T cells, which characterizes the progress of HIV infection to AIDS (Todd *et al.*, 1995).

2.2 Introduction to infection models of HIV-1 and neutralizing antibody assays

Measuring the immune responses specifically against HIV is critical for understanding the relationship between the virus and the immune system of the host. An understanding of this will support the development of efficient prophylactic vaccines. The humoral immunity is detected by neutralizing antibody assays, while cellular immunity is examined using a variety of techniques, which includes cytotoxic T-lymphocyte assays (Horton *et al.*, 2003).

Disease modeling is considered an extensive challenge due to the high tropism of HIV-1 for human cells. Methods for studying this have been developed to gain insight into the disease pathogenesis and proficient evaluation of new therapies. Models include *in vitro*, *in vivo* and *in silico* models and are challenging due to the species-specific nature of HIV (Stoddart and Reyes, 2006).

2.2.1 Neutralizing antibody assays

Neutralizing antibody assays require the presence of HIV-specific neutralizing antibodies. These antibodies prevent viral infection (without the aid of any antiretroviral or antiviral drugs) by means of binding to regions of the HIV envelope that are required for viral attachment and entry into the host cells. This antibody-mediated neutralization is measured by MT-2 cell killing assays. MT-2 is a $CD4^+$ human lymphoblastoid cell line that is highly accommodating to infection with CXCR4-utilizing strains of HIV (Horton *et al.*, 2003).

In this neutralizing assay, cell-free virus is incubated with multiple dilutions of serum samples at 37°C for one hour before MT-2 cells are added. If neutralization antibodies are present in the serum, it will bind to the virus and it will limit its ability to infect the MT-2 cells. Neutralization is measured as a function of absorbance after staining the cultures with Finter's neutral red when cytopathic effects in virus-control wells are greater than 70% and less than a 100% (Horton *et al.*, 2003).

2.2.2 Infection models of HIV-1

2.2.2.1 *In vitro* models

Using primary lymphocyte human cells, the causative agent of AIDS was successfully isolated and identified within *in vitro* culture systems. With the use of peripheral blood lymphocyte culture methods it was indicated that infection with HIV caused depletion in $CD4^+$ T cells. Primary human cells (other than peripheral blood lymphocytes) are also prone to HIV-1 infection and can be cultivated *in vitro* for studies of virus-host cell interactions. Primary human cells include monocyte-derived macrophages, plasmacytoid dendritic cells and myeloid dendritic cells. The use of primary human cells includes the isolation of new HIV-1 strains to increase virus stocks and to study the kinetics of viral replication (Stoddart and Reyes, 2006).

Lymphoblastoid T-cell lines including MT-4 and MT-2, CEM, Sup-T1, HuT 78 and H9 cells are also susceptible to HIV-1 infection. MT-2 cells were originally used to differentiate between syncytium and nonsyncytium inducing HIV strains (Stoddart and Reyes, 2006).

These types of infection models are relatively inexpensive, are accessible for most biohazard level II or III laboratories and have a turnaround time of about one week. Negative aspects of this model are the exclusion of cells that are infected *in vivo* and the lack of an interactive immune system (Stoddart and Reyes, 2006).

2.2.2.2 *In vivo* models

Several animal models have been used for the evaluation of antiretrovirals and vaccines and to gain insight into the pathophysiology of HIV-1 infection and immunodeficiency in a controlled invironment. Examples of these models are the humanized mouse model, the transgenic rodent model, the non-human primate model and the feline immunodeficiency virus (FIV) in cats (Stoddart and Reyes, 2006).

Advantages of this model are the similarity of non-human primates' immune complexities and physiology and the development of an immune deficiency which result in accurate assessment of the safety and efficacy of ARVs. The disadvantages of these models are the

high cost and the fact that nonhuman primates can't be productively infected with HIV (Stoddart and Reyes, 2006).

2.2.2.3 *In silico* models

These are models that mimic HIV-1 infection and disease. Such mathematical models are not currently available although attempts have been made to model HIV-1 biology, *in vivo* replication dynamics and virus evolution. The model only requires a computer and mathematical algorithms but outcomes are based on assumptions that might not be true (Stoddart and Reyes, 2006).

2.3 Viability assays

A number of methods have been developed to study cell viability and proliferation in cell culture (Cook and Mitchell, 1989). Colorometric- and luminescence-based assays are modern and convenient assays that have been optimized for the use of microtiterplates (48- and 96-well plate format). These cytotoxicity assays use a number of parameters associated with cell death (Weyermann *et al.*, 2005). Applications for *in vitro* cytotoxicity assays include the prediction of human toxicity and general chemical screening (Scheers *et al.*, 2001; Fotakis & Timbrell, 2006).

Four different assays will be discussed hereafter, which are generally being used to study cell viability and proliferation:

- One parameter for cell death is to determine the integrity of the cell membrane which can be measured by the cytoplasmic enzyme activity released by damage cells. Lactate dehydrogenase (LDH) is rapidly released into the culture supernatant upon damage of the cell membrane (Korzeniewski and Callewaert, 1983).

- Another parameter that can be measured is the metabolic activity of viable cells. Tetrazolium salts are converted (reduced) only by metabolically active cells: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) is reduced to blue formazan crystals (Mosmann, 1983) (Weyerman *et al.*, 2005).

- The neutral red (3-amino-*m*-dimethylamino-2-methylphenazine hydrochloride) assay quantifies the number of viable, uninjured cells, after exposure to a toxin (Finter, 1969).

- A bioluminescence measurement assay: this assay utilizes the enzyme, luciferase, which catalyses the formation of light from ATP and luciferin (Weyerman *et al.*, 2005).

As an endpoint for viral infection a variety of parameters can be examined. These parameters include:

- immunofluorescence;
- immunoprecipitation;
- antigen capture;
- immunoblotting for viral antigen expression;
- reverse transcriptase activity in conditioned culture fluids for virus production;
- syncytium formation;
- viable cell counts for cytopathic effect.

There are a number of limitations for the effective *in vitro* testing of antiretroviral drugs, which include man-hours for testing large quantities of samples, the high cost of immunological reagents and radioisotopes required. Avoiding this, rapid and sensitive 96-well plate assays have been developed that requires no immunological reagents or radioisotopes. The extensive application of these assays was demonstrated using known ARV's such as zidovudine (AZT) and 2', 3'-dideoxycytidine (ddCyd) as inhibitors of reverse transcription, interferon (IFN), double stranded RNA as biological response modifiers and amphotericin B as a direct inactivator of virions (Montefiori *et al.*, 1988).

The two major defense systems of the body against viruses are neutralizing antibodies and cytotoxic T lymphocytes. The greatest effect of HIV infection on MT-2 cells in culture is the formation of syncytia, which is thought to be indicative of cell death induced by virus infection (Wünschmann & Stapleton, 2000). Based on these cell reactions, the assays mainly used for the *in vitro* viability of cells are neutral red (NR) and MTT. (Zhang *et al.*, 2003).

Neutral red (Finter, 1969) and the tatrazolium dye (MTT) (Mosman, 1983) will be discussed as assays used in the determining of cell viability. Cytotoxicity assays (Fotakis & Timbrell, 2006) revealed neutral red and MTT assays to be the most affordable and sensitive cytotoxicity assays showing statistically significant differences between treated and control cells.

Working mechanisms of the two assays rely on the following:

- MTT enzyme based conversion of the tetrazolium dye in the mitochondria;
- The neutral red assay is a colorimetric assay measuring the uptake of the dye by functional lysosomes.

(Fotakis & Timbrell, 2006).

It was also found that treated cells have different viabilities depending on the cytotoxicity assay and test agent used (Weyermann *et al.*, 2005). It has therefore been suggested that more than one assay should be used to determine cell viability in *in vitro* studies, as it would increase the reliability of the results (Fotamis & Timbrell, 2006).

Both the MTT cell viability assay and the neutral red assay will be discussed in this chapter. Laboratory work and the cell viability assays were done at the National Institute for Communicable Diseases (South Africa) as part of a collaborative study due to the absence of a biohazard level II/III laboratory at the North-West University.

2.4 Development and application of the adapted neutral red assay

2.4.1 Introduction

This assay was developed as a general method for measuring the damage caused to cell cultures (thus indirectly measuring cell viability) by the growth of a cytocidal virus or the action of other noxious agents. Neutral red is concentrated within the lysosomes of cells, when taken up by living cells (Fotakis & Timbrell, 2006).

After surplus dye has been washed away, the degree of cell damage can be shown by the amount of dye bound in the cells, which can be judged or measured to determine cell viability (Finter, 1969).

Neutral red has also been used as a dye-uptake method for titrations of interferons (Billiau *et al.*, 1972) and as indicator of cytotoxicity in primary hepatocyte cultures and other cell lines.

2.4.2 Adapted neutral red assay for T-cell line adapted HIV-1 strain infection in MT-2 cells

The neutral red assay used in this study is based on the initial protocol by N.B. Finter (1969) as adapted by D.C. Montefiori (1988), and determines the viability of the examined cell culture due to an accumulation of the vital stain in lysosomes.

The adapted neutral red assay used by the NICD will be discussed (as used by the NICD), with changes from the assays developed by Finter (1969) and Montefiori (1988) to be indicated.

2.4.2.1 Introduction

This assay measures inhibition of viral infection and the level of toxicity of the ARV drugs in 96-well plates as a function of cell viability, after infection with the virus. The surviving fraction of cells is quantified by vital dye (neutral red) staining, with the intensity of staining being directly proportionate to the number of viable cells. MT-2 or PM1 cells infected with T cell line adapted strains of HIV-1 (SW7-TCL, IIIB and MN) can be used in this assay. A cell stock of 5×10^5 cells/ml as well as a virus stock of 1×10^5 $TCID_{50}$ HIV per ml must be prepared.

Purpose:

This assay is used to assess whether (a) drug(s) possesses activity against HIV-1. The toxicity of the specific drug(s) is determined in conjunction with the anti-HIV-1 test. This is necessary in order to assess whether a decrease in cell viability is linked to the drug(s)'s toxicity.

Principle:

This assay is based on the principle is that only healthy cells can take up a vital dye, such as neutral red (Finter, 1969), and accumulate it in lysosomes. After surplus dye has been washed away, cell damage in cultures is shown by the relative amounts of dye which accumulated in the lysosomes (Montefiori *et al.*, 1988), thus indicating cell viability.

The complete protocol for the neutral red assay as used by the NICD, South Africa is attached in annexure A.2, but a typical protocol will be briefly described below.

2.4.2.2 Protocol

2.4.2.2.1 Cell stock solution

A cell stock of 5×10^5 cells/ml need to be prepared in complete RPMI-1640 medium. A 100 µl of cells are added to each well to have a final concentration of 1×10^4 cells/well (the final volume per well is 250 µl). Refer to annexure A.2 for the preparation of the cell stock.

2.4.2.2.2 Preparation of virus

A virus stock of 1×10^5 $TCID_{50}$ (50% Tissue Culture Infective Dose) HIV strain per ml is prepared. A T-cell line adapted HIV-1 subtype C virus (SW7) that induces syncytia formation

amongst infected T-cells is used. It is advisable that the dilution of virus, that kills approximately 80% of cells after 7 days of culture, be determined beforehand. The virus is diluted in complete RPMI 1640 medium to 5 times higher than the final amount required.

Infective HIV-1 strains are used in this assay. The assay should be performed in a Biohazard Level II/III laboratory and all safety measures pertaining to this environment should be adhered to. Refer to NICD AIDS Unit Safety Manual (annexure A.1).

2.4.2.2.3 Preparation of the poly-L-lysine plates

To prepare a poly-L-lysine plate for use in this neutral red assay refer to annexure A.2. The plates must be prepared in advance before the cell staining procedure occurs. The prepared poly-L-lysine plates can be stored at 4 °C for up to 6 months.

2.4.2.2.4 Cell staining with Finter's neutral red

The cell staining procedure is described in annexure A.2. The mechanism of action of Finter's neutral red has been discussed in 2.3. Briefly, Finter's neutral red is added to plated cells. After a 75 minute incubation period, the dye is removed, the cells washed and fixed with acid alcohol, and the absorbance at 540 nm is determined.

2.4.2.3 Assay procedure

A typical plate layout used in this assay is reflected in figure 2.1. The procedure is enclosed in annexure A.2. The tested drug can be used in a concentration series or at desired concentrations or different drugs can be used. Sterility should be the main focus when cells are being handled or transferred. The assay has an incubation time of 7 days, thus cell viability can then be determined thereafter.

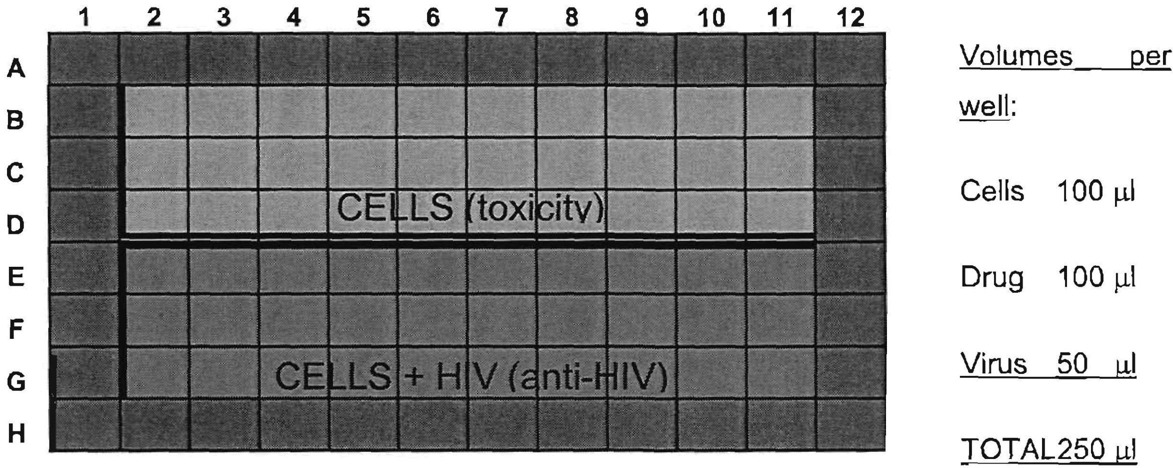

Figure 2.1 Plate layout (96-well plate)

CHAPTER 3

PHEROID™ TECHNOLOGY

3 Pheroid™ technology

3.1 Introduction

Insufficient drug delivery is the prime factor for delaying drug optimization of new molecular therapeutics, thus delaying optimized application and onset of action. Several drug delivery systems are available, but these are loaded with stability problems, high market cost, restricted field of application, low solubility and bioavailability (Grobler *et al.*, 2007).

The following list contains considerations for a good drug carrier or delivery system in liquid form: fine and uniform particle size, biocompatible and biodegradable qualities, good stability; it should be suitable for targeting and be pharmaceutical acceptable. The choice of an emulsion system to be used for drug delivery is dependant on the route of administration, drug characteristics and effects required. Specific bio-distribution may be achieved inertly by control of the physicochemical properties of the suggested carrier system, such as particle size and dose, surface charge and characteristics (Buszello & Muller, 2000).

Pheroid™ technology can improve the absorption and/or efficacy of a wide variety of active compounds, and can be manipulated according to size and type, depending on the drug molecules, which will be discussed in section 3.4. (Grobler *et al.*, 2007).

This technology was discovered when Piet Meyer developed a treatment for the management of his own psoriasis, with the basic ingredient found in banana peel extract, later discovered to be essential fatty acids (Steyn, 2006).

3.2 Pheroid™ classification and structural characteristics

The Pheroid™ delivery system is a dispersed medium with uniformly distributed lipid-based (without phospholipids or cholesterol) submicron- and micro-sized structures described as Pheroids™. It consist mainly of ethylated and pegylated poly unsaturated fatty acids, together with omega-3 and -6 fatty acids, excluding arachidonic acid. These Pheroids™ can be manipulated in terms of morphology, structure, size and function, with a diameter of between 200nm and 2µm. The purpose of the carrier system is to enhance the efficacy of pharmacological active compounds, while at the same time reducing side-effects (Grobler *et al.*, 2007).

3.2.1 Pheroid™ types

Pheroids™ can be divided into three types, namely:

- nano-and micrometer size lipid-bilayer vesicles (mean diameter of 200nm);
- microsponges (0.5 - 5.0 µm) (Steyn, 2006);
- pro-Pheroid™ containing depots or reservoirs (depot size is determined by the amount of pro- Pheroid™) (Uys, 2006).

3.2.2 Formation and characteristics

Pheroids™ are dispersed and equally distributed within a dispersed medium, consisting of two liquid phases, with a dispersed gas phase (associated with the dispersed fatty acid phase). The reservoir effect of these microspheres can be contributed to the ratio of pegylated to ethylated fatty acids used in the assembling of the Pheroids™ (Grobler *et al.*, 2007).

The Pheroid™ microspheres hold one inimitable component, namely nitrous oxide (N_2O), distributed within the dispersed phase throughout the continuous phase (Grobler *et al.*, 2007). The relationship of the N_2O with the dispersed phase has at least three functions:

- It contributes to the miscibility of the fatty acids in the dispersal medium (Grobler *et al.*, 2007);
- It contributes to the process of self-assembly of the Pheroids™ (C.E. Uys, 2006; Grobler *et al.*, 2007);
- It stabilizes the formed Pheroids™ (Grobler *et al.*, 2007).

The different sizes of Pheroids™ are pre-determined by the kind, ratios, saturation state and modification state of the fatty acids. The manufacturing process does also contribute to the size, but to a lesser extent (Grobler *et al.*, 2007).

The structures and functional characteristics of Pheroids™ can be controlled by the following:

- changing the fatty acid composition or concentrations;
- the addition of non-fatty acids or phospholipids such as cholesterol;
- the addition of cryoprotectants;

- the addition of charge-inducing agents;
- changing the hydration medium (ionic strength, pH);
- changing the method of preparation (subtle changes may have dramatic results);
- changing the character and the concentration of the active compound (Grobler *et al.*, 2007).

3.2.3 Functions of Pheroids™

Essential fatty acids are necessary for various cell functions, and cannot be manufactured by mammalian cells, thus it has to be ingested. Westernized diets often lack these important compounds (Grobler, 2004).

The Pheroid™ system has intrinsic therapeutic qualities, such as:

- the maintenance of mammalian cells membrane integrity;
- modulation of the immune system; and
- energy homeostasis.

This accounts for significant advantages of the Pheroid™ system over other delivery systems (Grobler, 2004).

3.3 Pheroid™ technology versus other lipid based delivery systems

Table 3.1 will compare some of the similarities, differences and advantages of the Pheroid™ and other lipid-based drug delivery systems. Only a few aspects will be highlighted in this table.

Table 3.1 Comparison of Pheroid™ to other lipid-based delivery systems (Grobler, 2004)

Pheroid™ delivery system	Lipid-based delivery systems
Pheroids™ can be formulated as a selection, depending on the manufacturing method chosen and composition.	An assortment of liposomes have been depicted: single- and multi-lamellar vesicles, multivesicular vesicles, nanosomes, etc.
Consist mostly of natural and essential ingredients of the body, namely essential fatty acids.	Other delivery system usually contains artificial substances, unknown to the body, e.g. artificial polymers, egg *phosphatidylcholine* and/or

	lysolecithin.
An entrapment efficiency of 85 – 100% was encountered in all compounds tested.	Problematic entrapment, due to charge and steric limitations, may decrease the efficiency.
Pheroids™ causes no cytotoxicity, because it is part of natural biochemical processes in the body, and it aids the maintenance of cell membranes.	Liposomal systems may cause cytotoxicity, but it may also decrease cytotoxicity.
Insoluble as well as drugs with different solubilities, can be entrapped	Most delivery systems are either lipo- or hydrophilic.
Drug resistance are reduced or eliminated in all *in vitro* studies with the use of Pheroid™ technology.	Some delivery systems are prone to drug resistance, while others may decrease drug resistance.
Pheroid™ technology improves oral, topical and buccal bioavailability and show an increase in absorption.	Other systems improve absorption, while some decreases absorption.
Pheroids™ inhibits the drug efflux mechanism in the intestinal lumen, which enhances absorption.	Liposomes do not possess this ability, with Cremophor to be co-administered to achieve this effect.

3.4 Anti-infective and other pharmaceutical applications of the Pheroid™ system

In this section, the Pheroid™ system will be discussed with some of its key advantages over other available delivery systems and its applicability to the enhancement of the efficacy of ARVs.

3.4.1 Increased delivery of active compounds

The Pheroid™ system showed promising results for the delivery of antifungal and antiviral active compounds to the site of action when investigated by membrane diffusion studies. The membrane used in these studies was skin. The active compounds released through the membrane were determined by HPLC methods according to the USP. Commercial preparations of acyclovir and miconazole nitrate (COM) and the same active compounds at identical concentrations entrapped in Pheroids™ were used (PHR) (Grobler, 2004).

0.5% active/product acyclovir was used in both the PHR and COM samples. The acyclovir-Pheroid™ sample indicated a release rate of 69.1533µg/cm2/h against the 54.0942µg/cm2/h of the acyclovir COM sample. With the same samples, the % release per label claims of the acyclovir PHR and COM were 0.1214% and 0.0952% respectively. Thus, the Pheroid™ sample of this antiviral compound had an increased diffusion rate as well as an increased %

release per label claim when compared to the commercial product investigated (Grobler, 2004).

Membrane diffusion studies were also performed on an antifungal agent, miconazole nitrate, where the results were as follow:

Miconazole nitrate (PHR) had a diffusion rate of 389.9238µg/cm^2/h and a release per label claim of 6.8155%. The percentage release per label claim of the Pheroid™-entrapped product indicated a much higher release when compared to that of the commercial product, which was 1.947%. Enhancement in the diffusion rates was also observed when the diffusion rate of the *miconazole nitrate* PHR compound of *389.9238* µg/cm^2/h was compared to that of the commercial (COM) miconazole nitrate of 111.222 µg/cm^2/h (Grobler, 2004).

3.4.2 Increased therapeutic effect and efficacy

According to Grobler *et al.* (2007), the effect of a novel delivery system should be measured by its contribution to therapeutic efficacy. This was tested with five commercially available anti-infective agents, using zone inhibition studies. The five pharmacological active compounds were: cloxacillin, erythromycin, ciprofloxacin, cotrimoxazole and itraconazole. Similar concentrations of the Pheroid™ entrapped compounds were tested against commercially available formulations or where no commercial preparations were available against the compound itself. All of the Pheroid™-entrapped compounds had an increase in efficacy when it was tested on *S. Aureus, P. Aerugin, B. Cereus, E. Coli, A.Niger and C. Albicans.* The compounds had pharmacological inhibitory effects on the micro-organisms accordingly (Grobler 2004).

3.4.3 Reduction in cytotoxicity

As discussed in table 3.1, section 3.3, Pheroids™ itself cause no cytotoxicity, due to the fact that it forms part of a natural biochemical process in the body and aids with the maintenance of cell membranes. Amongst other fatty acids (which is the major ingredient of Pheroids™), linolenic acid and linoleic (LA), are one of the main sources of energy in mammalian cells, and form integral structural components of cell membranes (Grobler *et al., 2007).* The enhancement of these normal cell integrity processes may counter some of the cellular damage that may occur due to exposure to harmful effects of active ingredients (Steyn, 2006).

3.4.4 Decreased time to onset of action

The Pheroid™ system crosses the majority of physiological barriers, due to its compositional similarity to cell membranes. This characteristic enables delivery of the pharmacological

active compound radically quicker than conservative carrier systems, which in turn will bring faster healing and/or relief from symptoms (Grobler 2004). This hypothesis was confirmed with a study of the anti-tuberculosis drug plasma levels of fourteen healthy volunteers using a pro-Pheroid™ formulation. The efficacy of rifampicin, pyrazinamide, isoniazid and ethambutol entrapped in the Pheroid™ delivery system (named Pyriftol) was measured against a commercial product named Rifafour-e200 (from Aventis). Pyriftol contained only 60% of the amount of the four antimicrobials contained in the commercial product. The time needed to achieve C_{max} in the case of rifampicin is halved by the entrapment of the compound in Pheroids™ (Pyriftol) when compared to that of the commercially available product, Rifafour-e200. (Grobler, 2004).

3.4.5 Reduction of the minimum inhibitory concentration (MIC)

Research indicated that it may be possible to use less of the active compound (as little as a 1/40th of the active compound) to reach an effective drug inhibitory concentration when the active is entrapped in Pheroids™. Reduction of the MIC could lead to cost effective treatment and side effects reduction (Grobler, 2004). This could be helpful in the treatment of HIV/AIDS, as a reduction in the variety of side effects could improve patient compliance and effective antiretroviral treatment, as discussed in chapter 1.

3.4.6 Reduction of drug resistance

A study was conducted between September 2000 and January 2004 in New York State on the prevalence of ARV drug mutations. Of the 151 people tested, seventeen (11.3%) had at least one drug-resistance mutation. Of the 17 people, 5 people had $CD4^+$ counts of less than 200, an indication of advanced infection. Results were as follow:

Table 3.2 Indication of the mutation resistance in 151 subjects

ARV compound	Percentage mutations in 151 subjects
NRTIs	6.6%
NNRTIs	5.3%
PIs	0.7%

The administration of combination antiretroviral therapy abridged mortality resulting from HIV infection, but it has started the beginning of a worrying era of drug-resistant HIV strains in infected patients (Parker *et al.*, 2007).

According to Grobler (2004), Pheroid™ technology has been shown to reduce or eliminate drug resistance *in vitro.* Studies have been done on drug resistant TB (resistance to rifampicin and isoniazid) and drug resistant malaria (*Falciparum* drug resistant reference strain W2). Both these drug-resistant infections showed promising inhibiting results when treated with well known, inexpensive, existing drugs entrapped in Pheroids™ (Grobler, 2004).

3.4.7 Immunological responses

Immunological responses may be induced by some drugs, such as proteins or peptides. The Pheroid™ delivery system is capable of masking these compounds, which reduces the recognition by the body's immune system. This would reduce the occurrence of adverse intolerance and immunological responses in turn (Grobler, 2004).

3.5 Clinical applications of Pheroid™ technology

3.5.1 Preventative applications and a possible HIV vaccine

The development of an HIV vaccine is one of the most challenging processes for the scientific community, with HIV-1 and HIV-2 so divergent in their genetic sequences, that their envelope glycoproteins don't show immunological cross-reactions. This results in high levels of viral replication that persist in the face of seemingly robust anti-viral antibody and cell-mediated immune responses (Letvin, 2002).

Promising approaches in this regard involve the use of plasmid DNA immunogens and live, recombinant vectors (Letvin, 2002).

Hanke & McMichael (2000) found that an ideal prophylactic vaccine should provoke sterilizing immunity, without virus detection after exposure.

By applying Pheroid™ technology, Grobler (2004) investigated the possible enhancement of a rabies vaccine in a comparative animal study. The Pheroid™-adjuvanted rabies vaccine showed a 9-fold increase in comparison with the commercial vaccine, with the usual aluminium-based adjuvant.

The development of an effective HIV vaccine is in its infancy, but by using Pheroid™ technology, future prospects of this vaccine may be brighter.

3.5.2 Pheroid™ technology and its possible impact on ARV treatment

The body cannot produce essential fatty acids which are required for numerous cell functions. Some of the functions of Pheroids™ have been stated in previous discussions, but I want to highlight the following: maintenance of membrane integrity of cells, energy homeostasis, modulation of the immune system and regulatory aspects of programmed cell death (Grobler, 2004).

Adverse reactions of ARV treatment have been discussed in chapter 1 in detail, but a few is mentioned here: liver failure, lactic acidosis, pancreatitis, lypodystrophy and hyperlactemia, fat redistribution, insulin resistance, diabetes and hyperlipidemia (DOH, 2004). Some of these adverse reactions could perhaps be alleviated with the use of this novel delivery system.

3.6 Conclusion

The Pheroid™ delivery system complies with all the properties of a good liquid drug delivery system, as stated in section 3.1. The key advantages of Pheroids™ over other lipid-based drug delivery systems have been highlighted. This carrier system is known to lessen adverse reactions, increase efficacy and assist cells in membrane integrity.

Efficacy enhancement properties on acyclovir showed promising results in the field of anti-viral drugs (Grobler, 2004). Applying the same principles may result in the enhancement of the efficacy of antiretrovirals and the possibility of effective HIV/AIDS treatment.

In my opinion, research on the Pheroid™ delivery system must intensify, but based on the previous research, the advantages of this delivery system over the rest are clear. An area which could be further investigated with regards to the Pheroid™ system is its interactions with drugs used.

CHAPTER 4

Article for publication in the Journal Cell Death and Differentiation

4 Introduction

The format chosen for this thesis was the article format. In this format the student needs to write an article on the scientific findings in his/her study according to the format prescribed by a selected journal. The article must be enclosed in the thesis.

The journal for *Cell Death and Differentiation* was chosen for this study as this journal publishes all articles devoted to i) cell biology, ii) molecular biology and biochemistry of cell death and differentiation both in normal tissue regulation and in disease.

Over 93,000 recipients will receive via e-mail a copy of the table of contents for the Cell Death & Differentiation issue in which the article is published. *Cell Death and Differentiation* has an impact factor of 7.463. The Guide for Authors is included in annexure I.

Cell Death and Differentiation

Cover letter

Contributing authors: MM Botha[1,2], AF Grobler[*,1], L-M Nieuwoudt[1,3], A Basson[4,5], L Morris[4,6] and AF Kotze[1,7]

[1] Unit of Drug Research and Development, Faculty of Health Science, North-West University, Potchefstroom Campus, Potchefstroom, 2520, South Africa.

* Corresponding author: AF Grobler, Unit of Drug Research and Development, Faculty of Health Science, North-West University, Potchefstroom Campus, Potchefstroom, 2520, South Africa.

Tel: +27-18-299-4467; Fax: +27-18-299-2251;

* E-mail: anne.grobler@nwu.ac.za

[2] E-mail: fmsmmb@nwu.ac.za

[3] E-mail: 20969120@nwu.ac.za

[4] The National Institute for Communicable Disease, Johannesburg, South Africa,

[5] E-mail: adriaanb@nicd.ac.za

[6] E-mail: lynm@nicd.ca.za

[7] E-mail: 10200142@nwu.ac.za

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Title page

The questionable reproducibility of the MTT cell viability assay

Running title: The questionable reproducibility of the MTT viability assay

MM Botha, AF Grobler*, L Nieuwoudt, A Basson, L Morris and AF Kotze

* Corresponding author: AF Grobler, Unit of Drug Research and Development, Faculty of Health Science, North-West University, Potchefstroom Campus, Potchefstroom, 2520, South Africa.

Tel: +27-18-299-4467; Fax: +27-18-299-2251;

E-mail: anne.grobler@nwu.ac.za

Abstract

The initial purpose of the study was to investigate the effect of interventions such as the use of a drug delivery system on the *in vitro* efficacy of antiretrovirals. However, the study resulted in addressing the reproducibility of MTT in cell viability assays. MTT reduction is a frequently used method for determining cell viability. The reproducibility of the results found with this assay was evaluated in the described study. MT-2 cells were infected with an HIV-1strain that induces the formation of syncytias known as gigantic multinucleated cells. The viability of these cells was determined using the MTT assay in the absence and presence of antiretrovirals. Absorbance levels at the concentrations used for lamivudine and stavudine fluctuated from one experiment to the next. In an effort to elucidate the cause of the experimental variation, the syncytias were investigated microscopically, which showed that the formed formazan crystals were present in both healthy and infected cells (syncytia). This aspecific crystal formation is probably the cause of the non-reproducibility of MTT in this study.

Keywords

HIV, antiretrovirals (ARV's), syncytia, MTT, mitochondria, reproducibility, 3TC, d4T

Abbreviations

3TC, lamivudine; d4T, stavudine; AIDS, acquired immunodeficiency syndrome; ARV, antiretroviral; ATP, adenosine 5'-triphosphate; DMF, dimethyl formamide; FCS, fetal calf serum; HIV-1, human immunodeficiency virus-1; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide; MT-2, human lymphoblast cells; NICD, National Institute for Communicable Diseases (South Africa); NR, Finter's neutral red; NRTI,

nucleoside reverse transcriptase inhibitor; PBS, phosphate buffered solution; pH, an inverse logarithmic representation of hydrogen proton (H^+) concentration (acidity); PLL, poly-L-lysine; SDS, sodium dodecyl sulphate.

Introduction

AIDS is clinically defined as the unavoidable depletion of $CD4^+$ T-cells, due to human immunodeficiency virus-1 (HIV-1 and HIV-2) infection. The depletion in $CD4^+$ T cells is associated with virus induced immunodeficiency (1). This epidemic is one of the greatest threats of our time, with 40 million people currently infected, 5 million newly infected cases and 3 million AIDS related deaths in 2005. The transmission of the virus occurs through contamination by i) contaminated blood products or syringes, ii) sexual intercourse, iii) mother to child transmission *in utero* or breastfeeding. Without effective treatment, HIV is consistently fatal within 5 – 10 years from infection (2). Models to study the pathology and disease progression in HIV-1 infection include *in vitro, in vivo* and *in silico* models (2).The relationship between the appearance of syncytium-inducing (SI) HIV variants, the rate of $CD4^+$ cell depletion and the clinical progression of HIV-1 infection was reported to be as follows: the appearance of SI variants is prognostic for a rapid decline of $CD4^+$ cell counts (3).

Several *in vitro* assays to study cell viability and proliferation in cell culture have been developed (4). These assays are mainly colorimetric, luminescence or cytotoxic based assays (5). Parameters for cell death include i) determination of the integrity of the cell membrane (lactate dehydrogenase release) (6), ii) measuring the metabolic activity of viable cells (using MTT as viable cell stain) (7), iii) neutral red staining for quantification of vital cells (5) and iv) a bioluminescent measurement of the amount of adenosine triphosphate (ATP) present (8).

The assay most frequently used for cell viability determination is the enzymatic reduction of MTT to formazan crystals as indicators of biological redox systems and viability of intact cells (9, 10, 11). MTT is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes in the mitochondria of viable cells. This water insoluble formazan can be solubilized using isopropanol or other solvents (formazan solution) and the dissolved material is measured spectrophotometrically yielding absorbance as a function of concentration of converted dye, hence the number of active mitochondria as a reflection of viable cells present in the culture (6, 11).

An MT-2 cell line was used in an attempt to study the effect of a novel drug delivery system on the antiviral efficacy of lamivudine and stavudine. HIV-1 infection in the above mentioned cell line induces cell fusion to form multinucleated giant cells called syncytia. Syncytia formation is mainly responsible for the depletion of cell numbers, rather than virus-induced cell death (12). Syncytium formation could thus serve as a significant marker in the progression of HIV-1 infection (13, 14).

The viability of the cells after infection was determined spectrophotometrically with the use of MTT based on the assumption that absorbance is directly proportionate to viability. The results obtained with this assay fluctuated using same concentrations of the ARVs on consecutive experiments. This fluctuation of results raised the question of the reproducibility of MTT and the exclusive role of mitochondria in the reduction of MTT to formazan crystals. The reproducibility of MTT was also raised by Liu *et al.* who questioned the generally accepted statement that MTT is reduced by active

mitochondria only (9). The definite proof for the association of mitochondria with MTT reduction is still lacking due to the reduction of MTT by dehydrogenases or flavin oxidases in cells without mitochondria (9).

Reminiscent to the MTT assay is Finter's neutral red assay, which is based on lysosomal uptake of the stain by viable cells (15). The purpose of the use of this assay was to assess a) whether (a) drug(s) possess(es) activity against HIV-1 infection and in conjunction with this, determining the toxicity of the same drug(s) and b) whether the non-reproducibility found for the MTT assay was indeed related to mitochondrial activity.

The purpose was then to determine whether a decrease in the cell viability was linked to the drug(s) toxicity.

Results

An MT-2, syncutium inducing cell line was used to analyze the toxic and protective properties of lamivudine and stavudine, using an MTT assay adapted from that described by Mosmann (7). The cells (uninfected and infected cells) were incubated with an antiretroviral (d4T or 3TC) for 1 hour after which the virus was added. After the incubation period, cells were stained and the viability was determined. The results obtained are presented in tables 1, 2, and 3, which summarize the absorbance thus, viability and protection of the specific drug used. Table 1 summarizes incubation with d4T at 4 µM and its obtained results. Table 2 and 3 is a summary of the absorbance obtained after incubation with 3TC at concentrations of 20 µM, 100 µM and 500 µM respectively. Absorbance was measured in optical density units at the applicable wavelength. The units as such are not shown in the tables or figures.

The protective properties of d4T were investigated using Finter's neutral red and MTT cell viability staining assays. Both these assays were used to determine the toxicity and protection of the drug in an effort to elucidate the role of mitochondria in the MTT cell viability assay.

All studies were done on different days and variables were eliminated as far as possible. All procedures were done using the same instruments i.e. incubator, microscope, flow hood, centrifuge, hemacytometer, plate reader and the research was executed by a single person only.

The results from d4T were obtained from 2 studies whereas the results obtained from incubation with 3TC were obtained from 6 studies. It should be noted that the assay used in studies 4, 5 and 6 were adapted to a 3 day incubation period in an effort to overcome cell depletion.

Discussion

The results will be discussed in terms of intra-repeatability and inter-reproducibility.

Table 1 represents the absorbance data obtained after incubation with stavudine and staining with MTT. The toxicity and the viability are represented in table 1 as are the absorbance obtained from the two studies. Study 1 had a lower absorbance than that of study 2, which indicated questionable reproducibility.

The same trend can be seen in the following two tables (table 2 and table 3). This absorbance data were obtained after infected and uninfected cells were incubated with lamivudine and stained with MTT. The calculated viability and percentage protection are also presented in these two tables. The non-reproducibility of the

assay is obvious from these results: if for instance, the protective properties of 3TC at a concentration of 20 μM are compared, the absorbance found differ significantly between the three studies, which resulted in protection values of 122.21%, 91.78% and 86.50% respectively.

Values from the 3 day incubation assay, using 3TC, indicated the same fluctuation in absorbance: 29.85%, -43.04% and 85.45%. Values from the negative control do not correlate either. These values from 3 studies have been compared and are illustrated in Figure 1. For the uninfected cells, there is intra-repeatability on the viability of 3TC, but there is no inter-reproducibility when the three studies are compared. For the infected cells, there is no intra- or inter-repeatability on the three samples when the absorbance results are compared. This suggests that these studies were neither repeatable nor reproducible when using the MTT assay.

Using Finter's neutral red to stain infected and uninfected cells, the effect of d4T was investigated. The results are illustrated in figure 2. The graph shows non-repeatability within the same experiment, as indicated by the fluctuation in the viability seen in figure 2a. Similarly, the protection of d4T shows a non-repeatability within the same study. Both the NR and MTT assays have been used with success for a number of years and are well known vital dyes (16). On the other hand, Liu *et al.* stated that definite proof has been lacking for exclusive mitochondrial MTT reduction, and found that MTT was reduced by other cell components (9).

As all variables were eliminated as far as possible, the only explanation could be that other cellular components were metabolically reducing MTT or accumulating NR or that the syncytia can not be regarded as metabolically dead with no active mitochondria or lysosomes present. Little is known about the metabolic state of giant

multinucleated cells, but it is generally assumed that these cells are metabolically impaired and that the formation of formazan crystals would not take place. Syncytium formation was induced by infecting MT-2 cells with HIV. The formed syncytia, as well as uninfected cells, were exposed to MTT and the formed crystals within the cells were investigated microscopically. Figure 3 is an illustration of the light micrographs from the infected and uninfected cells. These micrographs are self explanatory – both uninfected and infected (syncytias) cells had formed formazan crystals within its cell membranes. Therefore either mitochondria or other cellular components are metabolically active in these syncytia and are able to reduce MTT. The microscopy analysis showed that MTT is reduced in syncytia, but did not indicate whether the reduction was associated with the mitochondria.

A contributing factor to the non-reproducibility of the MTT assay could perhaps be the effects of antiretrovirals (ARVs) (nucleoside reverse transcriptase inhibitors – NRTIs) on mitochondrial DNA (mt-DNA) and the enzyme responsible for the replication of mt-DNA, polymerase gamma (14). The possibility of interactions between the administered drugs and the vital dye has not been excluded.

Finter's neutral red versus MTT

The protective properties of d4T were determined in two different infection studies, and on different days. d4T at a concentration of 0.75 µM showed a lack of reproducibility using both Finter's neutral red (NR) and MTT. The percentage protection obtained at this concentration using NR was 66.05% whereas with MTT, it was 76.47%. The only reproducibility obtained using the two different assays were that of the control groups. The absorbance obtained for the control group with the NR assay was 1.66, whereas it was 1.697 in the control group of the MTT assay. NR

have poor solubility in RPMI medium and have a series of complex experimental processes including plate coating with poly-L-lysine (PLL), the adhering of cells to the PLL-coated plate, wash procedure and the extraction of NR from cells (15). The reproducibility of the absorbance of the control groups suggest however that the problematic inter-reproducibility is not assay-specific but occurs only in infected cells.

In conclusion: the discussion above and the results obtained in this study suggest that neither MTT nor Finter's neutral red assays can be used with confidence in the determination of cell viability or the effect of ARVs on HIV-infection *in vitro* when using a syncytia-inducing viral infection model. The effect of ARV toxicity on mitochondria, the role of syncytias in MTT reduction and the degree of metabolic activity of the mitochondria in syncytias have been neglected in most studies. NR is based on lysosomal staining and the non-reproducibility is not only mitochondria specific. In future, this should be taken into consideration when viability staining assays are to be used in the evaluation of the toxicity of and protection by ARVs.

Materials and methods

Cell cultures

Syncytium inducing MT-2 cells (obtained from the NIH AIDS Reagent and Reference Programme, catalog number: 237) were cultured in complete RPMI-1640 medium (Highveld Biological, Sandringham, Johannesburg, South Africa), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). No antibiotics was added to the medium. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cultured MT-2 cells were collected by centrifugation at 1200 rpm. The cells were suspended in 1 – 2 ml of RPMI-1640 growth medium. The cell titer was

determined by trypan blue staining: 10 µl of the newly suspended cell suspension was transferred to 90 µl trypan blue solution, mixed and 10 µl was transferred to each chamber of a hemacytometer. The cells were counted using a light microscope (10 x magnification). Cells were then diluted with basic RPMI-1640 medium to a concentration of 1×10^5 cells/ml. 100 µl of the cells was transferred to the wells, resulting in a concentration of 1×10^4 cells/well.

The MT-2 cell line was used to assess the toxicity and inhibitory activity of d4T and 3TC.

Virus

A T-cell line adapted HIV-1 subtype C virus (SW7-TCL) that induces syncytium formation in T-cells was used for infection. The virus was obtained from patients: virus was isolated from plasma, cells added and the virus stock was cultured. Patient samples were collected, centrifuged and the plasma was used to infect MT-2 cells. All laboratory work was performed at the NICD in a Biohazard Level II/III laboratory. Safety measures were followed according to the AIDS Unit Safety Manual, NICD. Cell death of approximately 80% after infection of the cells per well was required. The stock dilution of virus used was determined beforehand and needed to be 5 times higher than the final amount required.

Staining with MTT

MTT and formazan solutions

A solution of 5 mg/ml MTT (Sigma, St. Louis, USA, ref. nr.: M6555) was dissolved and stored in PBS (Highveld Biological, South-Africa). The solution was sterilized by

filtration through a 0.2 µm membrane filter and stored in 800 µl aliquots at -20 °C, away from light. The MTT was prepared in advance.

To obtain a 20% SDS (w/v) (Sigma, St. Louis, USA) and a 50% DMF (v/v) (Sigma, St. Louis, USA) solution, 40 % SDS (w/v) solution was prepared in distilled water, adding an equal volume of DMF. This was mixed thoroughly. It is common for the SDS to precipitate during storage (room temperature). The solution needs to be re-warmed to 37 °C to re-dissolve the precipitates before use.

Assay procedure

The drugs used in this study (3TC and d4T) (obtained from Xiamem MCHEM Laboratories LTD., Batch no. for d4T: 0601002, for 3TC: 040801) were dissolved in RPMI-1640 medium without the fetal calf serum supplement. 96-well plates are numbered from A1 to H12 as indicated in the plate. To avoid fluctuations in the volume of the tested wells, 250 µl RPMI-1640 was added to the wells on the perimeter of a round bottom, 96-well plate. This inhibits evaporation from the test wells. The prepared cells at the concentration described above were seeded to all the inner wells (B2 – G11). 150 µl complete RPMI-1640 medium was added to wells B2 – D2, and 100 µl medium to wells E2 – G2. These were the uninfected and infected controls respectively. 100 µl of the drug was added from column 3 – 11. Different drug concentrations (in a serial dilution) were used. The initial concentration of the drug needed to be 2.5 times higher than the final concentrations. All concentrations were tested in triplicate. The plate was incubated for 1 hour at 37 °C, 5% CO_2. After the incubation period, 50 µl RPMI-160 complete media, and 50 µl virus (diluted in complete media) was added to rows B – D and E – G respectively. The plate was then incubated at 37 °C, 5% CO_2. This is day 0. On day 4 of the incubation

period, 125 µl of the supernatant was removed from all the test wells, and replaced with fresh complete medium or drug containing medium in identical fashion.

On day 7, 150 µl supernatant was removed from the test and control wells and 10 µl MTT solution was added to each well. The plate was incubated for 5 hours at 37 °C, 5% CO_2, after which the formed formazan crystals was dissolved by adding 100 µl formazan solution. The contents of each well were mixed thoroughly with a pipette, and the plate was read using a tunable plate reader (VERSAmax, Molecular Devices, USA) at 570 nm and 690 nm. Toxicity and protection were calculated using the following formulas (15, 17):

Toxicity: $$\frac{\text{Mean OD}_{\text{(cells + drug)}}}{\text{Mean OD}_{\text{(control cells)}}} \times 100$$

Protection: $$\frac{\text{Mean OD}_{\text{(cells + virus + drug)}} - \text{Mean OD}_{\text{(cells + virus)}}}{\text{Mean OD}_{\text{(control)}} - \text{Mean OD}_{\text{(cells + virus)}}} \times 100$$

Staining with Finter's Neutral red

Preparation of the poly-L-lysine

A poly-L-lysine (obtained from Sigma, St. Louis, USA) solution with a concentration of 50 µg/ml was prepared and 100 µl was added to the wells of a flat-bottom 96-well plate. After the plate was incubated (at 37 °C, 5% CO_2) for an hour with the poly-L-lysine solution, it was removed from the wells. The wells were washed twice with PBS, and the plate was drained upside-down on paper towel for 5 minutes.

Assay procedure

The assay procedure was followed according to the MTT assay. This assay differs from the MTT assay only in terms of cell staining on day 7.

For cell staining, the following solutions were prepared in advanced: Finter's neutral red diluted 1:10 times in RPMI-1640 media, PBS and an acid alcohol solution of 50% ethanol in 1% acetic acid. A 100 µl cell suspension of the test or control wells was transferred to the poly-L-lysine plates already containing 100µl neutral red in each well. The plate was incubated for 75 minutes. The contents of the wells were removed and the wells were washed twice with 150 µl PBS. A 100 µl of the acid alcohol solution was added to each well and the plate was read at an absorbance of 540 nm.

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Table 1 Summary of the absorbance, viability and protection of 4 µM d4T in two different studies

Study	Cell status	Group	Absorbance (570 nm – 690 nm)	Viability (%)	Protection (%)
1	Uninfected	Control	1.697	81.52	74.76
		Experimental	1.383		
	Infected	Control	0.399		
		Experimental	1.369		
2	Uninfected	Control	2.095	93.01	91.56
		Experimental	1.948		
	Infected	Control	0.606		
		Experimental	1.969		

Table 2 Summary of the absorbance, viability and protection of 20µM 3TC in six different studies

Study	Cell status	Group	Absorbance (570 nm – 690 nm)	Viability (%)	Protection (%)
1	Uninfected	Control	1.310	104.07	122.21
		Experimental	1.364		
	Infected	Control	0.419		
		Experimental	1.508		
2	Uninfected	Control	1.517	89.45	91.78
		Experimental	1.357		
	Infected	Control	0.478		
		Experimental	1.432		
3	Uninfected	Control	-	-	86.50
		Experimental	-		
	Infected	Control	0.395		
		Experimental	1.328		
4	Uninfected	Control	0.917	83.64	29.85
		Experimental	0.767		
	Infected	Control	0.465		
		Experimental	0.600		
5	Uninfected	Control	1.356	87.39	-43.04
		Experimental	1.185		
	Infected	Control	0.433		
		Experimental	0.793		
6	Uninfected	Control	1.051	107.52	85.45
		Experimental	1.001		
	Infected	Control	0.710		
		Experimental	1.130		

Table 3 Summary of the absorbance, viability and protection of 3TC at 100µM and 500 µM in three different studies

Concentration (µM)	Study	Cell status	Group	Absorbance (570 nm – 690 nm)	Viability (%)	Protection (%)
100	1	Uninfected	Control	1.310	106.16	106.51
			Experimental	1.391		
		Infected	Control	0.419		
			Experimental	1.368		
	4	Uninfected	Control	0.917	82.63	34.86
			Experimental	0.758		
		Infected	Control	0.465		
			Experimental	0.622		
	5	Uninfected	Control	1.356	85.52	-42.93
			Experimental	1.160		
		Infected	Control	0.433		
			Experimental	0.941		
500	1	Uninfected	Control	1.310	96.85	93.53
			Experimental	1.269		
		Infected	Control	0.419		
			Experimental	1.253		
	4	Uninfected	Control	0.917	80.41	27.12
			Experimental	0.737		
		Infected	Control	0.465		
			Experimental	0.587		
	5	Uninfected	Control	1.356	82.92	-42.95
			Experimental	1.124		
		Infected	Control	0.433		
			Experimental	0.903		

Titles and legends to figures

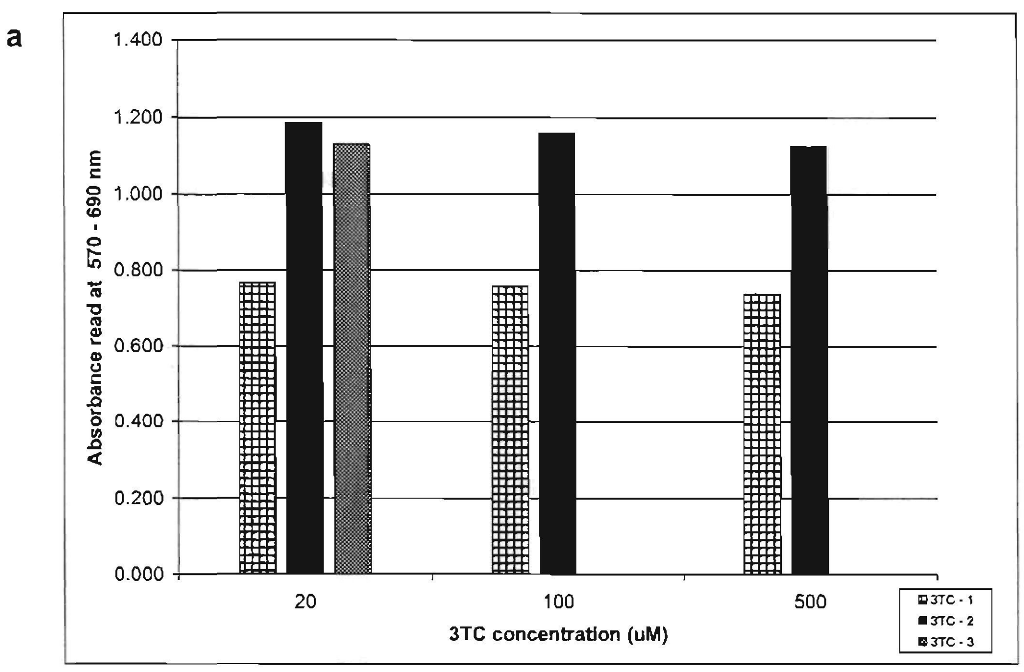
Figure 1 The absorbance of 3 studies using the MTT assay with 3TC concentrations of 20 µM, 100 µM and 500 µM respectively. **a)** The viability of MT-2 cells at the indicated three drug concentrations. The graph shows intra-repeatability, but not inter-repeatability of MTT. **b)** The graphs illustrate the protective properties of 3TC. Protection analysis shows lack of both intra- and inter-repeatability.

Figure 2 Finter's neutral red (NR) determination of cell viability and protection by d4T. The viability of the cells shows large variation, where linear decline was expected as the concentration of drug increased. There was no correlation between the viability and protective properties of d4T, as determined by staining the infected cells with NR.

Figure 3 Micrographs of formazan crystals in MT-2 cells. **a – c)** These micrographs show syncytias which were formed when MT-2 cells were infected with HIV and stained with MTT. Light microscopy captured images of purple, needle-like crystals in all the syncytias. **d – f)** These micrographs are uninfected MT-2 cells which was exposed to MTT. MTT was reduced, as expected, and purple water-insoluble formazan crystals formed in cells. Both uninfected cells and syncytias contained formazan crystals.

Figure 1

a



b

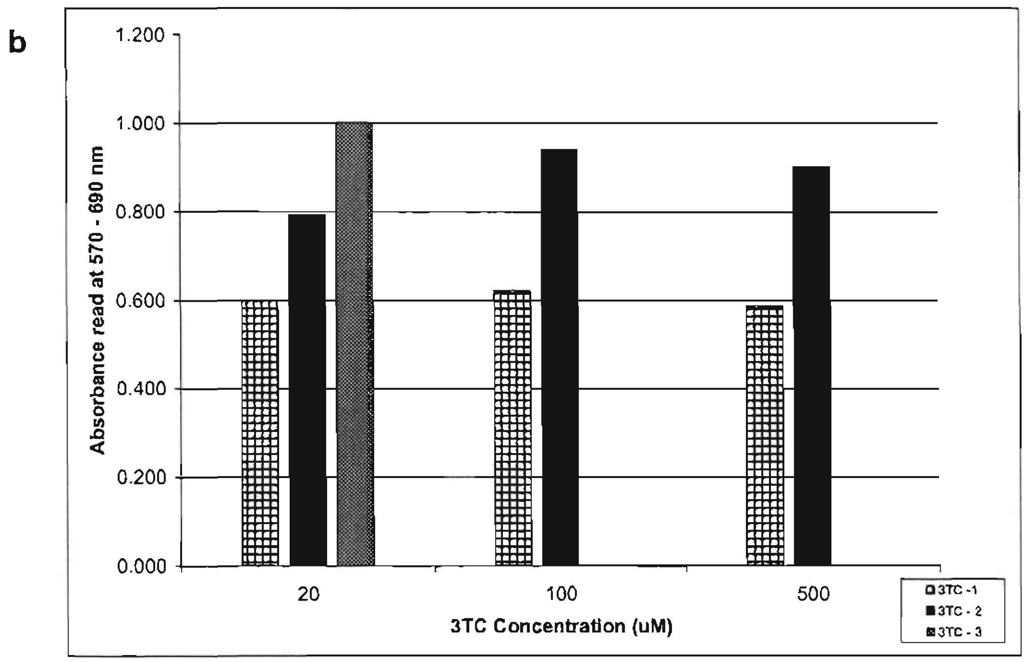


Figure 2

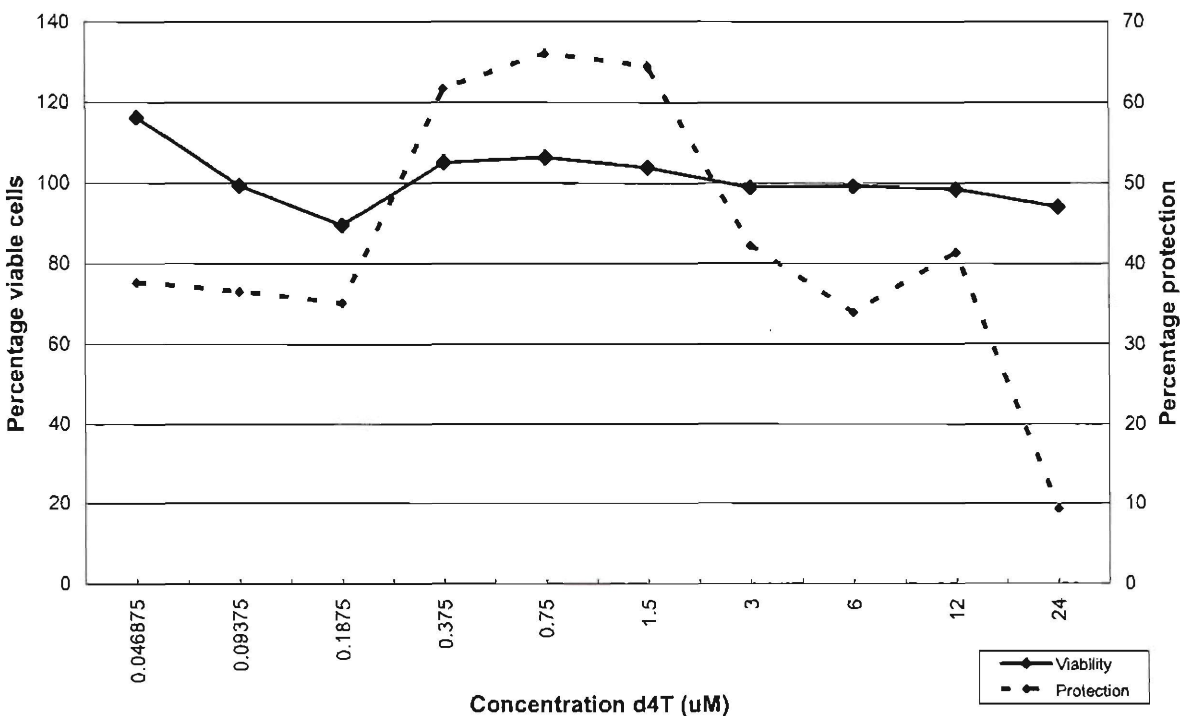
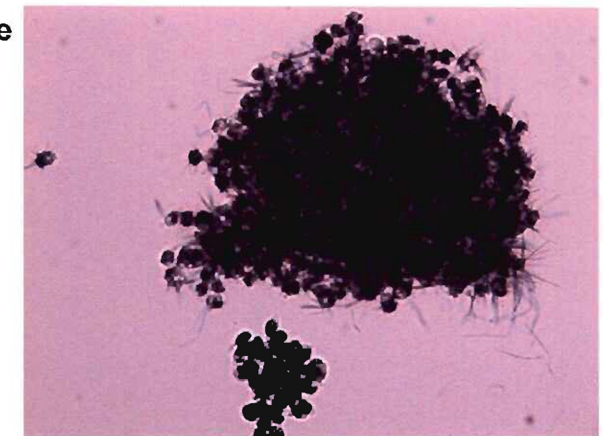


Figure 3

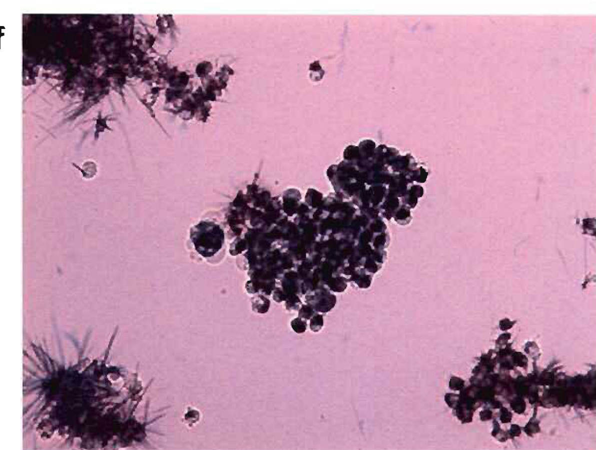
a

b

c



d



e

f

CHAPTER 5

The *in vitro* effect of the Pheroid™ drug delivery system

5 Introduction

The Pheroid™ drug delivery system as an efficacy enhancer was discussed in chapter 3 together with its main characteristics, functions and advantages. The following chapter is dedicated to the experimental results obtained by applying the Pheroid™ technology as an efficacy enhancer for antiretrovirals *in vitro*. The Pheroids™ used in the viability assays was manufactured by the Unit of Drug Research at the North-West University, Potchefstroom. Experimental research was performed at the NICD due to the absence of a biohazard level II/III laboratory at the North-West University.

5.1 Investigation of the possible enhancement of *in vitro* efficacy by Pheroid™ entrapment of antiretrovirals in a 7 day MTT assay

Using the Pheroid™ drug delivery system to enhance the efficacy of antiretroviral therapy could lead to a more effective therapy, as highlighted in chapter 3. Formulation problems and adverse effects could be overcome with the use of this delivery system. The viability and toxic properties of d4T and 3TC have been determined in the described *in vitro* model and are presented in annexure G.

At the time that this investigation was initiated, the lack of reproducibility of the MTT assay in this infection model, as described in chapter 4, has not yet been observed. Therefore, despite a current lack of confidence in the assay, some results observed using Pheroid™ technology and two ARVs are described in this chapter. The methodology used in this study is similar to that described in chapter 4 and is further clarified in the various annexures. The cell line and specific virus strain used in this chapter was selected after an investigation into the viability and protection of the cell lines and three viral strains in the infection model, which is described in annexure B.

5.1.1 Investigation of cell viability at different concentrations of Pheroids™.

5.1.1.1 Introduction

Before evaluating the enhancement properties of Pheroids™ on antiretrovirals, the effect of Pheroid™ on cell viability needed to be determined. Pheroids™ was diluted with N_2O gassed water in a serial dilution and incubated with MT-2 cells according to the adapted MTT assay as discussed in annexure D.

5.1.1.2 Method

A total volume of 100 µl Pheroid™ dilutions were added to each of the wells. The Pheroids™ were diluted with gassed water as illustrated in table 5.1 below. The absorbances of the wells were determined at 570 nm and 690 nm as an indication of the amount of viable cells present. Absorption was used to determine viability and was determined in optical density units, which will not be indicated as such in the results in this chapter.

Table 5.1 Serial dilution of Pheroids™

Pheroid™(µl)	0.002	0.004	0.008	0.016	0.031	0.063	0.125	0.250	0.500
Dilution	128000	64000	32000	16000	8000	4000	2000	1000	500

Pheroid™(µl)	1	2	3	4	5	6	7	8	9	10	12.5	15
Dilution	250	125	83.3	62.5	50	41.7	35.7	31.3	27.8	25	20	16.7

Pheroid™(µl)	20	50	100
Dilution	12.5	5	2.5

5.1.1.3 Results

The raw absorbance data (table 5.2 and 5.3) was plotted against the dilution of Pheroids™ used. The results obtained are presented below in figure 5.1.

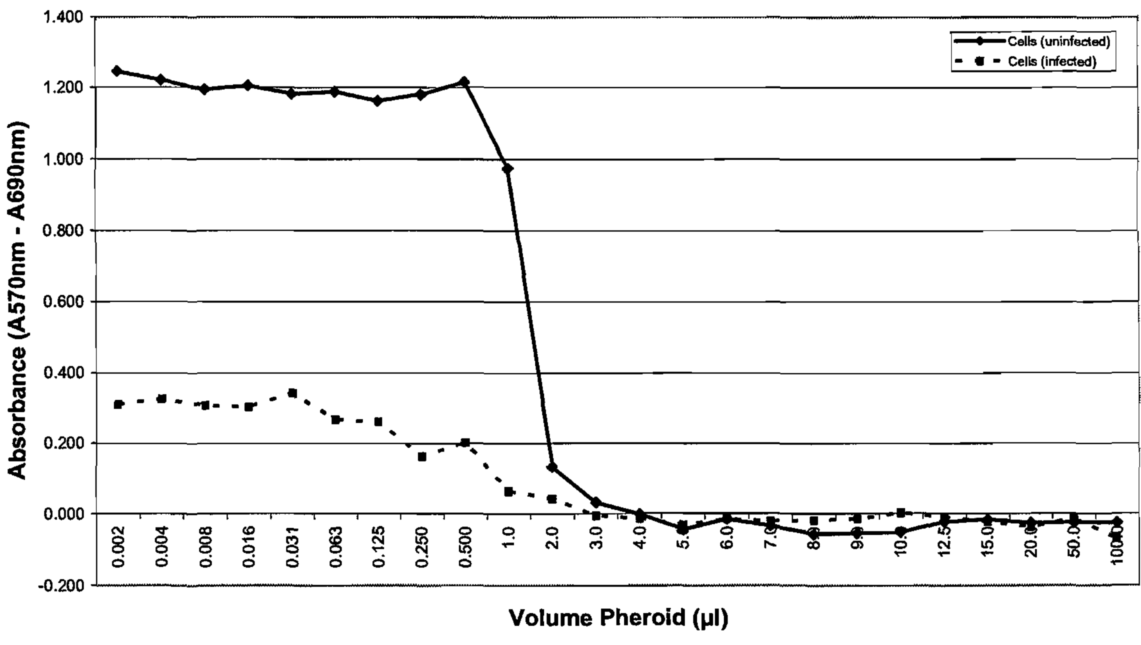


Figure 5.1 Absorbance reflecting the number of viable cells present against the dilution of Pheroids™ used. Absorbance was read at 570 nm – 690 nm.

Table 5.2 The absorbance data from uninfected, viable cells incubated with a serial dilution of Pheroids™

Pheroid™		Blanks				Samples			Samples-Blanks			
(µl)	Dilution	1	2	3	Average	1	2	3	1	2	3	Average
Control		**0.040**	**0.065**	**0.081**	**0.062**	**1.429**	**1.405**	**1.427**	**1.367**	**1.343**	**1.365**	**1.358**
0.002	**128000.0**	0.111	0.123	0.111	**0.115**	1.334	1.328	1.418	1.219	1.213	1.303	**1.245**
0.004	**64000.0**	0.107	0.108	0.108	**0.108**	1.309	1.326	1.348	1.201	1.218	1.240	**1.220**
0.008	**32000.0**	0.114	0.106		**0.110**	1.346	1.271	1.296	1.236	1.161	1.186	**1.194**
0.016	**16000.0**	0.103	0.112	0.107	**0.107**	1.280	1.330	1.325	1.173	1.223	1.218	**1.204**
0.031	**8000.0**	0.102	0.106	0.106	**0.105**	1.287	1.280	1.292	1.182	1.175	1.187	**1.182**
0.063	**4000.0**	0.111	0.108	0.107	**0.109**	1.286	1.280	1.323	1.177	1.171	1.214	**1.188**
0.125	**2000.0**	0.107	0.111	0.106	**0.108**	1.282	1.268	1.259	1.174	1.160	1.151	**1.162**
0.250	**1000.0**	0.112	0.110	0.105	**0.109**	1.273	1.273	1.321	1.164	1.164	1.212	**1.180**
0.500	**500.0**	0.117	0.125	0.109	**0.117**	1.323	1.351	1.323	1.206	1.234	1.206	**1.215**
1.0	**250.0**	0.116	0.108		**0.112**	1.122	1.119	1.020	1.010	1.007	0.908	**0.975**
2.0	**125.0**	0.117	0.121		**0.119**	0.306	0.199	0.249	0.187	0.080	0.130	**0.132**
3.0	**83.3**	0.120	0.118	0.116	**0.118**	0.131	0.146	0.171	0.013	0.028	0.053	**0.031**
4.0	**62.5**	0.121	0.107		**0.114**	0.120	0.104	0.114	0.006	-0.010	0.000	**-0.001**
5.0	**50.0**	0.125	0.113		**0.119**	0.061	0.060	0.104	-0.058	-0.059	-0.015	**-0.044**
6.0	**41.7**	0.126	0.114		**0.120**	0.113	0.101	0.102	-0.007	-0.019	-0.018	**-0.015**
7.0	**35.7**	0.129	0.117		**0.123**	0.058	0.105	0.104	-0.065	-0.018	-0.019	**-0.034**
8.0	**31.3**	0.129	0.113	0.110	**0.117**	0.062	0.061	0.059	-0.055	-0.056	-0.058	**-0.057**
9.0	**27.8**	0.124	0.112	0.122	**0.119**	0.066	0.065	0.063	-0.053	-0.054	-0.056	**-0.055**
10.0	**25.0**	0.117	0.114	0.116	**0.116**	0.062	0.064	0.066	-0.054	-0.052	-0.050	**-0.052**
12.5	**20.0**	0.120		0.123	**0.122**	0.071	0.111	0.115	-0.051	-0.011	-0.006	**-0.023**
15.0	**16.7**	0.134	0.134	0.137	**0.135**	0.121	0.121	0.115	-0.014	-0.014	-0.020	**-0.016**
20.0	**12.5**	0.161	0.151	0.141	**0.151**	0.130	0.121	0.125	-0.021	-0.030	-0.026	**-0.026**
50.0	**5.0**	0.146	0.133	0.154	**0.144**	0.117	0.123	0.121	-0.027	-0.021	-0.023	**-0.024**
100.0	**2.5**	0.185	0.183	0.162	**0.177**	0.159	0.152	0.144	-0.018	-0.025	-0.033	**-0.025**

Table 5.3 The absorbance data from infected cells incubated with Pheroids™

Pheroid™		Blanks				Samples			Samples-Blanks			
(µl)	Dilution	1	2	3	Average	1	2	3	1	2	3	Average
Control		**0.056**	**0.068**	**0.073**	0.066	**0.432**	**0.469**	**0.463**	**0.366**	**0.403**	**0.397**	0.389
0.002	**128000.0**	0.111	0.123	0.111	**0.115**	0.48	0.379	0.416	0.365	0.264	0.301	**0.310**
0.004	**64000.0**	0.107	0.108	0.108	**0.108**	0.409	0.388	0.503	0.301	0.280	0.395	**0.326**
0.008	**32000.0**	0.114	0.106		**0.110**	0.416	0.39	0.449	0.306	0.280	0.339	**0.308**
0.016	**16000.0**	0.103	0.112	0.107	**0.107**	0.369	0.363	0.499	0.262	0.256	0.392	**0.303**
0.031	**8000.0**	0.102	0.106	0.106	**0.105**	0.448	0.388	0.505	0.343	0.283	0.400	**0.342**
0.063	**4000.0**	0.111	0.108	0.107	**0.109**	0.302	0.326	0.499	0.193	0.217	0.390	**0.267**
0.125	**2000.0**	0.107	0.111	0.106	**0.108**	0.287	0.414	0.404	0.179	0.306	0.296	**0.260**
0.250	**1000.0**	0.112	0.110	0.105	**0.109**	0.274	0.255	0.283	0.165	0.146	0.174	**0.162**
0.500	**500.0**	0.117	0.125	0.109	**0.117**	0.283	0.480	0.195	0.166	0.363	0.078	**0.202**
1.0	**250.0**	0.116	0.108		**0.112**	0.172	0.194	0.158	0.060	0.082	0.046	**0.063**
2.0	**125.0**	0.117	0.121		**0.119**	0.157	0.181	0.143	0.038	0.062	0.024	**0.041**
3.0	**83.3**	*0.120*	*0.118*	*0.116*	***0.118***	*0.120*	*0.116*	*0.102*	*0.002*	*-0.002*	*-0.016*	***-0.005***
4.0	**62.5**	0.121	0.107		**0.114**	0.105	0.099	0.096	-0.009	-0.015	-0.018	**-0.014**
5.0	**50.0**	0.125	0.113		**0.119**	0.106	0.060	0.096	-0.013	-0.059	-0.023	**-0.032**
6.0	**41.7**	0.126	0.114		**0.120**	0.115	0.098	0.104	-0.005	-0.022	-0.016	**-0.014**
7.0	**35.7**	0.129	0.117		**0.123**	0.107	0.102	0.096	-0.016	-0.021	-0.027	**-0.021**
8.0	**31.3**	0.129	0.113	0.110	**0.117**	0.116	0.120	0.060	-0.001	0.003	-0.057	**-0.019**
9.0	**27.8**	0.124	0.112	0.122	**0.119**	0.108	0.106	0.101	-0.011	-0.013	-0.018	**-0.014**
10.0	**25.0**	0.117	0.114	0.116	**0.116**	0.122	0.120	0.111	0.006	0.004	-0.005	**0.002**
12.5	**20.0**	0.120		0.123	**0.122**	0.117	0.111	0.109	-0.004	-0.011	-0.013	**-0.009**
15.0	**16.7**	0.134	0.134	0.137	**0.135**	0.119	0.114	0.104	-0.016	-0.021	-0.031	**-0.023**
20.0	**12.5**	0.161	0.151	0.141	**0.151**	0.118	0.115	0.107	-0.033	-0.036	-0.044	**-0.038**
50.0	**5.0**	0.146	0.133	0.154	**0.144**	0.131	0.130	0.133	-0.013	-0.014	-0.011	**-0.013**
100.0	**2.5**	0.185	0.183	0.162	**0.177**	0.106	0.107	0.109	-0.071	-0.070	-0.068	**-0.069**

5.1.1.4 Discussion

The uninfected cells with Pheroids™ showed a high absorbance for dilutions between 83.3 and 128000 times. A dilution of 500 times showed no cytotoxicity. The Pheroid™ itself shows some protection of the MT-2 cells against HIV-1 (SW7-TCL strain) infection at the same dilutions. A 1:1000 times dilution was determined as the optimal dilution for future experimental work. Cell death occurred as a result of lack of gas exchange due to surface occlusion of wells at higher Pheroid™ concentrations, since the composition of Pheroids is mainly oil-based.

5.1.2 The *in* vitro efficacy of Pheroid™-entrapped stavudine (d4T)

5.1.2.1 Introduction

A seven day MTT assay as described in annexure D was used for determining the possible *in vitro* enhancement of the efficacy of stavudine. The Pheroid™ dilution of 1:1000

Pheroids™ in gassed water was used. The viability and protective properties of d4T itself were evaluated beforehand (see annexure G 1.1 for results obtained).

5.1.2.2 Method

Lower concentrations of d4T were used than that described in annexure G, due to the possible enhancement in efficacy by Pheroid™ entrapment. The concentration series of d4T is presented in table 5.4. The assay was performed accordingly. Absorbance of each well was read as an indication of viability.

5.1.2.3 Results

The absorbance results at the indicated wavelengths from d4T alone and d4T entrapped in Pheroids™ are presented below in table 5.4.

Table 5.4 Absorbance (viability) of the cells incubated with d4T and Pheroid™-entrapped d4T

d4T (µM)	570 nm – 690 nm							
	d4T				d4T plus Pheroids™			
	1	2	3	Average	1	2	3	Average
0.05	0.859	0.908		0.884	0.310	0.314	0.347	0.324
0.10	1.215	1.138		1.177	0.387	0.455	0.388	0.410
0.50	1.290	1.497	1.438	1.408	0.399	0.415	0.310	0.375
0.75	1.467	1.240	1.454	1.387	0.364	0.289	0.324	0.326
1.00	1.301	1.418	1.532	1.417	0.401	0.298	0.349	0.349
1.50	1.550	1.465	1.543	1.519	0.427	0.283	0.361	0.357
2.00	1.329	1.441	1.568	1.446	0.325	0.280	0.414	0.340
2.50	1.705	1.687	1.631	1.674	0.340	0.387	0.211	0.313

The two sets of data are illustrated in figure 5.2 to demonstrate the possible enhancement of the protective properties of stavudine by Pheroid™ technology.

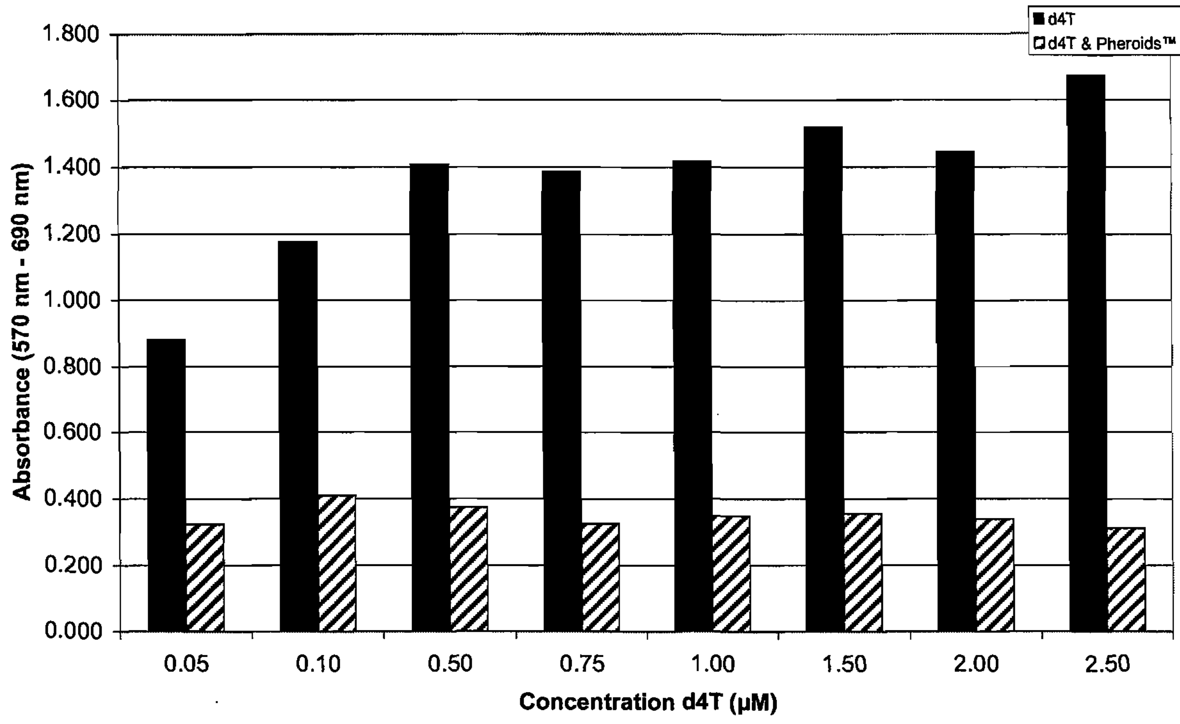


Figure 5.2 Illustration of the protective properties of d4T in the presence and absence of Pheroids™.

5.1.2.4 Discussion

The results of d4T (without Pheroids™) indicated protection as expected. The protective properties of the d4T/Pheroid™ samples were low, indicating cell death. The cell death could be explained by the following arguments:

1. The Pheroid™ drug delivery system carried an excessive amount of drug into the cells, which led to toxic levels, promoting cell death.

2. The concentration of d4T used was too high.

3. Stavudine broke down to one of its breakdown products (thymine), which is cell toxic, resulting in cell death.

4. Or the Pheroid™ prevented absorption of d4T by the cell.

5.1.3 The *in vitro* efficacy of Pheroids™-entrapped lamivudine (3TC) in a 7 day MTT assay

5.1.3.1 Introduction

The seven day incubation study was based on the adapted MTT assay as described in annexure D. The protective and toxic properties of 3TC were determined previously (annexure G), which enables a comparison with the same properties when the drug was entrapped in Pheroids™.

5.1.3.2 Method

A dilution series of lamivudine (3TC) were entrapped in Pheroids™ before it was incubated with MT-2 cells. The adapted MTT assay was used. The Pheroid™ dilution was a 1:1000 Pheroid™ : gassed water. In the negative control, no virus or drug was added to the cells.

5.1.3.3 Results

Table 5.5 Absorbance (viability) of the cells incubated with 3TC and Pheroid™-entrapped 3TC

3TC (µM)	570 nm – 690 nm							
	3TC				3TC plus Pheroids™			
	1	2	3	Average	1	2	3	Average
Control	1.452	1.512	1.458	1.474	-	-	-	-
0.000	0.411	0.328	0.446	0.395	0.411	0.328	0.446	*0.395*
0.001	0.434	0.585	0.462	0.494	0.362	0.489	0.748	0.395
0.005	0.370	0.424	0.489	0.428	0.398	0.509	0.597	0.533
0.010	0.639	0.589	0.486	0.571	0.380	0.460	0.464	0.501
0.050	0.452	0.667	0.442	0.520	0.424	0.538	0.542	0.435
0.100	0.562	0.595	0.548	0.568	-	0.564	0.531	0.501
0.500	0.633	0.723	0.582	0.646	0.435	0.412	0.488	0.548
1.000	0.512	0.375	0.614	0.500	0.475	0.489	0.541	0.445
10.000	1.256	1.343	1.388	1.329	0.088	0.088	0.082	0.502
20.000	1.197	1.404	1.384	1.328	0.122	0.110	0.099	0.086

The results obtained from the incubation with 3TC only and 3TC entrapped in Pheroids™ are presented in table 5.5.

5.1.3.4 Discussion

The viability of the cells incubated with 3TC and Pheroids™ had low but almost equal absorbance readings to that of the cells incubated with 3TC only. At a 3TC concentration of 10 µM the viability of the cells incubated with 3TC only were significantly higher than that of the cells incubated with 3TC entrapped in Pheroids™.

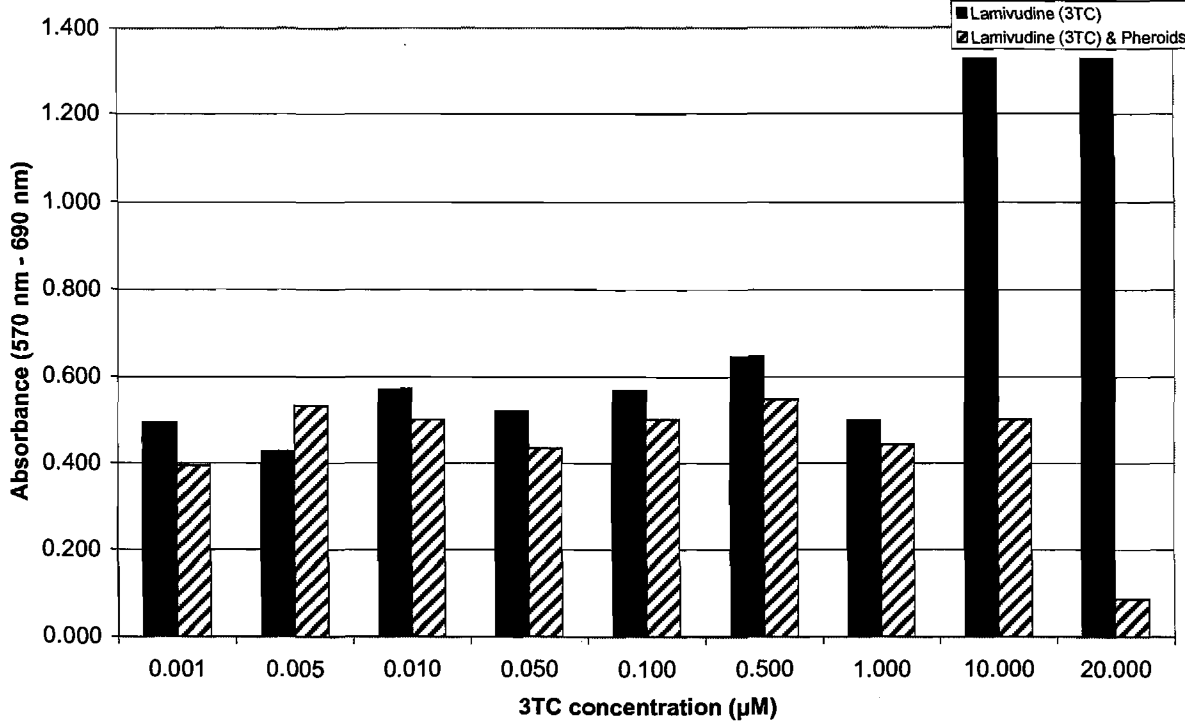


Figure 5.3 The possible enhancement properties of 3TC in Pheroids™

At 20 µM the absorbance levels of the cells (when incubated with 3TC and Pheroids™) were almost zero, indicating major cell death. Since nearly identical protection of cells were found at the lower concentrations, the only possible explanation could be that the cells died due to an increased uptake of drugs and not a decreased uptake. This confirmed the hypothesis of the Pheroid™ drug delivery system – the enhanced uptake of drugs into cells.

As described in chapter 4, infection led to syncytium formation. Since syncytia were observed from day 1, it was decided to shorten the incubation period to prevent the cell loss due to accumulation of toxic levels of the antiretrovirals in the cells. It was concluded from this experiment that the contact time between Pheroids™-entrapped drugs and the cells needed to be shortened.

5.2 The possible *in vitro* enhancement in efficacy by Pheroid™ entrapment during a 3 day incubation period

Results from the previous experiments suggested that the incubation period needed to be shortened. Before any studies could continue, the MTT assay needed to be adapted to an incubation time of 3 days. It was therefore necessary to determine the cell and virus dilutions to be used to achieve the required viral infection and syncytium formation again.

5.2.1 Optimization of the cell and virus concentrations for a three day incubation period

The procedure and experimental results of this experiment are presented in annexure K. The optimal cell concentration and virus dilution were 1 x 10^4 cells/well with a 10 times virus dilution.

5.2.2 Determination of cell viability and protection by 3TC over a three day incubation period

5.2.2.1 Introduction

The toxicity and efficacy of lamivudine was determined for the new cell concentration and virus dilution.

5.2.2.2 Method

3TC was incubated with an optimal cell concentration of 1 x 10^4 cells/well and a 10 times virus dilution. The adapted 3 day MTT assay was used to determine the absorbance of all the wells. This experiment was also repeated with an extended concentration series of 3TC, due to the lack of information from the first experiment. These results are presented in figures 5.4 and 5.5 respectively.

5.2.2.3 Results

The results obtained from the incubation with lamivudine on the new cell concentration and virus dilution are presented below.

Table 5.6 Absorbance (viability) of the cells incubated with 3TC over a three day incubation period (initial concentration series)

3TC (µM)	570 nm – 690 nm							
	Uninfected				Infected			
	1	2	3	Average	1	2	3	Average
Control	0.906	0.922	0.923	0.917	0.470	0.453	0.471	0.465
0.001	0.812	0.856	0.850	0.839	0.451	0.459	0.459	0.456
0.006	0.865	0.874	0.858	0.866	0.452	0.454	0.457	0.454
0.032	0.817	0.838	0.856	0.837	0.438	0.448	0.456	0.447
0.160	0.816	0.862	0.836	0.838	0.451	0.445	0.468	0.455
0.800	0.837	0.822	0.810	0.823	0.479	0.479	0.482	0.480
4.000	0.832	0.837	0.821	0.830	0.516	0.565	0.561	0.547
20.000	0.766	0.775	0.760	0.767	0.597	0.603	0.599	0.600
100.000	0.725	0.769	0.779	0.758	0.612	0.645	0.610	0.622
500.000	0.757	0.731	0.724	0.737	0.575	0.577	0.610	0.587

Table 5.7 Absorbance (viability) of the cells incubated with 3TC over a three day incubation period (extended concentration series)

3TC (µM)	570 nm – 690 nm							
	Uninfected				Infected			
	1	2	3	Average	1	2	3	Average
Control	1.366	1.343	1.359	1.356	0.433	0.424	0.442	0.433
0.010	1.288	1.303	1.302	1.298	0.409	0.425	0.416	0.417
0.050	1.249	1.301	1.253	1.268	0.419	0.425	0.439	0.428
0.100	1.296	1.249	1.242	1.262	0.407	0.451	0.418	0.425
0.500	1.223	1.187	1.202	1.204	0.463	0.446	0.441	0.450
1.000	1.175	1.160	1.139	1.158	0.478	0.497	0.477	0.484
2.500	1.283	1.200	1.113	1.199	0.596	0.592	0.575	0.588
5.000	1.202	1.203	1.134	1.180	0.67	0.647	0.676	0.664
10.000	1.203	1.169	1.178	1.183	0.89	0.765	0.791	0.815
20.000	1.207	1.175	1.173	1.185	0.789	0.802	0.787	0.793
30.000	1.284	1.274	1.302	1.287	1.051	1.06	1.064	1.058
40.000	1.306	1.254	1.292	1.284	0.965	1.062	1.059	1.029
50.000	1.192	1.178	1.163	1.178	0.982	0.933	0.98	0.965
60.000	1.197	1.194	1.240	1.210	0.918	0.959	0.977	0.951
70.000	1.133	1.141	1.147	1.140	0.848	0.942	0.931	0.907
80.000	1.170	1.115	1.101	1.129	0.81	0.926	0.964	0.900
90.000	1.306	1.118	1.153	1.192	0.909	0.913	0.932	0.918
100.000	1.140	1.162	1.177	1.160	0.901	0.933	0.988	0.941
250.000	1.090	1.153	1.127	1.123	0.882	0.936	0.984	0.934
500.000	1.129	1.120	1.124	1.124	0.843	0.911	0.954	0.903

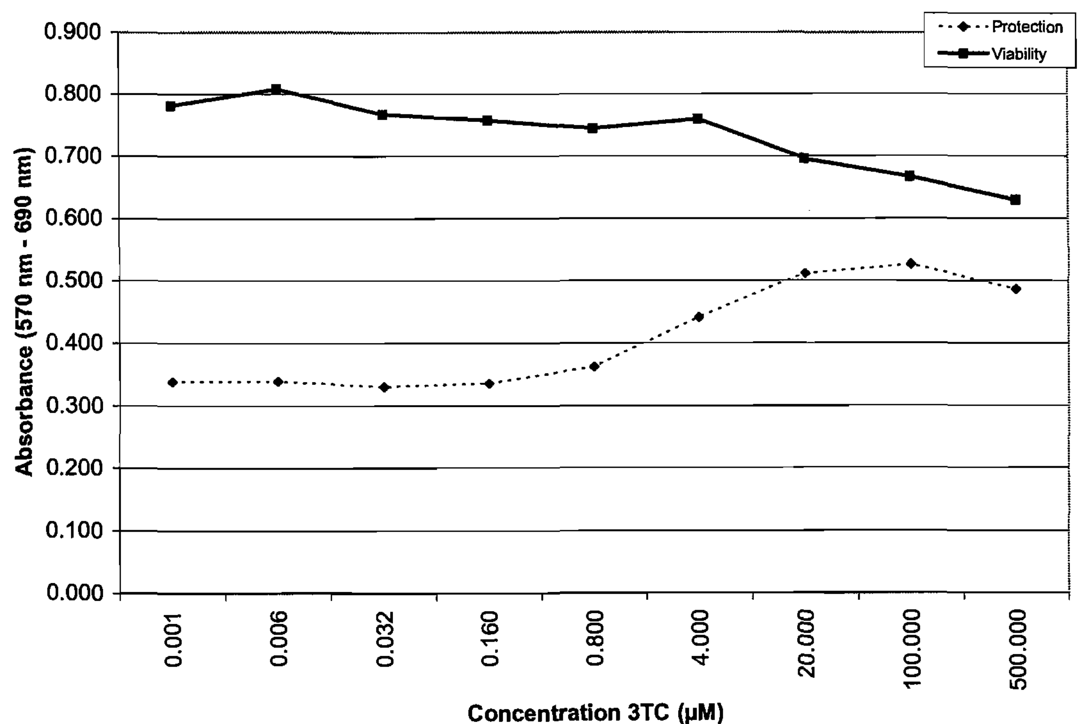


Figure 5.4 Absorbance data, reflecting cell viability from 3TC (serial dilutions)

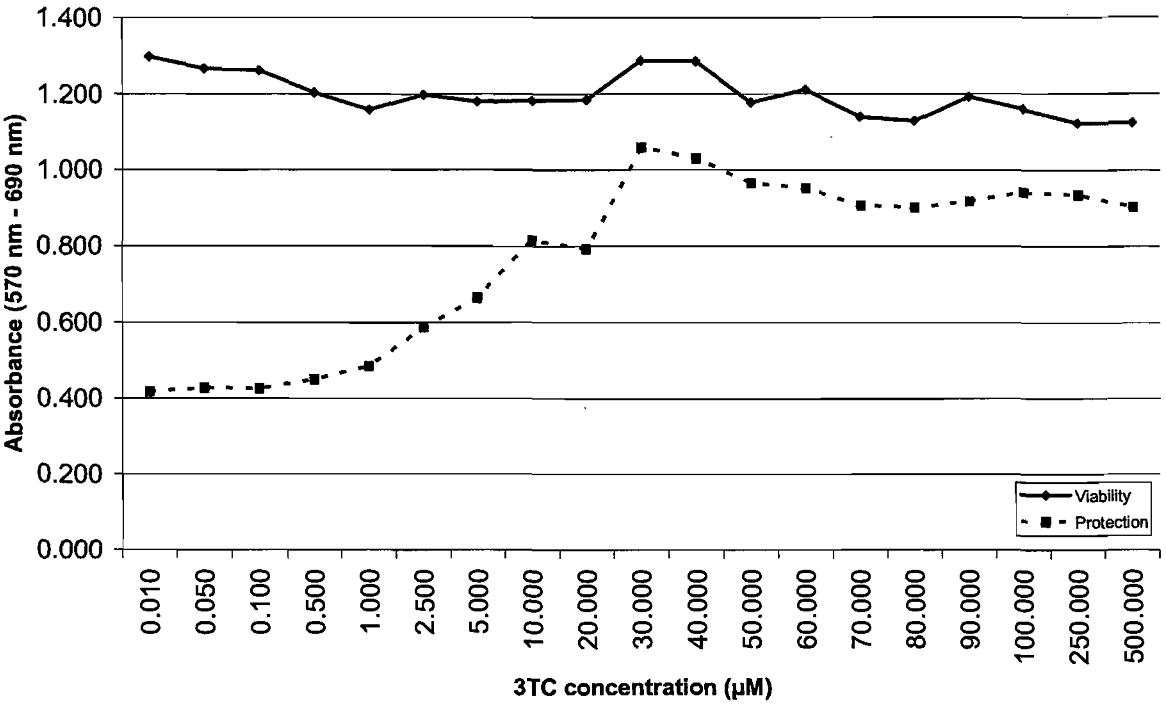


Figure 5.5 Extended 3TC concentration series

5.2.2.4 Conclusion

Lamivudine showed both toxic and protective properties over the concentration ranges used. Optimal protection was not obtained but with the Pheroid™ technology, this protection could perhaps be optimized. The lack of reproducibility (see chapter 4) in this assay is obvious from a comparison of the negative control of uninfected cells. The protection of lamivudine did not reach optimum levels; perhaps the infection limited cell growth and healthy cells were not given enough time to multiply.

5.2.3 The cell viability and protection of lamivudine entrapped in Pheroid™ (using a 3 day incubation period)

5.2.3.1 Introduction

With the information and data obtained from the previous experiments the 3 day incubation period was optimized to lessen the cell toxicity as a result of the enhanced drug delivery properties of Pheroids™. The aim of this experiment was to illustrate the possible enhancement properties of Pheroid™ technology without cell loss due to toxicity.

5.2.3.2 Method

The protocol followed for this experiment was the assay adapted to a 3 day incubation period (annexure K). The Pheroid™ was diluted with N_2O gassed water to a dilution of 1:1000 Pheroids™ to gassed water. Lamivudine was entrapped within the Pheroids™ and this was incubated for one hour with the cells. After this incubation, the virus was added and the well plate was incubated for three days.

5.2.3.3 Results

The obtained results are presented in table 5.8. The cell control had an absorbance of 1.051 and the virus control an absorbance of 0.710.

Table 5.8 Absorbance (viability) of the cells incubated with 3TC and Pheroid™-entrapped 3TC

3TC (μM)	570 nm – 690 nm							
	Uninfected				Infected			
	1	2	3	Average	1	2	3	Average
control	0.702	0.718	0.709	0.710	0.702	0.718	0.709	0.710
0.01	0.692	0.652	0.680	0.710	0.686	0.686	0.699	0.690
0.05	0.677	0.648	0.648	0.658	0.706	0.742	0.696	0.715
0.1	0.667	0.640	0.678	0.662	0.709	0.709	0.722	0.713
0.5	0.729	0.700	0.705	0.711	0.692	0.715	0.685	0.697
1	0.751	0.766	0.853	0.790	0.657	0.769	0.692	0.706
2.5	0.857	0.825	0.856	0.846	0.817	0.818	0.767	0.801
5	0.958	0.845	0.922	0.908	0.878	0.858	0.865	0.867
10	0.963	0.979	1.014	0.985	0.900	0.854	0.842	0.865
20	0.984	0.980	1.040	1.001	0.911	0.896	0.845	0.884
30	1.001	1.012	1.086	1.033	0.889	0.870	0.846	0.868

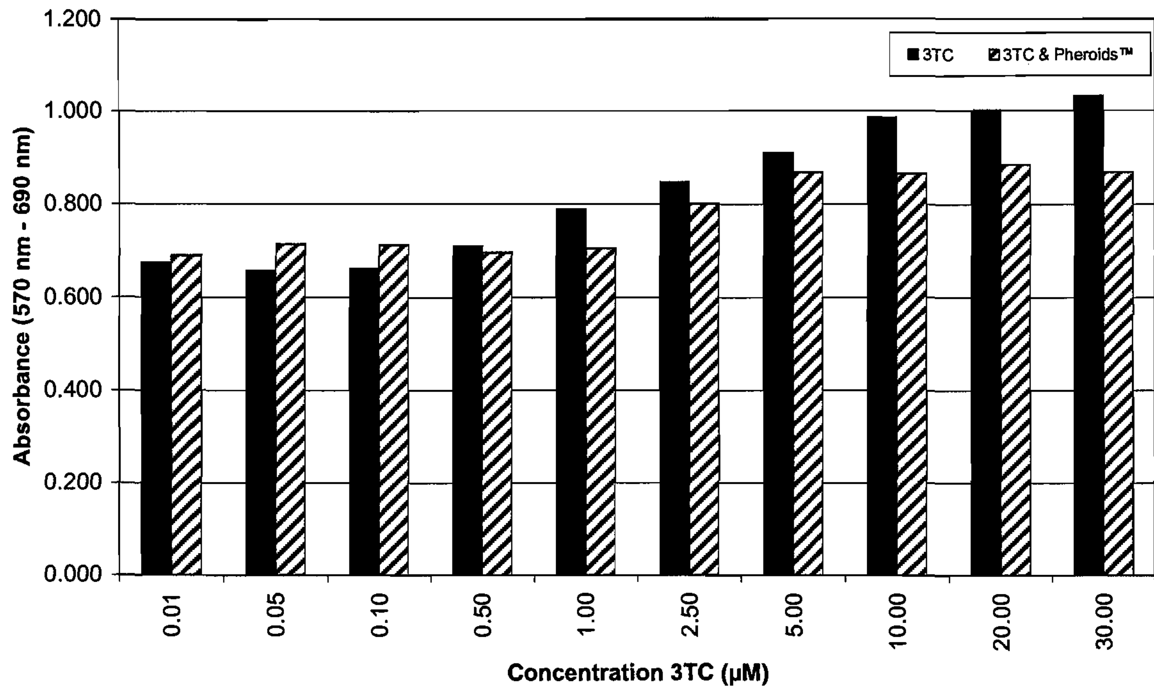


Figure 5.6 Illustration of the absorbance data, reflecting protection with and without Pheroids™.

5.2.3.4 Conclusion

This was the only study in which the cells were still viable and alive at the end of treatment. Thus, the shorter incubation time was successful.

The viability was slightly enhanced at lower drug concentrations by the Pheroid™, as indicated in figure 5.6. Enhancement was present at only two concentrations, 0.05 µM and 0.1 µM 3TC. At the concentrations higher than 0.1 µM 3TC there is a trend of increased cell death; the higher the concentration of Pheroid™-entrapped lamivudine, the bigger the difference between entrapped and un-entrapped 3TC. This confirmed previous findings. Due to the proven degradation of stavudine in the experimental set up (results not shown), the study was not repeated with stavudine.

Thus, Pheroid™ technology has the ability to enhance drug delivery, but more accurate viability assays is a must to ensure valid data analysis. An alternative approach is to use specific neutralizing antibody assays rather the aspecific viability and toxicity assays to determine the impact of Pheroid™ on drug efficacy.

CHAPTER 6

Summary and Conclusion

6 Introduction

After a detailed literature study was conducted (Chapter 1), areas that could be improved in the treatment of HIV/AIDS were identified. Obstacles that influence antiretroviral treatments and the adherence to it are strict dosage regimes, as well as an endless list of side effects. If these complications could be overcome, the treatment of HIV/AIDS will be improved.

A novel drug delivery system based on Pheroid™ technology was used in an effort to improve the efficacy of selected antiretrovirals, as previous research suggested that such enhancement of efficacy of compounds by its entrapment into Pheroids™ is possible.

6.1 Summary

The aim of this study was to enhance the efficacy of the chosen antiretrovirals: stavudine (d4T), lamivudine (d4T) and nevirapine. Current and frequently used assays, Finter's neutral red and Mosmann's MTT viability assays, were adapted to study the possible enhancement of the above mentioned drugs. The infection model consisted of an MT-2 cell line that was infected with an HIV-1 virus strain, SW7-TCL. Virus infection induces syncytia formation in this infection model. The absorbance determined for the cells at the end of the assays was converted into the viability of the cells and protection offered by the drugs against the infective virus.

As expected, both toxicity and protection were observed for the antiretrovirals used. Stavudine was found to be more toxic than lamivudine, due to the formation of thymine which caused cell death in the control and experimental groups. Lamivudine showed a promising lack of toxicity, as the cells were still viable after being exposed to high concentrations. Nevirapine presented with solubility problems and was not investigated any further.

Applying Pheroid™ technology with the prescribed 7-day incubation period resulted in cell death at all the concentrations used. An assay containing a dilution series with only Pheroids™ and the diluent (N_2O gassed water), showed that cells remained viable in the presence of Pheroids™ diluted five hundred times or more. Applying Pheroid™ technology at a 1:1000 dilution with the antiretroviral, cytotoxic levels were reached and cell death

occurred whereas no cell death occurred with only the Pheroid™ at the dilution used. The only explanation, which confirmed the delivery characteristic of Pheroids™, was an enhanced uptake of the drugs into the cells, resulting in toxic levels. Thus, Pheroid™ technology did indeed enhance the uptake of drugs into the cells.

To decrease the cytotoxicity, the MTT assay was adapted to a three day incubation assay. New virus dilutions and cell concentrations were optimized and the three day incubation modification was found to be successful, as cells were still viable after applying the Pheroid™-entrapped lamivudine.

During the assay optimization, non-repeatability and non-reproducibility of the results were observed with the two viability assays used. Absorbance fluctuated with both these assays, when identical concentrations of drugs in different studies were compared. The experimental set up, the researcher and the equipment used were identical in each study. Intra-day repeatability could not be shown with Finter's neutral red assay, while the MTT assay showed no inter-day reproducibility. Both these assays are based on the staining of intra-cellular organelles. It was thought possible that the formation of syncytia interferes with the specificity of viability staining. Indeed, microscopic investigation of uninfected cells and syncytia showed that both these cell types presented with water insoluble formazan crystals. These results thus do not contradict the proposed mechanism of MTT whereby only active cells reduce MTT to formazan crystals, but challenge the assumption that syncytia are not regarded as active cells. The exact degree by which a syncytium is still active varies and is difficult to estimate, and is probably the cause of the respective non-repeatability and non-reproducibility of the Finter's red and the MTT assay in this study.

6.2 Conclusion

The effective treatment of HIV and AIDS in this day and age is dependent on three factors, namely, effective medicinal treatment, uncomplicated dosage regimes and patient compliance. By applying Pheroid™ technology, these factors can be improved and side effects can be at least partially eliminated.

It was concluded from this study that the viability assays used produced non-repeatable and non-reproducible results in this specific infection model. Additional assays which may assist in obtaining repeatable and reproducible results include the p24 antigen assay and the reverse transcriptase assay. The two viability assays used in this study should be used with caution in the future.

Since the Pheroid™ has been shown to enhance drug uptake in this study, an effort should be made to continue the investigation. The following factors should be taken into account in such continued development:

- Design and development of accurate viability assays;
- Comprehensive stability and solubility studies on the drugs used;
- The metabolic state of syncytia;
- Shortening the time of the cell-drug-Pheroid™ contact to prevent cell death due to the accumulation of toxic levels of the drug in the cells;
- The use of the p24 antigen or reverse transcriptase assays, as these assays are specific and should show better repeatability and reproducibility of results;
- The reproducibility of the MTT assay, which should be evaluated within the context of each *in vitro* infection model used.

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ANNEXURES

ANNEXURE A

Annexure A.1 is a copy of the AIDS unit safety manual and the indemnity form for the AIDS unit at the NICD. These documents are a summary of the standard operating procedures to be followed when working in the unit.

Annexure A.2 is the protocol for the neutral red assay as used by the NICD.

ANNEXURE A.1

STANDARD OPERATING PROCEDURE

Title: Safety in the AIDS Unit

Document number: NIC0100

Version number: 2

(Changes from previous version highlighted)

Written by: S. Cohen

Checked by: N/A

Approved by: Prof L. Morris

Active date: 07-03-2007

Date of next review	*Date reviewed*	*Reviewed by*	*Action*
07-03-2008			

Date withdrawn: ………………

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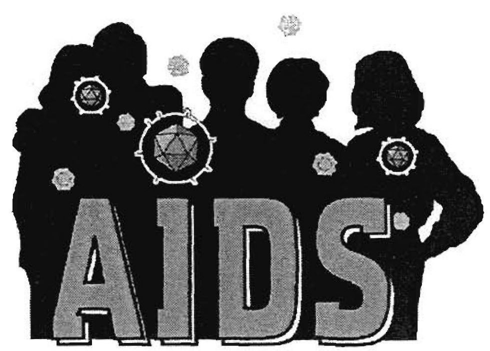

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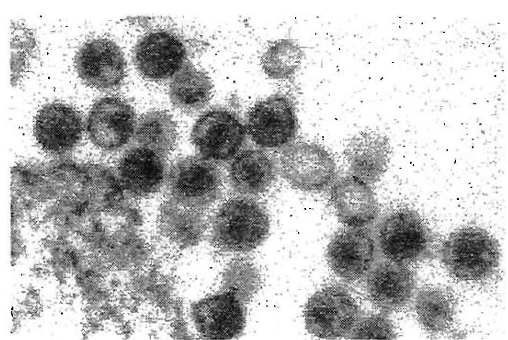
AIDS UNIT SAFETY MANUAL

Compiled by Prof Lynn Morris
National Institute for Communicable Diseases
September 2001
Revised: August 2005
Updated: February 2007



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1. GENERAL LABORATORY RULES

1.1 Only work with HIV-infected material in designated HIV culture laboratories. (i.e. BSL3) Follow strict guidelines (see pages 3-8) when working in these areas.

1.2 Perform pre-PCR and post-PCR procedures in separate areas to prevent contamination (see pages 10-13). Do not take PCR products and plasmids into clean laboratories and culture laboratories.

1.3 Every sample, included those stored in fridges and freezers, must be labelled with:

Patient Sample identification
Date of sample receipt
Contents
Harvest date (virus isolates only)
Barcode number (if applicable)

Keep samples in a container with YOUR name on it. Unlabelled samples will be discarded.

1.4 Wear face-shield and cryogen-gloves and closed shoes when working with liquid N_2.

1.5 Weigh out harmful chemicals (eg acrylamide, SDS) inside a fume hood.

1.6 Wear gloves when handling ethidium bromide and discard liquid ethidium bromide into destaining bag container. (GPL0094)

1.7 Keep laboratory areas and equipment clean and tidy. Discard unwanted samples and reagents on a regular basis, particularly in communal areas. Report faulty equipment to the Laboratory Manager for repair by Workshop Department. The Laboratory Manager must keep an error log action sheet to record all faults reported, and keeps regularly updated records of all maintenance of equipment.

1.8 Be considerate of others that you are sharing the space with. This includes keeping noise levels down and radios off if they are disturbing others.

1.9 No eating, drinking or smoking or running in the laboratories.

1.10 Always wear a lab coat and wash your hands with disinfectant soap when you leave the laboratory.

2. BIOSAFETY IN LABORATORIES WORKING WITH INFECTIOUS HIV (from UNAIDS guidelines)

The major hazard to laboratory workers working with HIV-infected blood and body fluids is contamination of hands and mucous membranes of the eyes, nose and mouth. HIV has not been shown to be transmitted via aerosolization. However, the generation of aerosols should be minimized. The use of a biosafety cabinet and other containment device must be used whenever the creation of an aerosol is possible. Contamination, if it occurs, is usually the result of penetrating injuries caused by sharp objects and from the spilling and splashing of specimen materials. Therefore, the most important elements of biosafety guidelines are:

2.1 • to avoid penetrating injuries and to prevent direct contact of skin or mucous membranes with HIV-infected blood

2.2 • to prevent contamination of an individual or their clothing by wearing a laboratory coat, gloves and safety glasses and good basic hygiene practices, including regular hand

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washing, control of surface contamination by containment and disinfection, and safe disposal of contaminated waste

The role of training in laboratory safety is vital and must be continually monitored and strengthened. Poor laboratory practice and human error can negate all safety standards and render equipment hazardous. Continual on-the-job training and monitoring in safety measures is essential for all laboratory and support staff.

2.3 Laboratory facilities

All procedures involving infected cell culture manipulation should be performed in a designated biological safety cabinet and preferably with sealed centrifuge buckets or rotors.

- work in a laboratory room devoted exclusively to work with HIV-infected materials
- biological safety cabinets (Class II) should be used with HIV infected-material. A class II biological safety cabinet is a partially open-fronted work chamber that provides protection for personnel and the surrounding laboratory space by means of a barrier air flow at the working opening. The cabinet also provides product and/or experiment protection against contamination by means of HEPA-filtered air flowing in a downwards, uniform, unidirectional manner (laminar air flow). These cabinets must be properly installed and routinely checked and serviced every 6 months: failure to do so may render the cabinet ineffective and dangerous.
- sealed centrifuge buckets or rotors should be regularly checked for integrity, to prevent the accidental dispersion of any material from the centrifuge. They should be loaded and unloaded inside a biosafety cabinet
- the windows in the laboratory should be closed and sealed
- the walls, ceilings and floor should be smooth, easily cleaned, impermeable to liquids and resistant to chemicals
- the bench tops should also be impermeable to liquids and resistant to chemicals
- the laboratory furniture should be sturdy and easily cleaned (no cloth chairs)
- washbasins should be provided in each laboratory room, preferably near the exit
- laboratory room doors should be self-closing and have vision panels, and have a "Biohazard - No Admittance" sign posted
- an autoclave for the decontamination of laboratory material and waste should be available in the same building as the HIV laboratory.
- facilities for storing clothes and items for eating, drinking and smoking should be provided outside the laboratory
- It is recommended that viral isolation and characterization involving cell lines be physically separated, that is, carried out in two different laboratory rooms. This will minimize the risk of contamination of primary isolates by a virus strain replicating in cell lines. In addition, it will prevent contamination by mycoplasma - a problem often encountered when working with cell lines. It is mandatory that laboratories working with cell lines regularly carry out mycoplasma PCR testing of all cell lines used and, when positive, remove the mycoplasma from cell lines. Cell supernatants should regularly be given to be lab manager who will arrange for PCR testing.

2.4 Precautions for laboratory workers

- wear gloves for all manipulations of potentially infectious materials. Discard gloves whenever they are thought to have become contaminated or damaged, wash your hands with soap and water and put on new gloves
- wear a laboratory gown; wrap-around gowns are preferable. Remove this protective clothing and leave it in the laboratory when leaving.
- never use mouth pipetting
- eliminate the use of glassware as much as possible, since broken glassware may be the source of exposure

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- perform all technical procedures in a way that minimizes the creation of aerosols, droplets, splashes or spills. People handling clinical samples should pay attention to the particular geographical origin of the samples, since other pathogens, requiring specific precautions, may be present.
- access to the laboratory should be restricted to essential personnel. A baseline serum sample should be obtained from each of these personnel every 12 months and stored for future reference.

2.3 Spills and accidents

- spills of blood or other body fluids should first be covered with paper towelling or other absorbent materials. A disinfectant - a hypochlorite (bleach) solution - should be poured around the spill area and then over the absorbent material and left for 10 minutes. The solution should then be removed with absorbent material and placed in a container for contaminated waste. The surface should then be wiped again with the disinfectant. Avoid direct contact of gloved hands with the disinfected spill. Broken glass or fractured plastic should be collected with a dustpan and brush.
- needle stick or other skin-piercing wounds, cuts and skin contamination by spilled or splashed specimen material should be immediately disinfected and thoroughly washed with soap and water. Bleeding from such a wound should be encouraged
- all spills, accidents and overt or possible exposure to infected or potentially infected material should be reported immediately to the laboratory supervisor. A written record should be prepared and maintained
- a spill kit, for major spills, is available in the reagent laboratory.

2.4 Handling and disposal of contaminated material and waste

- disposable contaminated equipment, e.g. needles, syringes and other sharp instruments or objects, must be placed in a puncture-resistant metal or plastic container at the work station. The container should be autoclaved or chemically disinfected prior to incineration
- used laboratory gowns, coats and other protective clothing should be placed in a separate container located within the laboratory. Before reuse, lab coats should be autoclaved and washed
- all general laboratory waste should be placed into sealed and labelled Sanumed boxes
- waste generated from BSL3 should be autoclaved prior to being placed in Sanumed waste containers

3. SAFETY PROCEDURES IN HIV CULTURE LABS AT THE NICD (this is in addition to the UNAIDS guidelines)

Prior to commencing work in an HIV culture laboratory staff must visit the Clinic to receive the appropriate immunizations including that for Hepatitis B. Staff must also be closely supervised when first working with HIV to ensure that they adhere to strict safety measures.

3.1 Always wear a lab coat and work in a Class II biohazard hood. Confine work with infectious virus to HIV culture laboratories. The use of overshoes is also recommended to maintain cleanliness of laboratory floors.

3.2 Wear double gloves, glasses when handling blood, virus stocks and infected cultures. Remove gloves <u>before</u> leaving the laboratory if you have been handling infectious material. Wash hands at the end of the procedure.

3.3 Do NOT use glass syringes, needles or glass pasteur pipettes when working with HIV.

3.4 Clean up spills in biosafety cabinets, centrifuges, benchtops and floors with bleach.

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3.5 Place cultures on tray of incubator labelled BIOHAZARD in a secondary container in case of leakage or spilling. Be careful not to tilt flasks and petri dishes.

3.6 In the event of any accident/ incident, complete Accident/ Incident form with the assistance of the Laboratory Manager. In case of HIV accident report immediately to the Laboratory Manager and Clinic Staff for assessment of injury and further medical management. (See Procedures in the event of an accident page 8)

3.7 Decontaminate haemocytometers by soaking them in a staining dish with 70% alcohol. Coverslips placed in petri dish with 70% alcohol.

3.8 Swab down hood, benchtops and microscope stage with 70% alcohol regularly. Clean up at the end of every day and switch off equipment.

3.9 Label all infected materials (in fridge, freezer and incubator) with sample name and date.

3.10 Perform rapid HIV and HBV on plasma from all normal donor units as per SOP0051, and record results in the lab book. Only process if both results are negative.

DISPOSAL

3.11 Dispose of flasks, dishes and other plasticware including Gilson tips inside the hood in BIOHAZARD bags. Place bags in metal bins, tape up and place on trolley for autoclaving. Transfer to SanuMed boxes, after autoclaving, for disposal.

3.12 Dispose of plastic pipettes in BIOHAZARD bags inside buckets in hood. Transfer to metal bins for autoclaving/ to SanuMed boxes for disposal.

3.13 Dispose of liquid waste inside hood by either tipping into milk bottles or sucking through suction line. Make sure milk bottles are filled with ±100 ml bleach (to prevent bacterial growth and inactivate virus) and are clearly labelled BIOHAZARD WASTE. When ½ full, seal with foil and autoclave tape and label BIOHAZARD WASTE. DO NOT OVERFILL THESE BOTTLES.

3.14 Place bottles, including all media and other bottles inside metal container - don't leave them on the floor. Tape up container and place them on the trolley for autoclaving and washing

NOTE: DO NOT REMOVE ANY HIV-INFECTED MATERIALS FROM THE HOOD WITHOUT IT BEING INACTIVATED OR PLACED INSIDE A BIOHAZARD BAG. PLACE BAGS IN METAL BINS (DON'T SEAL BAGS). TAPE UP METAL BIN WITH AUTOCLAVE TAPE AND TAKE TO WASH-UP TO BE AUTOCLAVED AND WASHED. DO NOT MIX PLASTIC AND GLASSWARE.

This applies to BSL3 Laboratory. All other laboratory waste to be placed in labelled and sealed Sanumed boxes to await collection.

3.15 All staff to know location of the following:
First Aid Kit
Fire fighting equipment
Eye-baths
PEP

4. WORKING WITH PSEUDOVIRUSES

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Principle:
The performance of neutralizing antibody assays under properly standardized, optimized and validated conditions requires accurate titration of virus infectivity. The use of molecularly cloned pseudoviruses has advantages over uncloned virus for greater reagent stability, improved assay reproducibility and precision. The first step is the production of molecularly cloned Env-pseudotyped HIV-1 in 293T cells by co-transfection with an ENV-expressing plasmid plus a backbone plasmid lacking Env. Co-transfection generates pseudovirus particles that are able to infect cells but, due to the absence of a complete genome, they are unable to produce infectious progeny virions. This single cycle infection can be detectable in suitable cell lines that contain a Tat-regulated reporter gene, such as luciferase.

Safety precautions when working with pseudoviruses include the following:
- Wear gloves for all manipulations of potentially infectious materials
- Wear laboratory coats when working in laboratory areas
- Never pipette by mouth
- Eliminate the use of glassware whenever possible
- Perform all technical procedures in a way that minimises the creation of aerosols, droplets, splashes or spills
- Clean up spills immediately with bleach
- Follow SOPs for disposal of laboratory waste
- Clean incubators and replace distilled water in trays on a regular basis
- Clean work surfaces with 70% alcohol on a regular basis
- Label all flasks, tubes and reagents clearly
- Make sure all instrumentation is regularly serviced and calibrated

5. PROCEDURE IN THE EVENT OF AN ACCIDENT: POST-EXPOSURE PROPHYLAXIS GUIDELINES

5.1 Stay calm

5.2 Assess the accident and act accordingly:
HIV-containing fluid (spill or splash)
Wash off as much of the HIV-containing fluid as possible. Use water and soap followed by 10% bleach. In the event of an eye splash use an eye wash station or shower head available in the labs to rinse the eye several times
Penetrating injury
Wash off as much of the HIV-containing fluid as possible. Use water and soap and encourage the wound to bleed. Do this repeatedly. Cover with absorbent tissue

5.3 Assess the risk
Low risk: Exposure of intact skin or mucous membranes to blood from an asymptomatic patient.
High risk: Exposure through a deep injury or on broken skin or mucocutaneous exposure (eye, nose, mouth) to blood from patients with high viral loads (AIDS or acutely infected individuals) or with tissue culture fluid from p24 antigen positive cultures.

5.4 If you have had any exposure contact the following individuals (all hours):
- Dr Terry Marshall: 555-0484 (w); 614-74717 (h); 082 909 1792
- Dr Lucille Blumberg: 386-6337 (w); 082 807 6770
- Dr Adrian Puren: 386-6328 (w); 648-1200/1152 (h); 082 908 8048
- Prof BD Schoub: 386-6137(w); 440-4691 (h); 082 908 8049

If they consider that you have had a significant exposure they will recommend that you receive post-exposure prophylaxis (PEP). There is an emergency 3 day supply in Dr Caroline

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Tiemessen's section; in Mrs Sue Herrmann's office; in First Aid box in Prof Lynn Morris' receiving area, and in the First Aid box in Dr Clive Gray's section. Otherwise all three of the above doctors have supplies as has the Clinic. All exposures (whether PEP is recommended or not) must also be reported immediately to NHLS Safety Officer: 386-6142 (w); 082 809 5992

5.5 If you start this course it is important to continue for a further 25 days. This will be provided to all members working in the AIDS Unit. You will also be required to have baseline blood samples drawn and will receive additional clinical management and counselling.

6. PCR GUIDELINES TO PREVENTING CONTAMINATION (from UNAIDS guidelines)

6.1 Prevention of sample contamination

Sample contamination is a critical problem for genetic screening protocols due to the extreme sensitivity of polymerase chain reaction (PCR). Nested PCR can detect single molecules of the viral genome, and after amplification, a 100 μl volume can contain 10^{12}-10^{13} viral molecules. Thus, if 1 μl is aerosolized, 10^{10}-10^{11} molecules are released. To dilute 10^{10} molecules to less than 1 molecule per 100μl (volume of a typical PCR reaction) requires 10^6 litres of diluent, or a space of 10m x 10m x 10m!

Sample contamination can occur in a number of ways. **Carry-over** contamination occurs when amplified product enters the PCR tube and then is re-amplified; it usually results **from reagent contamination** and is especially problematic with nested PCR. Contamination can also result from external sources such as **contaminated surfaces** and **aerosols**. Tests for contaminated reagents should be included in each PCR experiment and strict methodological guidelines should be observed to prevent contamination by experimental design.

Note: the most powerful and effective means of preventing PCR carry over contamination is careful and well thought-out laboratory techniques.

Guidelines for prevention of sample contamination:

If possible, establish separate pre-PCR and post-PCR rooms and limit access to the pre-PCR room. The pre-PCR laboratory should not be used by anyone after they have handled PCR reactions on a given day.

Pre-PCR:

- The pre-PCR room is used for two procedures only - reagent preparation and PCR reaction set up. Nucleic acid extraction should not be performed in the pre-PCR room. Nucleic acids should be added to the reaction in the post-PCR room/ nested hood.
- You should prepare all buffers and reaction mixes and aliquot these products in this room.
- Pipettes and other instruments needed are kept in this room and used exclusively for pre-PCR activities.
- No thermocyclers, plasmids, PCR products, or nucleic acid templates are allowed to enter this room, no reagents are allowed back into this room after being removed.
- Keep cleaning supplies and brooms etc. in this room and do not allow cleaning personnel in, to prevent them from tracking PCR products in from other rooms.

Laboratory clothing:

- No lab coats or gloves used in a laboratory where PCR products or plasmid preparations are handled (e.g., the post-PCR room), should enter the pre-PCR lab.
- Dedicated lab coats are used in the pre-PCR lab and should not leave the room.

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Equipment and tube handling:

- All pipetting devices used should have barrier plugs (cotton plugs in disposable tips for micropipets, or in the barrel of large pipets), or positive displacement pipette tips should be used.

- Always allow tubes to thaw completely, and mix the contents well before pipetting, since the distribution of dissolved solutes becomes non-uniform during the freezing process.

- Spin all tubes briefly before opening the tubes

Reaction setup and reagent handling:

- Use negative controls (PCR reactions without template DNA) for each PCR, and an additional negative control interspersed with patient samples for each 10-20 samples
- Reagents used in the master mixes described below should be aliquoted in amounts suitable for one experiment and stored frozen, and then thawed only once (any left over should then be discarded).
- Reagents should not be shared with anyone
- Prepare master mixes
- Add reagents in the following order to reaction tubes:
    1. 10X buffer plus dNTPs
    2. H_2O
    3. Primers
    4. DNA polymerase
    5. Template (NOT to be added in Pre-PCR Room)

Note: Put away each reagent before working with the next reagent on the list to rule out contamination of the preceding reagent with subsequent solutions.

- If second round PCR is to be conducted the same day as the first round, master mixes for both rounds should be prepared at the same time and the latter moved to the post-PCR room for later handling. Store mastermixes that have been prepared for later use at 4°C.

Post-PCR room(s)

- Ideally, two post PCR rooms or areas should be used. One for the pipetting associated with the second round of nested PCR, and a second for post PCR steps such as gel electrophoresis.
- Wear lab coats, gloves, and overshoes
- Wash hands and remove coats before leaving the room
- Try not to enter the pre-PCR room after working in the post-PCR room (PCR products stick to clothes). It is highly preferable to not return to the pre-PCR room the same day after working with PCR products in the post-PCR room.
- Always centrifuge reaction tubes before opening to prevent liquid near the top of the tube or on the cap from being released
- Use a paper towel fragment to hold and open each tube then discard the towel. This prevents gloved fingertips from getting contaminated easily and can trap droplets that are released when the tube is opened. It also creates a barrier between your glove and the next tube you handle.
- Use dilute bleach (followed by a thorough water rinse), 70% ethanol, or soap and water to decontaminate surfaces before and after each experiment, dry with towelling.

6.2 Sentinel test for PCR contamination:

The sentinel test is a very simple and useful method that should be used periodically for detecting PCR fragments that may be contaminating a laboratory. Identification of contamination through the sentinel test is useful for identifying an existing problem, and are indicative of the need for changes in laboratory procedures to prevent aerosol contamination.

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- Fill several PCR tubes with 50ul of distilled water and place them around the lab at different locations (on the laboratory bench, sink, centrifuge, etc.)
- At the end of the day close the tubes.
- The next day test 10ul of the contents of the tubes with the same primers used for fragments handled the previous day.

6.3 Reaction "sterilization":

The most powerful means of preventing PCR carry over contamination is careful and well thought out laboratory techniques. However, contamination can and still does occur at times and may be extremely difficult to recognize. Multiple inactivation or sterilization protocols have been developed that use chemical, photochemical and enzymatic methods for eliminating contaminating molecules. None of the methods are, however, 100% efficient and should not be relied upon to replace appropriate techniques and careful reagent handling.

6.4 Safe handling of phenol/$CHCl_3$ used for DNA extraction:

- **Always** wear gloves, eye protection and a lab coat
- Work in a fume hood and exercise extreme care when handling phenol.
- Rinse glassware carefully before putting into dirty glassware trays.

Skin contamination should be extensively washed with soap and water (to avoid substantial penetration) and in severe cases the individual should be taken a physician for burn treatment.

7. WORKING SAFELY WITH RADIOACTIVITY

The NICD is authorized to use certain sources of radioactivity including:
3H - low energy beta radiator. Half-life of 12.6 years
32P - high energy beta radiator with high penetrative capacity. Half-life of 14.3 days.
35S - intermediate energy beta radiator. Half-life of 87.4 days
51Chromium - intermediate energy gamma radiator. Half-life of 27.8 days.
125I - intermediate energy gamma radiator with high penetrative capacity. Half-life of 59.6 days.

Note: General laboratory rules apply.

7.1 Work in a designated area, preferably a fume hood in a radioactive room. Employ strict procedures to prevent spills and contamination of surfaces.

7.2 Wear a laboratory coat, gloves, eye shield and a dosimeter. Work behind plexiglass when using high energy beta-emitters and use an lead apron when working with gamma-radiation.

7.3 All radioactive materials to be clearly labelled "RADIOACTIVE" including those being used outside of the radioactive room.

7.4 Perform "swipe tests" at regular intervals and use a Geiger counter to monitor surfaces and clothing after each experiment. The Geiger counter must be left on continually during labelling.

7.5 Dispose of radioactive materials promptly and appropriately. Check with the Safety Officer.

7.6 Report any incidents involving radioactivity to the Radioactive Safety Officer, Dr Clive Gray and complete an Accident/ Incident form which must be sent to Sr. Henley.
Refer SAF0010 for safety rules regarding work with radioactive substances.

8. WORKING WITH VACCINIA VIRUS

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Vaccination is no longer a requirement when working with vaccinia. However, it is still recommended that those working with large stocks be immunised.
Note: General laboratory rules apply.

8.1 When growing large quantities of vaccinia virus (i.e. growing up and titrating stocks) work in designated laboratory area.

8.2 When working with small quantities of vaccinia (i.e. infecting cultures or doing FACS analysis) employ strict procedures to avoid accidents and spillage.

8.3 Adhere to the strict laboratory rules including safe disposal.

9. AIDS UNIT PROCEDURE IN THE EVENT OF AN ACCIDENT WITH INFECTIOUS HIV

1. Stay calm.

2. Assess the accident and act accordingly:

   HIV-containing fluid (spill or splash)
   Wash off as much of the HIV-containing fluid as possible. Use water and soap.
   In the event of an eye splash use an eye wash station or shower head available in the labs to rinse the eye several times.

   Penetrating injury
   Wash off as much of the HIV-containing fluid as possible. Use water and soap and encourage the wound to bleed. Do this repeatedly. Cover with absorbent tissue.

3. Assess the risk
   Low risk: Exposure of intact skin (superficial) or mucus membranes to blood from an asymptomatic patient (is known).

   High risk: Exposure through a deep injury or on broken skin or mucocutaneous exposure (eye, nose, mouth) to blood from patients with high viral loads (AIDS or acutely infected individuals) or with tissue culture fluid from p24 antigen positive cultures.

4. If you have had any exposure contact the following individuals (all hours):

   Dr Terry Marshall: 555-0484 (w); 614-74717 (h); 082 909 1792
   Dr Adrian Puren: 386-6328 (w); 648-1200/1152 (h); 082-908-8048
   Prof BD Schoub: 386-6137 (w); 440-4691 (h); 082-908-8049
   Dr Lucille Blumberg: 386-6337 (w); 082-807-6770

   If they consider you have had a significant exposure they will recommend you receive post-exposure prophylaxis (PEP). Otherwise all three of the above doctors have supplies as has the clinic.

5. If you start this course it is important to continue for a further 25 days. This will be provided to all members working in the AIDS Unit. You will also be required to have baseline blood samples drawn and will receive additional clinical management and counselling.

Prof Lynn Morris
Head, AIDS Unit

10. DECLARATION

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I, the undersigned, declare that I have read and understood the content of the **AIDS Unit Safety Manual** and that I agree to abide by these rules.

Name: Mario Botha

Signature:..............................

Date: 2/07/2007

Signature of section Head:..............................

Please return to Sarah Cohen

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Wits Health Consortium (Pty) Ltd

8 Blackwood Avenue, Parktown, 2193, South Africa
Tel: +27-11-274 - 9200, Fax: +27-11-274 - 9360
Postnet Suite 189, Private Bag x2600, Houghton, 2041

ndemnity Form for AIDS Unit for Visiting Student/Scientist

Background and rationale

Students/scientists may visit the facilities of the AIDS Unit (under the auspices of the Wits Health Consortium (Pty) Ltd (WHC) or the National Health Laboratory Services (NHLS) (including the National Institute of Communicable Diseases (NICD). Access is provided to these students/ scientists to the facilities/personnel so as to enable them to achieve their objectives. In addition hey may receive training in order to gain practical experience for their chosen degree

Purpose of this agreement:

The purpose of this agreement is to outline the roles and responsibilities of the various parties and to indemnify the Wits Health Consortium (Pty) Ltd from any accident, injury or harm that may occur during the student/scientist's visit.

The student/scientist undertakes the following:

- To conduct himself/herself in accordance with the rules and regulations of the AIDS Unit as stipulated in the AIDS Unit Safety Manual
- To adhere to the safety and health regulations of the NHLS/NICD as stipulated in general lab safety rules.
- That he/she shall not intentionally or carelessly interfere with, damage or misuse the laboratory and the premises on which it is contained, or any of the instruments, equipment or other property kept in the laboratory, or on the laboratory premises.

Risks and liability

The student/scientist enters the premises of the AIDS Unit at his or her own risk.
The AIDS Unit shall not be liable for any loss, damage or harm suffered by the student/scientist, as a result of his or her entry onto the premises or his or her use of the laboratory.

The AIDS Unit may act on behalf of students/scientists in cases of emergency and such authority s herby granted.

I Mario Matthew Botha hereby certify that I understand fully, the contents of this indemnity form, and accept the conditions thereof.

Signed at NICD on this 2nd day of July 2007.

A wholly owned subsidiary of the University of the Witwatersrand

Directors: Prof. M.R. Price (Chairman); Mr. A.J. de Wet; Mr. D.C. Arnold; Mr. P.C. Desai; Mr. A.A. Farrell; Prof. J.M. Pettifor; Prof. S. Bhangwanjee; Prof. S. Chandiwana; Dr. J. Fisher; Prof. B. Bozzoli

ANNEXURE A.2

1 The adapted neutral red assay

1.1 Supplies and reagents

A list of the supplies and reagents to be used will follow:

- Automatic pipette;
- Single channel pipettes (2-20 μl, 20-200 μl, 10-1000 μl) and tips;
- Eight- or twelve-channel pipettes (50 μl, 200 μl) and tips;
- Hemacytometer with cover slide;
- Microscope;
- Tally counter;
- Serological pipettes (5 ml & 10 ml);
- Conical tubes (15 ml & 50 ml);
- Microfuge tubes (1.5 ml);
- 96-well flat-bottom microtiter plates with lids (cell culture grade);
- Reagent reservoirs;
- Syringe and filter (0.2 μm);
- Balance and spatula;
- RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum;
- Trypan blue viability dye;
- Finter's Neutral Red (NR);
- Poly-L-lysine
- Phosphate-buffered saline (PBS);
- Acid Alcohol: 50% ethanol in 1% acetic acid;
- Swing-arm bucket centrifuge (Rotana 46R)
- VERSAmax tunable microplate plate reader (filters 570nm & 690 nm) (molecular devices, USA)
- MT2 cells (suggested cells to be used after 2.1.3), maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (NIH AIDS Reference Programme Cat nr: 237);
- SW7 (T-cell line adapted) strain HIV-1 (suggested virus strain to be used after 2.1.3);
- CO_2 water jacketed incubator (Forma Scientific, USA)
- Light microscope (Nikon TMS-F)

1.2 Protocol

Absorbance is read as optical density units (OD), which will not be indicated as such in the results.

1.2.1 Cell stock solution

Prepare a cell stock solution of 5 x 10^5 cells/ ml in RPMI 1640 medium with 10% FCS:

1. Transfer maintained cell suspension to a 50ml conical tube
2. Pellet the cells in the swing-arm bucket centrifuge at 1200rpm for 10min.
3. Re-suspend cell pellet in 3ml RPMI 1640 medium
4. Transfer 10µl of the suspension in nr. 3 and add 90µl trypan blue
5. Pipette 10µl to each side of the hemacytometer and count living cells under a light microscope
6. Thus, (cell count)(10)(10 000) = x
7. A total cell stock (1 x 10^5 cells/ml) volume of 8ml must be prepared:
   (8ml)(1 x 10^5) / x = the volume (µl) of cells (in nr. 3) to be re-suspended in 8ml of RPMI.

1.2.2 Preparation of the virus

Prepare a virus stock solution of 1 x 10^5 $TCID_{50}$ (50% Tissue Culture Infective Dose) HIV strain per ml. The dilution should kill 80% of cells after 7 days of incubation. The virus should be diluted to 5 times higher than the final amount required.

1.2.3 Preparation of the poly-L-lysine plates

1. Prepare a 50 µg/ml poly-L-lysine solution (MW = 90 000, Sigma) in PBS (pH 7.3).
2. Add 100 µl to the wells of a flat-bottom 96-well plate.
3. Incubate at room temperature for 1 hour.
4. Remove poly-L-lysine solution from plate.
5. Wash plate twice with PBS.
6. Place plate upside-down on paper towel to drain for 5 minutes.
7. Cover plate with lid.
8. Store at 4°C for up to 6 months.

1.2.4 Cell staining with Finter's neutral red

1. Prepare staining solutions
   a. Finter's neutral red: dilute 1:10 in media

b. PBS

c. Acid alcohol: 50% ethanol in 1% acetic acid

2. Transfer 100 μl cell suspension to poly-L-lysine plate
3. Add 100 μl Finter's neutral red
4. Incubate at 37°C for 75 minutes
5. Remove well contents
6. Wash wells with 150 μl PBS (2x)
7. Add 100 μl acid alcohol
8. Incubate at room temperature for 30 minutes
9. Read absorbance at 540 nm

1.2.5 Assay procedure

1. Dissolve the drug(s) in either RPMI 1640 medium without the fetal calf serum supplement.
2. Add 250 μl complete RMPI 1640 medium to the wells on the perimeter of the plate.
3. Add 100 μl of cells to all the inner wells (B2-G12).
4. Add 100 μl medium to wells B2-G2. These are the control wells.
5. Add 100 μl drug(s) from column 3 through to column 11. These may be different drugs or different concentrations of the same drug. Remember that the concentration(s) of the drug(s) added should be 2.5 times higher than the wanted concentration.
6. Incubate the plate at 37°C 5% CO_2 for 1 hour.
7. Add 50 μl SW7 (T-cell line adapted HIV-1).
8. Incubate the plate at 37°C 5% CO_2 (DAY 0).
9. On day 4, remove 125 μl of supernatant from the wells and replace with complete medium containing the same concentration of drug.
10. Incubate the plate at 37°C 5% CO_2.
11. On day 7, remove 150 μl of supernatant from the wells.
12. Stain cells according to 1.2.4 as described above.

ANNEXURE B

1 Titration of HIV-1 (SW7-TCL, IIIB, MN) in MT2 and PM1 cells

1.1 Introduction

Clinical isolates of the HIV-1 virus namely, SW7-TCL, IIIB and MN viruses with MT2 and PM1 cells were used in a titration experiment for the determination of virus and cell types to be used in the cell viability assays to follow. No drug was used in this experiment and results were determined by infection of virus in healthy cells and staining thereafter.

1.2 Method

Using the adapted neutral red assays as described in 2.5:

- PM1 and MT2 cell line were used in the titration, and infected with viruses SW7-TCL, IIIB and MN.
- Incubate according to protocol.
- Stain cells according to protocol.

The cell plate layout as well as the poly-L-lysine plate layout is presented in figures B.1 and B.2

	1	2	3	4	5	6	7	8	9	10	11	12
A	media	control	virus	4 x 10^5	df	virus	4 x 10^5	df	virus	4 x 10^5	df	media
B		cells	virus	78125	df	virus	78125	df	virus	78125	df	
C			virus	15625	df	virus	15625	df	virus	15625	df	
D			virus	3125	df	virus	3125	df	virus	3125	df	
E			virus	625	df	virus	625	df	virus	625	df	
F			virus	125	df	virus	125	df	virus	125	df	
G			virus	25	df	virus	25	df	virus	25	df	
H			virus	5	df	virus	5	df	virus	5	df	
				SW7-TCL			IIIB			MN		

Figure B.1 Illustration of the cell plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	media	control		virus	4 x 10^5	df	virus	4 x 10^5	df	virus	4 x 10^5	df
B		cells		virus	78125	df	virus	78125	df	virus	78125	df
C				virus	15625	df	virus	15625	df	virus	15625	df
D				virus	3125	df	virus	3125	df	virus	3125	df
E				virus	625	df	virus	625	df	virus	625	df
F				virus	125	df	virus	125	df	virus	125	df
G				virus	25	df	virus	25	df	virus	25	df
H				virus	5	df	virus	5	df	virus	5	df
				SW7-TCL			IIIB			MN		

Figure B.2 Illustration of the poly-L-lysine plate layout

1.3 Results

Results obtained from the MT2 and PM1 cells are presented in tables B.1 and B.2 respectively.

Table B.1 Absorbance of MT2 cells (df = dilution factor)

MT2 cells:	**A540nm**				
	1	**2**	**3**	**Average**	**Standard deviation**
Medium	0.158	0.113	0.161	**0.144**	**0.027**
Control cells	1.445	1.441	2.019	**1.635**	**0.332**
SW7-TCL - 5x df	0.505	0.474	0.440	**0.473**	**0.033**
SW7-TCL - 25x df	0.727	0.505	1.007	**0.746**	**0.252**
SW7-TCL - 125x df	1.155	1.221	1.113	**1.163**	**0.054**
SW7-TCL - 625x df	1.074	0.936	0.899	**0.970**	**0.092**
SW7-TCL - 3125x df	1.020	0.827	0.826	**0.891**	**0.112**
SW7-TCL - 15625x df	1.026	0.871	1.407	**1.101**	**0.276**
SW7-TCL - 78125x df	1.763	1.071	1.751	**1.528**	**0.396**
SW7-TCL - 390625x df	1.486	1.199	1.736	**1.474**	**0.269**
IIIB - 5x df	0.433	0.474	0.337	**0.415**	**0.070**
IIIB - 25x df	0.873	0.626	0.586	**0.695**	**0.155**
IIIB - 125x df	0.994	0.910	0.766	**0.890**	**0.115**
IIIB - 625x df	0.936	0.747	0.910	**0.864**	**0.102**
IIIB - 3125x df	1.120	0.733	0.961	**0.938**	**0.195**
IIIB - 15625x df	1.148	1.114	0.635	**0.966**	**0.287**
IIIB - 78125x df	1.755	1.524	1.449	**1.576**	**0.159**
IIIB - 390625x df	1.657	1.537	1.187	**1.460**	**0.244**
MN - 5x df	0.318	0.539	0.694	**0.517**	**0.189**
MN - 25x df	0.395	0.311	0.447	**0.384**	**0.069**
MN - 125x df	0.538	0.373	0.423	**0.445**	**0.085**
MN - 625x df	0.668	0.339	0.471	**0.493**	**0.166**
MN - 3125x df	0.757	0.522	0.495	**0.591**	**0.144**
MN - 15625x df	1.251	0.498	0.699	**0.816**	**0.390**
MN - 78125x df	1.831	1.127	1.265	**1.408**	**0.373**
MN - 390625x df	2.068	0.949	0.810	**1.276**	**0.690**

Table B.2 Absorbance of PM1 cells (df = dilution factor)

PM1 cells:	A540nm				
	1	2	3	Average	Standard deviation
Medium	0.252	0.204	0.193	**0.216**	**0.031**
Control cells	1.889	1.764	1.824	**1.826**	**0.063**
SW7-TCL - 5x df	0.765	1.283	1.200	**1.083**	**0.278**
SW7-TCL - 25x df	1.286	1.090	1.219	**1.198**	**0.100**
SW7-TCL - 125x df	1.142	1.456	1.334	**1.311**	**0.158**
SW7-TCL - 625x df	0.971	1.244	1.173	**1.129**	**0.142**
SW7-TCL - 3125x df	1.282	1.402	1.344	**1.343**	**0.060**
SW7-TCL - 15625x df	1.246	1.396	1.288	**1.310**	**0.077**
SW7-TCL - 78125x df	0.823	1.557	1.040	**1.140**	**0.377**
SW7-TCL - 390625x df	1.493	1.673	1.471	**1.546**	**0.111**
IIIB - 5x df	1.378	1.945	1.517	**1.613**	**0.296**
IIIB - 25x df	1.816	1.923	1.644	**1.794**	**0.141**
IIIB - 125x df	1.621	1.815	1.621	**1.686**	**0.112**
IIIB - 625x df	1.666	1.583	1.482	**1.577**	**0.092**
IIIB - 3125x df	1.690	1.735	1.563	**1.663**	**0.089**
IIIB - 15625x df	1.777	1.677	1.556	**1.670**	**0.111**
IIIB - 78125x df	1.794	1.718	1.692	**1.735**	**0.053**
IIIB - 390625x df	1.601	1.456	1.557	**1.538**	**0.074**
MN - 5x df	1.648	1.641	1.602	**1.630**	**0.025**
MN - 25x df	1.684	1.597	1.686	**1.656**	**0.051**
MN - 125x df	1.629	1.739	1.601	**1.656**	**0.073**
MN - 625x df	1.609	1.582	1.391	**1.527**	**0.119**
MN - 3125x df	1.603	1.645	1.517	**1.588**	**0.065**
MN - 15625x df	1.581	1.613	1.330	**1.508**	**0.155**
MN - 78125x df	1.493	1.620	1.373	**1.495**	**0.124**
MN - 390625x df	1.393	1.388	1.234	**1.338**	**0.090**

Dilutions of the MT2 and PM1 cells were plotted against the corresponding absorbance and plotted with the minimum, maximum and mean values. The graphs are presented below (Figure B.3 and figure B.4).

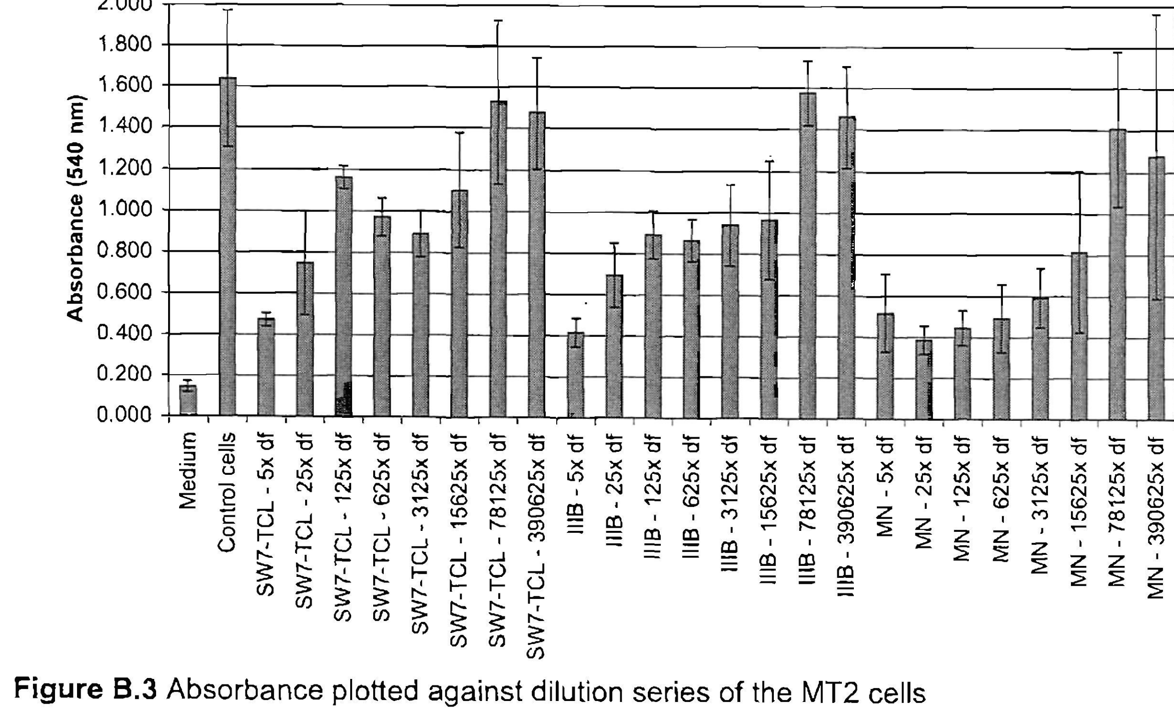

Figure B.3 Absorbance plotted against dilution series of the MT2 cells

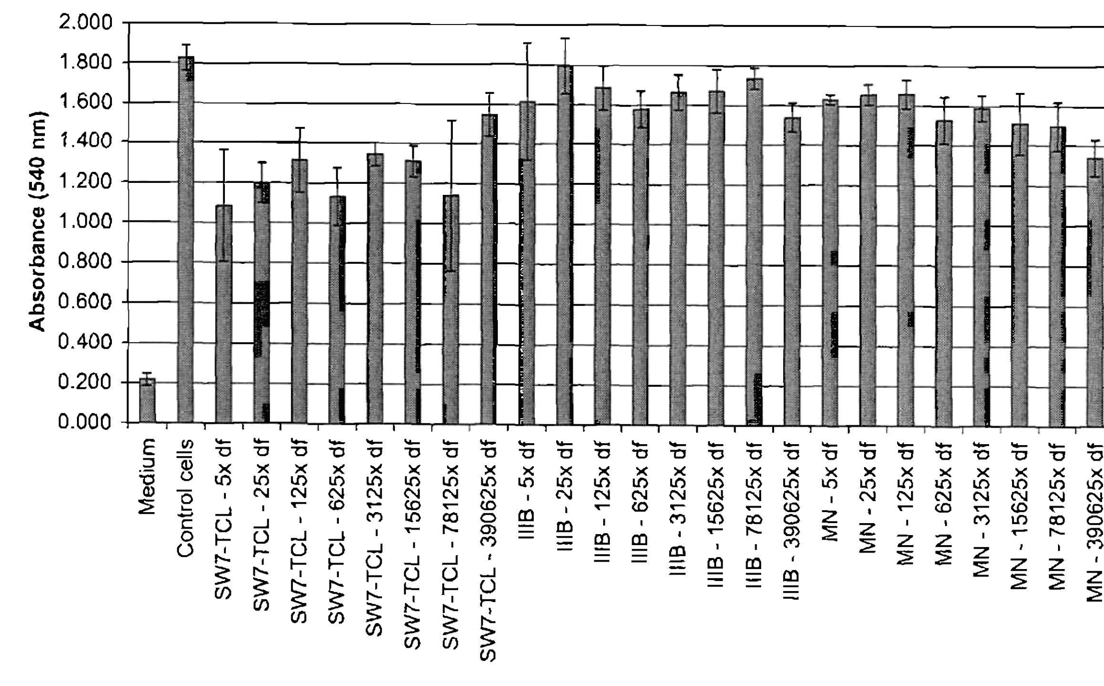

Figure B.4 Absorbance plotted against dilution series of the PM1 cells

Incubation of the MT2 cells with the three virus strains indicated a significant drop in cell viability by infection.

Viability of the PM1 cells was high, with little or no cell death. Infection with the SW7-TCL virus strain showed the highest depletion with a proportionate increase in the virus dilution factor. The IIB strain showed nearly identical absorbance from a virus dilution of 125 times up to a virus dilution of 15625 times. This indicated insufficient definition in virus infection. Absorbance were unusually high with all three viruses.

The best results were obtained by infection with the SW7-TCL and MN strains.

1.4 Conclusion

- The MT2-cells titrated with the three different viruses showed better variation in absorbance values than PM1-cells.
- With regards to the titration with PM1-cells and the three clones of HIV, the SW7-TCL virus strain had lower absorbance, which indicated good infection.
- The SW7-TCL and MN virus strains produced lower absorbance, indicative of better infection within the MT2-cell cultures.

This experiment showed that the MT2 cell line infected with SW7-TCL or MN HIV strains may be used in future experiments and titrations for this study.

Annexure C

1 Toxicity and anti-HIV properties of d4T in MT2 cells infected with SW7-TCL and MN virus strains

1.1 Introduction

Using the adapted neutral red assay (as described in 2.5), SW7-TCL and MN viruses were incubated with MT2 cells for a seven day incubation period. See figure C.1 for an illustration of the plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B			0.047	0.094	0.188	0.375	0.750	1.500	3.000	6.000	12.000	24.000
C			µM	µM	µM	µM	µM	µM	µM	µM	µM	µM
D			d4T	d4T	d4T	d4T	d4T	d4T	d4T	d4T	d4T	d4T
E												
F			0.047	0.094	0.188	0.375	0.750	1.500	3.000	6.000	12.000	24.000
G			µM	µM	µM	µM	µM	µM	µM	µM	µM	µM
H			d4T	d4T	d4T	d4T	d4T	d4T	d4T	d4T	d4T	d4T

Figure C.1 Illustration of the plate layout

1.2 Method

The cultures were incubated with d4T to analyze its toxicity and protective properties. The highest drug concentration needed was 24 µM (an estimated concentration which is higher than the IC_{100}). The total incubation volume was 250 µl/well. 100 µl/well drug solution was used, which implies that the drug will be diluted 2.5 tmies. Thus, a stock solution of 60 µM was needed. 0.0135g d4T was weighed and dissolved in 1 ml of RPMI growth medium. It was further diluted 1000 times to obtain a 60 µM solution, and filter sterilized with a 0.2 µm filter.

The dilution series of d4T:

- Pipette 200 µl 60 µM d4T into wells of column 12;
- Pipette 100 µl medium into remaining wells;
- Transfer 100 µl d4T from first wells (column 12) to the next set of wells (column 11), and mix;

- Transfer 100 µl d4T from wells in column 11 to the next set of wells (column 10), and mix;
- Continue diluting in series up to column 3, and discard.

The assay was adapted in the following manner:

- On day three of incubation, 125µl supernatant was removed and replaced with 125µl of growth medium or drug (at final concentration).
- The plate was then incubated for two days and supernatant was replaced with growth medium or drug as described above.
- A further two days of incubation took place.
- Day 7: Contents of each well were mixed and 190µl were transferred to a round-bottom plate.
- 10µl of undiluted neutral red staining was added.
- The plate was incubated at 37°C, 5% CO_2 for 1 hour and 15 minutes.
- After incubation, the plate was centrifuged at 1000 rpm for 10 minutes.
- The supernatant was removed.
- Cells were washed twice with 150µl PBS.
- 100µl acid-alcohol was added and the plate was incubated a last time at room temperature for 30 min.
- The absorbance of each well of the plate was read at a wavelength of 540 nm.

1.3 Results

In both instances low absorbance were obtained (figures C.2 and C.3 and tables C.1 and C.2). The explanation could be cell loss due to the washing step incorporated in this assay. Viability and protection were plotted as percentage against concentration (µM). Only the mean values are shown in the tables.

Table C.1 Absorbance obtained for the MT2 cells incubated with SW7-TCL virus strain.

d4T (µM)	Uninfected		Infected	
	A540 nm			
	Absorbance	% Viability	Absorbance	% Protection
Control	1.660	-	0.962	-
0.046875	1.930	116.27	1.225	37.68
0.09375	1.650	99.40	1.217	36.53
0.1875	1.487	89.58	1.207	35.10
0.375	1.745	105.12	1.393	61.75
0.75	1.766	106.39	1.423	66.05
1.5	1.725	103.92	1.412	64.47
3	1.642	98.92	1.257	42.26
6	1.648	99.28	1.199	33.95
12	1.635	98.49	1.251	41.40
24	1.562	94.10	1.027	9.31

Table C.2 Absorbance obtained for the PM1 cells incubated with SW7-TCL virus strain.

d4T (µM)	Uninfected		Infected	
	A540 nm			
	Absorbance	% Viability	Absorbance	% Protection
Control	1.254	-	1.061	-
0.046875	1.238	100.00	1.24	92.75
0.09375	1.249	100.89	1.158	50.26
0.1875	1.126	90.95	1.098	19.17
0.375	1.093	88.29	0.985	-39.38
0.75	1.160	93.70	1.079	9.33
1.5	1.155	93.30	1.054	-3.63
3	1.405	113.49	1.134	37.82
6	1.140	92.08	1.060	-0.52
12	1.219	98.47	1.152	47.15
24	1.003	81.02	1.059	-1.04

1.3.1 SW7-TCL strain

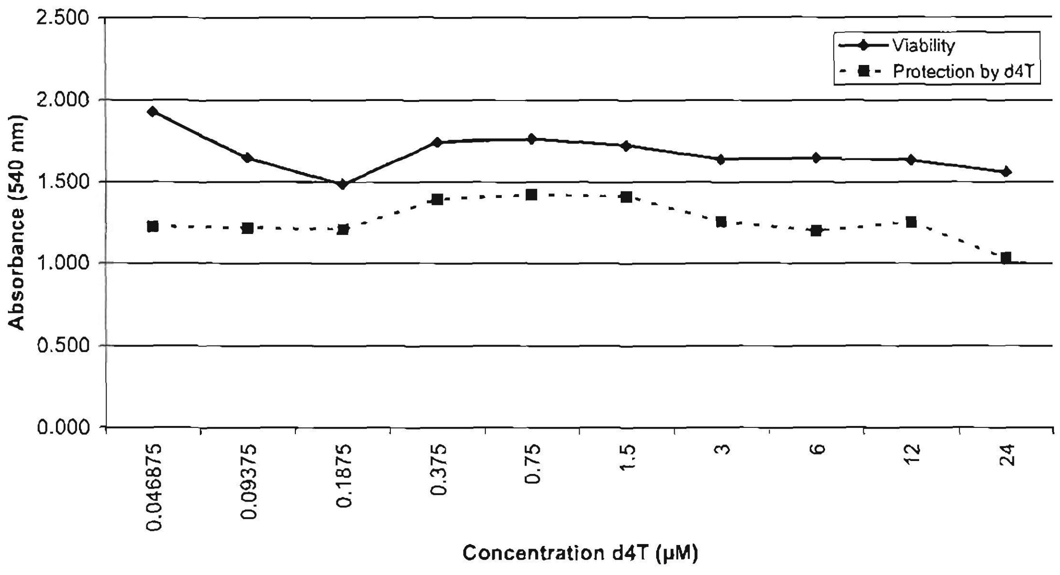

Figure C.2 Absorbance of the SW7-TCL strain plotted against d4T concentrations

Results of the MT2 cells incubated with SW7-TCL indicated viability and protection as expected, with some exceptions. A viability reading of 116.27% were obtained at 0.046875 µM which then decreased to 89.58% at 0.1875 µM, followed by an increase from that point forward. Protection was similar to what was expected, with a maximum of 66.05%.

The absorbance values are plotted against the concentration of d4T in figure C.2.

1.3.2 MN strain

All the readings on this plate were extremely low, which was worrying. The results of the cells that were infected with the MN virus were not expected, and could not even be used for further analysis. This graph (figure C.3) indicated a rollercoaster-like effect, with half of the absorbance values at zero and below

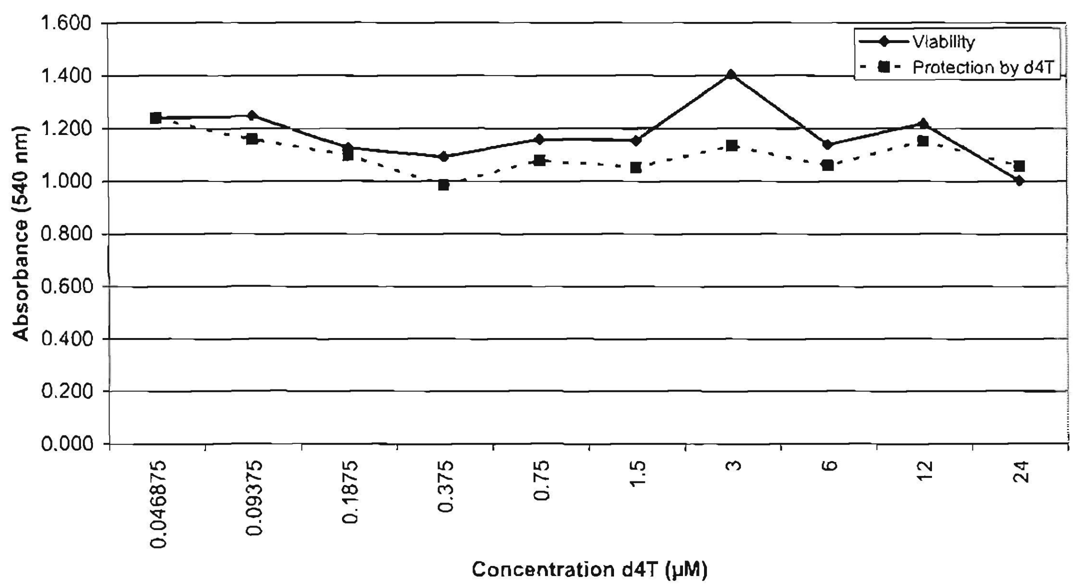

Figure C.2 Absorbance of the MN strain plotted against d4T concentrations

1.4 Conclusion

Neutral red (NR) has a poor solubility in growth- or RPMI-1640 medium (Zhang *et al.*, 2003) and its use requires a series of complex experimental processes: plate coating with poly-L-lysine, adherence of cells to the poly-L-lysine coated plates, washout procedures and a NR extracting step from the cells (Montefiori *et al.*, 1988).

All the steps mentioned above, which were used in this assay, could have a possible negative effect on the absorbance results. In our experience, the washout procedures of the cells were not responsible for the loss of cells (see figure C.2).

Neutral red is an inexpensive assay for the measurement of cell death, but it may in some cases be a less sensitive assay (Weyermann *et al.*, 2005), than the MTT assay. According to the literature, MTT on the other hand has good solubility and has simple experimental processes (Mosmann, 1983). Zhang *et al.* (2003) found the MTT viability/protection assay to be superior to the NR assay.

These statements in conjunction with the results described were convincing enough to change to the use of the MTT assay and experimental procedures.

ANNEXURE D

1 Development and application of the adapted MTT assay

1.1 Introduction

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is simple to use and is believed to be based on the metabolic activity of the mitochondria in viable cells (Weyermann *et al*, 2005.). The mechanism of action is the following: the respiratory chain and electron transport system (Liu *et al.*, 1997) reduce MTT and other tetrazolium salts to form non-water-soluble violet formazan within the cell (Altman, 1976; Mosmann, 1983). The formazan product is impermeable to cell membranes and accumulates in living, healthy cells (Fotakis & Timbrell, 2006).

These crystals can be quantified spectrophotometrically, which serves as an indication of the number of mitochondria and therefore the number of cells in the sample (Denizot & Lang, 1986).

The assay used in this study is based on the protocol by Mosmann (1983), adapted and optimized for use in MT2 cells, infected with the SW7-TCL HIV-1 virus strain.

1.2 The toxicity and anti-HIV-1 activity of drugs in MT2 cells infected with an HIV-1 strain, SW7-TCL, using the MTT viability dye.

This assay is relevant in the measurement of cell activation, cell growth and cell survival of mammalian cells, the bacterial activity of macrophages, the determination of chemo-sensitivity of tumor cell lines, viability of fungi (Mosmann, 1983; Bounous *et al.*, 1992) and is also used to determine cytotoxicity of cells after exposure to toxic substances (Fotakis & Timbrell, 2006).

1.2.1 Introduction

This assay measures the protection afforded by and the level of toxicity of ARVs in 96-well plates as a function of cell viability, after infection with the virus. It differs from the neutral red assay only in that the vital dye used is MTT and not neutral red. MT2-cells were infected with HIV-1 strain, SW7-TCL. Similar to the neutral red assay, a cell stock of 5 x 10^5 cells/ml as well as a virus stock of 1 x 10^5 $TCID_{50}$ HIV per ml were used.

<u>Purpose:</u>

As discussed in chapter 2 section 5

Principle:

MTT is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes in viable cells. This water insoluble formazan can be solubilized using isopropanol or other solvents and the dissolved material is measured spectrophotometrically yielding absorbance as a function of amount of converted dye.

Routinely, MTT stock solution (5 mg/ml) is added to each culture being assayed to equal one tenth the original culture volume and incubated for 3 to 4 hr. At the end of the incubation period the medium can be removed if working with adherent cells and the converted dye may be solubilized. Absorbance of converted dye is measured at a wavelength of 570 nm with background subtraction at 630-690 nm.

1.2.2 Protocol

1.2.2.1 Cell stock solution

Prepared as discussed in 2.5.2.3.1

1.2.2.2 Preparation of virus

The virus stock of 1 x 10^5 $TCID_{50}$ was prepared according to the procedure in 2.5.2.3.2

1.2.2.3 Cell staining

1.2.2.3.1 Preparation of the MTT solution

(Prepare in advance)

Prepare a solution of 5 mg/ml MTT(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) powder (Sigma, ref. nr.: M5655) in PBS. Make sure that the MTT has dissolved properly. Under sterile conditions, filter the MTT solution through a 0.2 μm filter. Divide into 800 μl aliquots and store at -20°C. Protect from light.

MTT is a carcinogen. Avoid contact with the skin and eyes. It may be harmful if inhaled or swallowed. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Dispose in a biological hazard container after transferring the MTT solution to a sealable container (e.g. Eppendorf tube). Seal 96-well plates containing MTT with adhesive film before disposing it in a biohazard container.

1.2.2.3.2 Preparation of the formazan solution

(Prepare in advance)

Prepare a 40% SDS (w/v) solution in distilled water. Add an equal volume of DMF (see Chapter 4) and mix properly. This solution contains 20% SDS (w/v) and 50% DMF (v/v). Store at room temperature.

NOTE: It is not uncommon for the SDS to precipitate during storage. Heat the solution to 37°C to re-dissolve the precipitates.

Sodium dodecyl sulfate (SDS) is a respiratory, skin and eye irritant, harmful if swallowed, inhaled or absorbed through the skin. It is a nuisance dust, but may also cause sensitization if inhaled.

Dimethyl formamide (DMF) is harmful by inhalation, ingestion or skin contact; it may act as a carcinogenic substance. Ingestion or absorption through skin may be fatal. Exposure may result in fetal death. Long-term exposure may result in kidney or liver damage.

1.2.3 Assay procedure

The assay procedure is the same as in 2.1.2.4., with the following differences, starting from point 12 in the described assay, after the incubation period:

12. Add 10 µl MTT solution to each well.
13. Incubate the plate at 37°C 5% CO_2 for 5 hours.
14. Dissolve the formazan crystals by adding 100 µl of formazan solvent (isopropanol: Mosman, 1983 or SDS/DMF : Zhang *et al.*, 2003) to the wells and mix the well contents with a pipette. Make sure that all the formazan crystals are dissolved before reading the plate.
15. Read the plate at 570 nm and 690 nm.
16. Seal the plate with adhesive film and store at room temperature (optional).
17. Remove the film and read the plate again (optional).

1.2.4 Data processing

1. Subtract the A690 nm values from the A570 nm values (i.e. OD = A570nm-A690nm).

2. Obtain the averages for the cell (cells + medium) and virus (cells + medium + virus) controls.

3. Calculate the cell viability, which indicates the drug(s)'s toxicity:

$$\% \text{ Cell viability} = \frac{OD_{cells+drug}}{OD_{cells+medium}} \times 100$$

4. Calculate the protection of the drug(s) against HIV-1:

$$\% \text{ Protection} = \frac{OD_{cells+drug+virus} - OD_{cells+virus}}{OD_{cells} - OD_{cells+virus}} \times 100$$

5. Plot both curves (% toxicity and % protection) on the same graph.

1.2.5 Test acceptance criteria

The following could be used as acceptance criteria:

1. Medium blank values should not exceed 0.250 (A570nm-A690nm).

2. Cell control values should be above 1.000 (A570nm-A690nm).

3. Virus control values should be approximately ¼ of the cell control values.

1.2.6 Interpretation of results

1. Toxicity: A viability of 100% indicates no toxicity. A decrease in viability indicates an increase in toxicity.

2. Protection: A value of 0% indicates no protection against HIV-1. An increase in values indicates an increased protection against HIV-1.

3. Protection vs. toxicity: be sure to correlate the protection results to the toxicity results, e.g. a lower amount of protection in the infected cells might be linked to an increased toxic effect of the compound on the cells.

ANNEXURE E

1 The effect of different cell concentrations on the MTT assay using two different solvents for formazan crystals

1.1 Introduction

Different concentrations of cells were tested to determine the cell concentration to be used in the adapted MTT assay (Mosmann, 1983). Two solvents were tested: acidic isopropanol (Mosmann, 1983) and SDS/DMF (Zhang *et al.*, 2003).

1.2 Method

The assay and procedure used are according to the assay discussed in annexure D, without virus infection. Cells (100 µl) were seeded (from column 2 to 11) in a concentration series: 1 x 10^6, 5 x 10^5, 2.5 x 10^5, 1.25 x 10^5, 6.25 x 10^4, 3.125 x 10^4, 1.5625 x 10^4, 7.8125 x 10^3, 3.963 x 10^3 and 1.3 x 10^3 cells/well. The plate was incubated for four hours at 37°C with 10 µl MTT (5mg/ml). 100 µl solvent was added and the results were obtained, reading the plate at 570 nm and 690 nm.

1.3 Results

The absorbance of the wells with SDS/DMF as solvent was higher than the cells using isopropanol as solvent for the formazan crystals. The results were as expected: the higher the concentration of the cells, the higher the absorbance. Leaving the plate overnight and reading it the following day, gave slightly higher absorbances, with a plato between the cell concentrations of 1 x 10^6 and 5 x 10^5 cells/well.

Tables E.1 and E.2 give the absorbance obtained from the plate readings on the same day and the following day respectively. The two sets of absorbances' that were obtained, were from the two different formazan solvents. Rows B, C and D are the values obtained using the SDS/DMF as solvent and rows E, F and G the values obtained using isopropanol as solvent.

Figure E.1 illustrates the absorbance of the two solvents plotted against cells/well, with figure E.2 illustrating the effect of leaving the crystals to stand overnight in the optimal solvent.

Table E.1 Absorbance obtained with two different MTT solvents (the same day)

570nm	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	0.114	2.789	2.824	2.367	1.645	1.126	0.678	0.413	0.271	0.195	0.157	
C	0.109	3.603	4	2.586	1.729	1.156	0.711	0.437	0.277	0.199	0.155	
D	0.109	2.686	3.371	2.573	1.638	1.158	0.731	0.45	0.286	0.204	0.158	
E	0.141	2.605	2.109	1.691	1.229	0.929	0.664	0.452	0.306	0.238	0.192	
F	0.138	1.779	2.42	1.803	1.182	0.883	0.656	0.461	0.313	0.243	0.193	
G	0.154	2.665	2.157	1.687	1.244	0.936	0.663	0.478	0.319	0.269	0.214	
H												

690nm	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	0.037	0.07	0.103	0.05	0.042	0.042	0.034	0.031	0.032	0.037	0.032	
C	0.039	0.083	0.071	0.056	0.049	0.045	0.042	0.041	0.04	0.039	0.039	
D	0.036	0.067	0.066	0.051	0.041	0.04	0.038	0.036	0.0365	0.035	0.036	
E	0.05	0.721	0.418	0.251	0.123	0.083	0.072	0.06	0.056	0.06	0.048	
F	0.042	0.864	0.392	0.201	0.099	0.074	0.065	0.055	0.052	0.058	0.045	
G	0.062	0.718	0.434	0.233	0.12	0.093	0.083	0.08	0.072	0.082	0.069	
H												

570nm-690nm	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	0.077	2.719	2.721	2.317	1.603	1.084	0.644	0.382	0.239	0.158	0.125	
C	0.07	3.52	3.929	2.53	1.68	1.111	0.669	0.396	0.237	0.16	0.116	
D	0.073	2.619	3.305	2.522	1.597	1.118	0.693	0.414	0.2495	0.169	0.122	
E	0.091	1.884	1.691	1.44	1.106	0.846	0.592	0.392	0.25	0.178	0.144	
F	0.096	0.915	2.028	1.602	1.083	0.809	0.591	0.406	0.261	0.185	0.148	
G	0.092	1.947	1.723	1.454	1.124	0.843	0.58	0.398	0.247	0.187	0.145	
H												

SDS/DMF	**Media**	**1000000**	**500000**	**250000**	**125000**	**62500**	**31250**	**15625**	**7812.5**	**3906.25**	**1953.125**
Average	0.073	2.953	3.318	2.456	1.627	1.104	0.669	0.397	0.242	0.162	0.121
Standev	0.004	0.494	0.604	0.121	0.046	0.018	0.025	0.016	0.007	0.006	0.005

Isopropanol	**Media**	**1000000**	**500000**	**250000**	**125000**	**62500**	**31250**	**15625**	**7812.5**	**3906.25**	**1953.125**
Average	0.093	1.582	1.814	1.499	1.104	0.833	0.588	0.399	0.253	0.183	0.146
Standev	0.003	0.578	0.186	0.090	0.021	0.021	0.007	0.007	0.007	0.005	0.002

Table E.2 Absorbance obtained with two different MTT solvents (the following day)

570nm	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	0.215	3.361	3.075	2.371	1.624	1.137	0.742	0.492	0.348	0.201	0.168	
C	0.229	4	4	2.645	1.865	1.25	0.816	0.542	0.381	0.247	0.181	
D	0.23	3.603	3.483	2.715	1.798	1.302	0.835	0.561	0.394	0.285	0.205	
E												
F												
G												
H												

690nm	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	0.042	0.225	0.116	0.053	0.044	0.043	0.039	0.037	0.037	0.035	0.031	
C	0.042	0.213	0.073	0.058	0.052	0.048	0.05	0.051	0.047	0.04	0.039	
D	0.04	0.219	0.071	0.057	0.05	0.048	0.047	0.047	0.045	0.042	0.037	
E												
F												
G												
H												

570nm-690nm	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	0.173	3.136	2.959	2.318	1.58	1.094	0.703	0.455	0.311	0.166	0.137	
C	0.187	3.787	3.927	2.587	1.813	1.202	0.766	0.491	0.334	0.207	0.142	
D	0.19	3.384	3.412	2.658	1.748	1.254	0.788	0.514	0.349	0.243	0.168	
E												
F												
G												
H												

SDS/DMF	Media	1000000	500000	250000	125000	62500	31250	15625	7812.5	3906.25	1953.125
Average	0.183	3.436	3.433	2.521	1.714	1.183	0.752	0.487	0.331	0.205	0.149
Standev	0.009	0.329	0.484	0.179	0.120	0.082	0.044	0.030	0.019	0.039	0.017

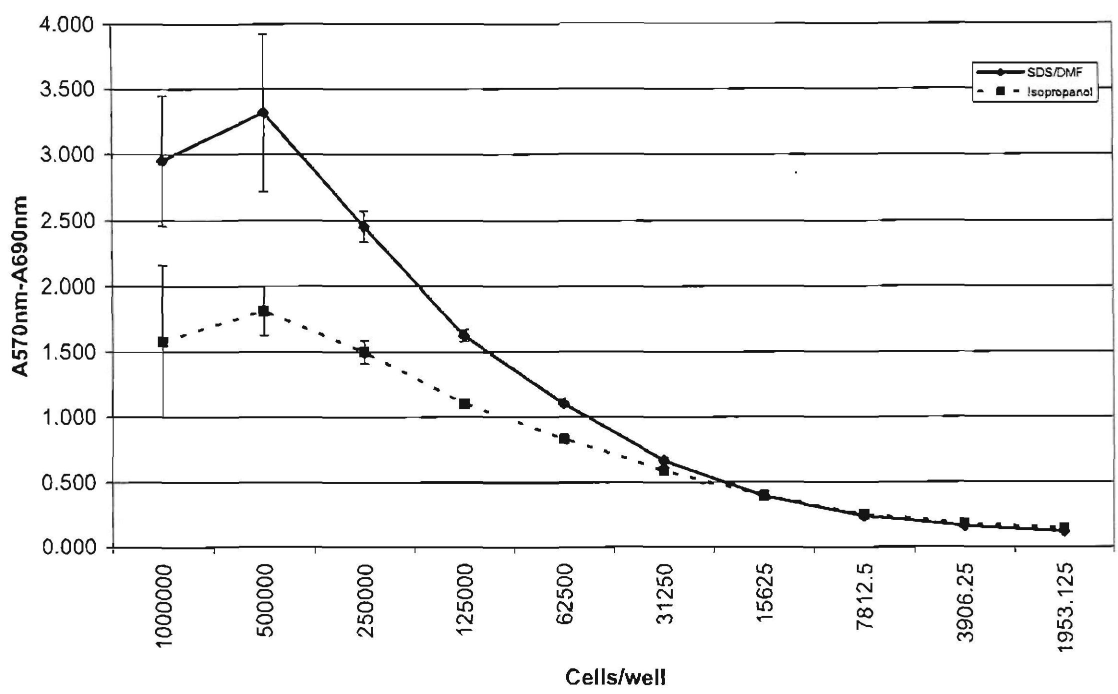

Figure E.1 The absorbance against different cell concentrations, using two different MTT solvents

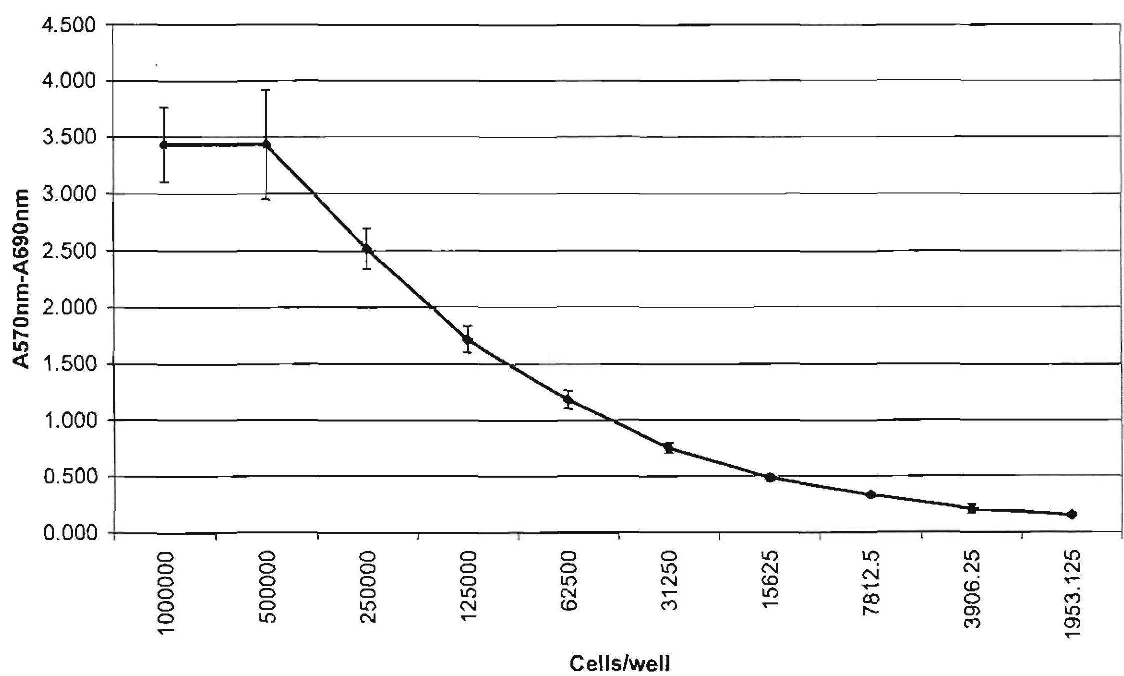

Figure E.2 The effect of leaving the solvent (SDS/DMF) overnight

1.4 Conclusion

SDS/DMF was chosen as the preferred formazan solvent as it gave higher absorption than the isopropanol solvent. Leaving the plate overnight produced slightly higher, but not significant, increases in the intensity of the absorption than immediately after the solvent has been added.

ANNEXURE F

1 Investigation of different cell and virus concentrations, using the MTT assay.

1.1 Introduction

Part of optimizing the MTT assay for this study, is the determination of the optimal cell and virus concentrations to be used in subsequent experiments.

1.2 Method

Cells (in different concentrations, to be discussed hereafter) were incubated with virus in a concentration range of dilutions. Starting from columns 2 to 12, virus was added in the following manner: no virus, 256-, 128-, 64-, 32-, 16-, 8-, 4-, 2 times diluted and undiluted. Given that only 50 µl of virus was added to each well, the final virus dilution was:

Column	2:	no virus
Column	3:	1280 times diluted
Column	4:	640 times diluted
Column	5:	320 times diluted
Column	6:	160 times diluted
Column	7:	80 times diluted
Column	8:	40 times diluted
Column	9:	20 times diluted
Column	10:	10 times diluted
Column	11:	undiluted

Cells were seeded from rows B – G as follows:

Rows B & C: 1×10^4 cells/well; rows D & E: 5×10^4 cells/well; rows F & G: 1×10^5 cells/well.

1.3 Results

Results are presented in tables F.1, F.2 and F.3. A cell concentration of 1×10^4 cells/well had the highest absorption. The cell concentrations of 1×10^5 cells/well and 5×10^4 cells/well showed almost no absorbance with the 5×10^4 cells/well showing an increase in absorbance from the 640 and 1280 virus dilution.

The virus dilution of 1280 (final well dilution) indicated an increase of 20% in viability against the control during a 7 day incubation period.

1.4 Conclusion

It can be concluded from this experiment that the cell and virus concentration to be used are 1 x 10^4 cells/well and a 10 times final dilution (per well) of the virus stock respectively.

Table F.1 Absorbance of different virus dilutions incubated with 1 x 10^5 cells/well

Virus dilution	1	2	**Average**	**Standev**
1280	0.025	0.026	**0.026**	**0.001**
640	0.025	0.027	**0.026**	**0.001**
320	0.026	0.026	**0.026**	**0.000**
160	0.027	0.026	**0.027**	**0.001**
80	0.026	0.025	**0.026**	**0.001**
40	0.027	0.026	**0.027**	**0.001**
20	0.027	0.025	**0.026**	**0.001**
10	0.025	0.025	**0.025**	**0.000**

The cell control obtained an absorbance average of 0.0277.

Table F.2 Absorbance of different virus dilutions incubated with 5 x 10^4 cells/well

5 x 10^4 cells/well	A570nm-A690nm				Average	Standev	Percent control				Average	Standev
Cells	0.783	0.74	0.825	0.74	**0.772**	**0.040735**	101.4249	95.85492	106.8653	95.85492	100	5.276543

					Percent control			
Virus dilution	1	2	**Average**	**Standev**	1	2	**Average**	**Standev**
1280	0.282	0.141	**0.212**	**0.100**	36.5285	18.26425	27.39637	12.91477
640	0.127	0.032	**0.080**	**0.067**	16.45078	4.145078	10.29793	8.701444
320	0.027	0.025	**0.026**	**0.001**	3.497409	3.238342	3.367876	0.183188
160	0.025	0.026	**0.026**	**0.001**	3.238342	3.367876	3.303109	0.091594
80	0.023	0.023	**0.023**	**0.000**	2.979275	2.979275	2.979275	0
40	0.022	0.025	**0.024**	**0.002**	2.849741	3.238342	3.044041	0.274782
20	0.038	0.038	**0.038**	**0.000**	4.92228	4.92228	4.92228	0
10	0.042	0.044	**0.043**	**0.001**	5.440415	5.699482	5.569948	0.183188

Table F.3 Absorbance of different virus dilutions incubated with 1 x 10^4 cells/well

1 x 10^4 cells/well	A570nm-A690nm				Average	Standev	Percent control				Average	Standev
Cells	1.309	1.309	1.367	1.337	**1.3305**	**0.027683**	98.38407	98.38407	102.7433	100.4885	**100**	**2.080626**

					Percent control			
Virus dilution	1	2	**Average**	**Standev**	1	2	**Average**	**Standev**
1280	0.247	0.303	**0.275**	**0.040**	18.564	22.773	**20.669**	**2.976**
640	0.179	0.245	**0.212**	**0.047**	13.454	18.414	**15.934**	**3.508**
320	0.152	0.162	**0.157**	**0.007**	11.424	12.176	**11.800**	**0.531**
160	0.124	0.143	**0.134**	**0.013**	9.320	10.748	**10.034**	**1.010**
80	0.105	0.106	**0.106**	**0.001**	7.892	7.967	**7.929**	**0.053**
40	0.093	0.097	**0.095**	**0.003**	6.990	7.290	**7.140**	**0.213**
20	0.078	0.083	**0.081**	**0.004**	5.862	6.238	**6.050**	**0.266**
10	0.072	0.074	**0.073**	**0.001**	5.411	5.562	**5.487**	**0.106**

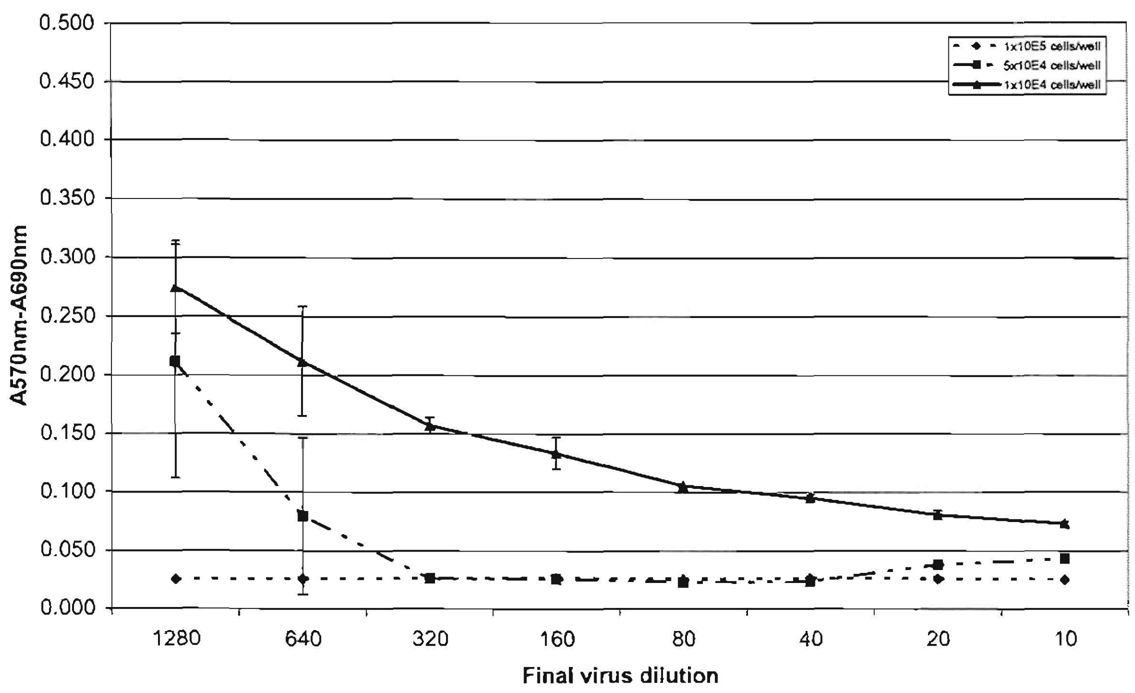

Figure F.1 Absorbance of different cell concentrations plotted against different virus dilutions

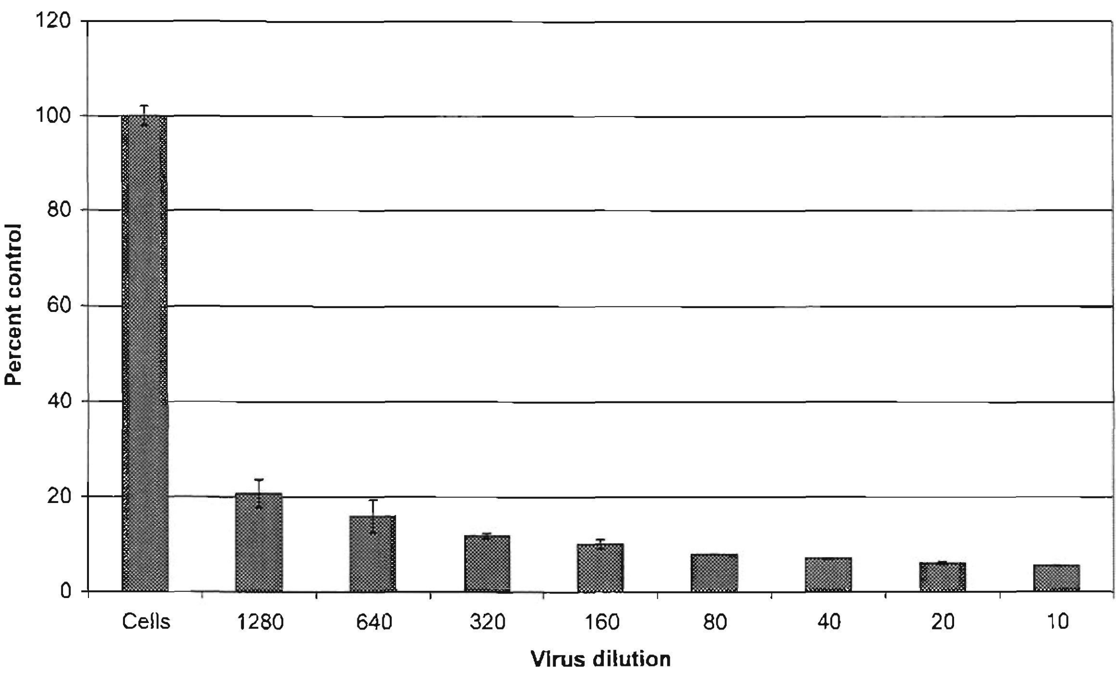

Figure F.2 MT-2 cells incubated with different virus dilutions

Figure F.2 illustrates the viability of the cells at the optimal virus dilution. The more the virus is diluted, the higher was the standard deviation.

ANNEXURE G

1 Investigation of the viability and toxicity properties of the chosen antiretrovirals (d4T, 3TC and NVP)

The use of the Pheroid™ drug delivery system as enhancer of the efficacy of antiretroviral therapy could result in effective therapy, as highlighted in chapter 3. Formulation problems and adverse effects may be overcome with this delivery system.

To investigate this, the viability and toxicity of the selected three drugs were determined. Using the adapted MTT assay as discussed above, wide concentration ranges were used, resulting in areas of unexpected change (in the graphs), which required a repeat of the assay in terms of the concentrations affected.

The MTT assay should indicate a viability range where the drug would demonstrate an area of toxicity, as the drug concentration increases. It should also indicate the concentrations where no, little or sufficient protection against virus infection is found.

1.1 The viability and protective properties of different concentrations of d4T

1.1.1 Introduction

This experiment was based on the adapted MTT assay. The EC_{50} and IC_{50} were used according to the NICD standards.

1.1.2 Method

d4T was used in a concentration range of 0.001 µM up to 500 µM. The drug was incubated with the cells for one hour, with 50 µl of virus dilutions added thereafter. The well plate was incubated for 4 days and 125 µl supernatant was removed from the wells, replaced with 125 µl medium containing the appropriate dilution d4T. 150 µl supernatant was removed from the well on day 7, and 10 µl MTT was added. It was incubated for 5 hours, the formed crystals were dissolved with SDS/DMF and the plate was read immediately.

1.1.3 Results

The results are presented in tables G.1 and G.2 and illustrated in figures G.1 and G.2.

Table G.1 Absorbance obtained from d4T (viability and protection)

d4T (µM)	Uninfected cells				Infected cells			
	570nm-690nm							
	1	2	3	Average	1	2	3	Average
Control	1.642	1.718	1.731	1.697	0.397	0.487	0.312	0.399
0.001	1.605	1.599	1.518	1.574	0.346	0.359	0.398	0.368
0.006	1.622	1.578	1.604	1.601	0.411	-	0.424	0.418
0.032	1.479	1.610	1.618	1.569	-	0.456	0.425	0.441
0.160	1.560	1.576	1.641	1.592	0.535	0.573	0.584	0.564
0.800	1.487	1.477	1.567	1.510	1.324	-	1.216	1.270
4.000	1.366	1.407	1.377	1.383	1.322	1.388	1.398	1.369
20.000	0.851	0.867	0.894	0.871	0.923	-	0.940	0.932
100.000	0.106	0.101	0.101	0.103	0.166	0.168	0.169	0.168
500.000	0.083	0.076	0.074	0.078	0.071	0.074	0.073	0.073

Table G.2 Extended concentration series of d4T

d4T (µM)	Uninfected cells				Infected cells			
	570nm-690nm							
	1	2	3	Average	1	2	3	Average
0	2.058	2.200	2.026	2.095	0.710	0.434	0.673	0.606
0.010	1.929	2.032	1.920	1.960	0.770	0.842	0.596	0.736
0.100	1.880	2.075	2.076	2.010	0.577	0.969	0.790	0.779
1.000	1.902	2.136	2.024	2.021	1.398	1.454	1.655	1.502
2.000	1.840	2.127	1.994	1.987	1.908	2.082	2.069	2.020
4.000	1.780	2.038	2.027	1.948	1.895	2.103	1.909	1.969
5.000	1.757	1.988	1.854	1.866	1.835	1.877	1.962	1.891
6.000	1.800	1.996	1.951	1.916	1.843	1.897	1.968	1.903
7.000	1.806	1.870	1.930	1.869	1.706	1.885	1.931	1.841
8.000	1.762	1.902	1.798	1.821	1.684	1.725	1.879	1.763

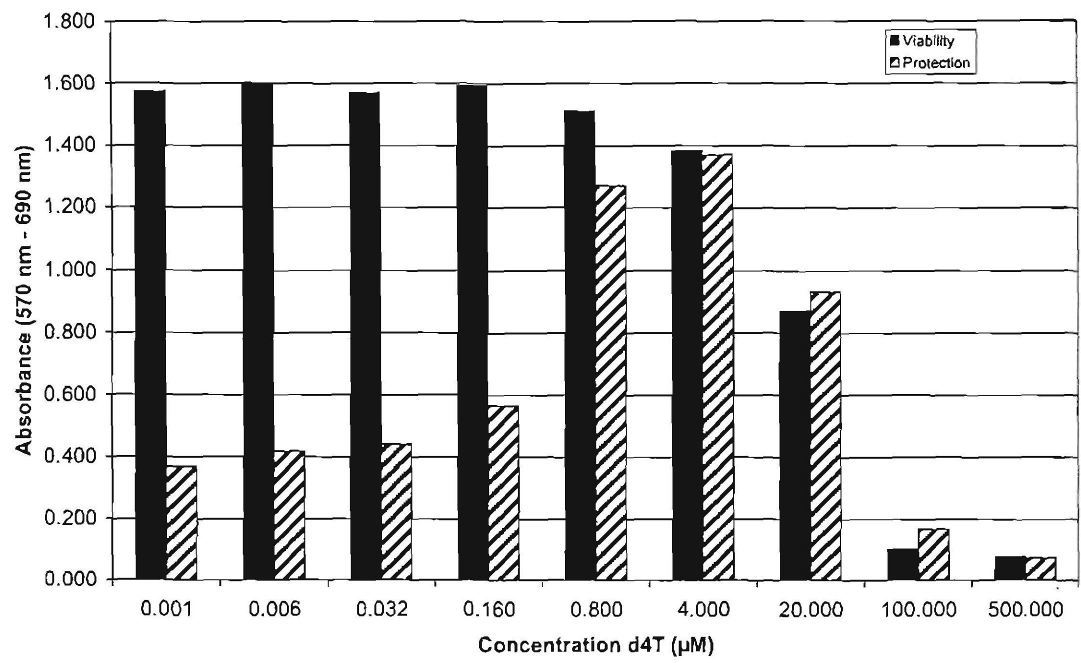

Figure G.1 Absorbance reflecting cell growth, plotted against a series of d4T concentrations, indicating viability and protective properties of stavudine.

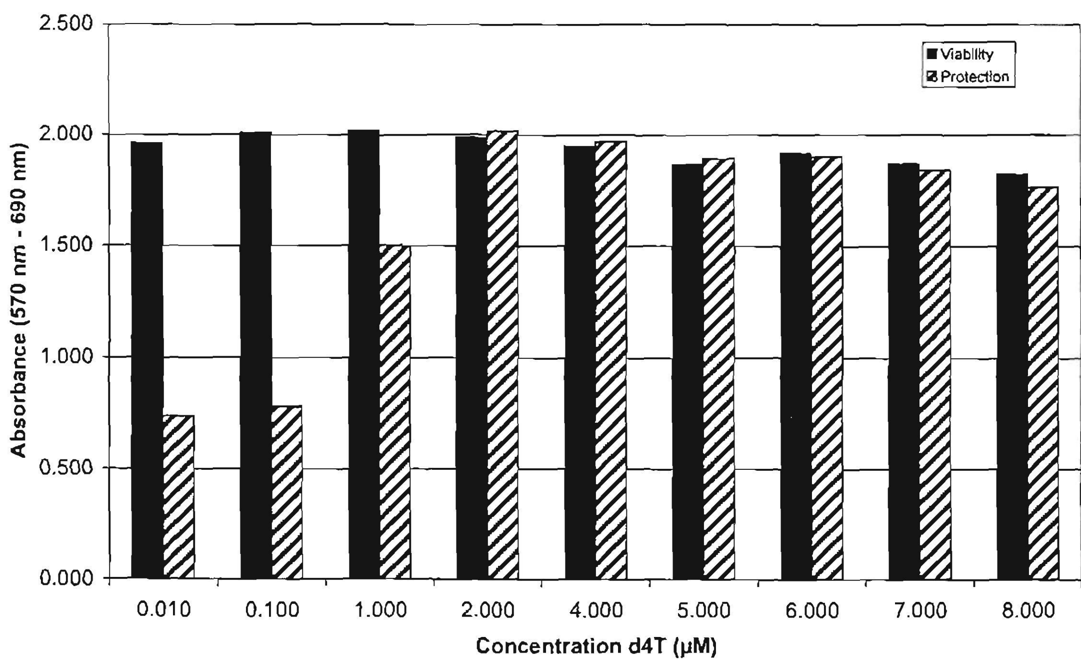

Figure G.2 Absorbance reflecting cell growth, plotted against an extended series of d4T concentrations, indicating viability and protective properties of stavudine.

1.1.4 Discussion

The viability and toxicity obtained were as expected. d4T showed viability of over 90% for most of the concentrations used; the viability decreases from a concentration of 2 µM and reaches nearly zero at a concentration of 100 µM (as indicated by the data plotted in figures G.1 and G.2).

The protection increased from a concentration of 0.032 µM and platoes at 2 µM, giving a maximum protection with an average of 94.963%. From 2 µM the protection decreased slowly giving zero percent protection when approaching a concentration of 100 µM. At this concentration the drug is completely cytotoxic.

1.2 The viability and protective properties of different concentrations of 3TC

1.2.1 Introduction

Lamivudine (3TC) in a wide concentration range was incubated with HIV-1 and MT-2 cells to investigate its protective and toxic properties.

1.2.2 Method

The assay used for this experiment is adapted from the assay by Mosmann (1983), as described above. A cell stock of 1 x 10^5 cells/ml was prepared, to give a cell concentration of 1 x 10^4 cells/well. The drug (3TC) concentration series was prepared to give a final well concentration of 0.001, 0.0064, 0.032, 0.16, 0.8, 4, 20, 100 and 500 µM in columns 2 to 11 respectively.

1.2.3 Results

Absorbance was obtained with the plate reader at the described wavelength and plotted as percentages against the corresponding concentration. See table G.3 and figure G.3 for experimental results.

Table G.3 Lamivudine absorbance obtained after staining with MTT

3TC (μM)	Uninfected cells 570nm-690nm				Infected cells			
	1	2	3	Average	1	2	3	Average
0.000	1.450	1.197	1.284	1.310	0.384	0.451	0.421	0.419
0.001	1.292	1.347	1.324	1.321	0.447	0.508	0.506	0.487
0.006	1.252	1.150	1.275	1.226	0.436	0.449	0.434	0.440
0.032	1.202	1.100	1.661	1.321	0.529	0.445	0.474	0.483
0.160	1.414	1.436	1.138	1.329	0.508	0.527	0.462	0.499
0.800	1.144	1.081	1.418	1.214	0.742	0.818	0.713	0.758
4.000	1.365	1.539	1.380	1.428	1.094	1.326	1.304	1.241
20.000	1.360	1.423	1.308	1.364	1.628	1.578	1.319	1.508
100.000	1.289	1.514	1.370	1.391	1.544	1.075	1.486	1.368
500.000	1.283	1.297	1.227	1.269	1.088	1.221	1.449	1.253

Table G.4 Extended concentration series of lamivudine: Absorbance

3TC (μM)	Uninfected cells			570nm-690nm		Infected cells		
	1	2	3	Average	1	2	3	Average
0.000	1.470	1.584	1.497	1.517	0.472	0.532	0.430	0.478
0.005	1.292	1.534	1.346	1.391	0.561	0.578	0.417	0.519
0.050	1.225	1.385	1.298	1.303	0.650	0.655	0.571	0.625
0.500	1.229	1.366	1.337	1.311	0.466	0.830	0.667	0.654
5.000	1.238	1.412	1.351	1.334	1.169	1.267	1.222	1.219
10.000	1.186	1.403	1.216	1.268	1.402	1.468	1.370	1.413
20.000	1.359	1.424	1.288	1.357	1.445	1.459	1.391	1.432
30.000	1.273	1.340	1.376	1.330	1.493	1.470	1.359	1.441
40.000	1.221	1.362	1.379	1.321	1.438	1.459	1.415	1.437
50.000	1.281	1.352	1.302	1.312	1.341	1.440	1.433	1.405

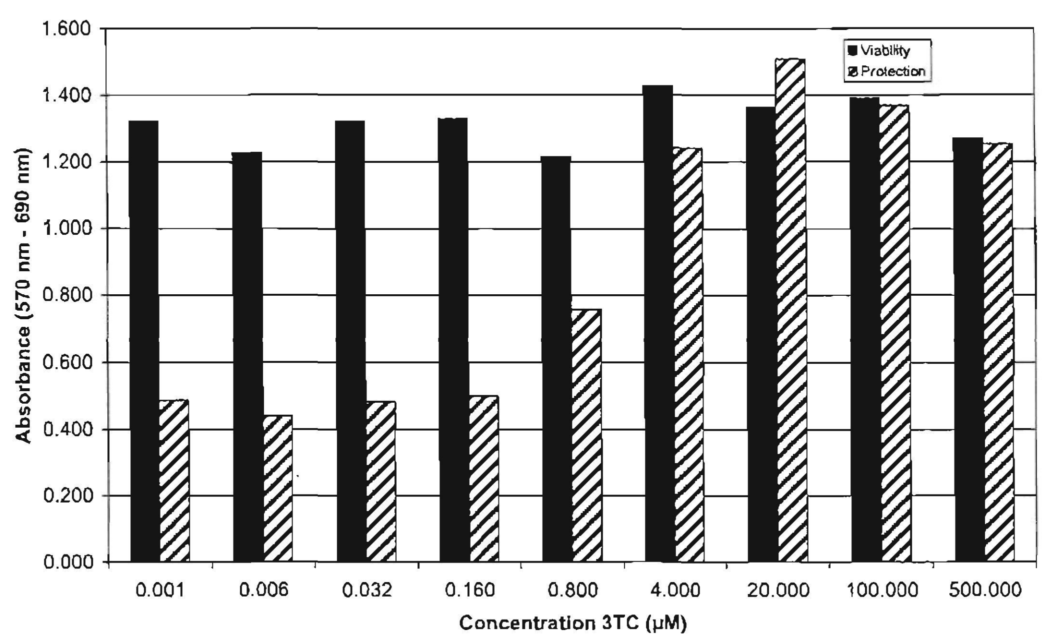

Figure G.3 Absorbance reflecting cell growth plotted against a 3TC concentration series, indicating viability and protection.

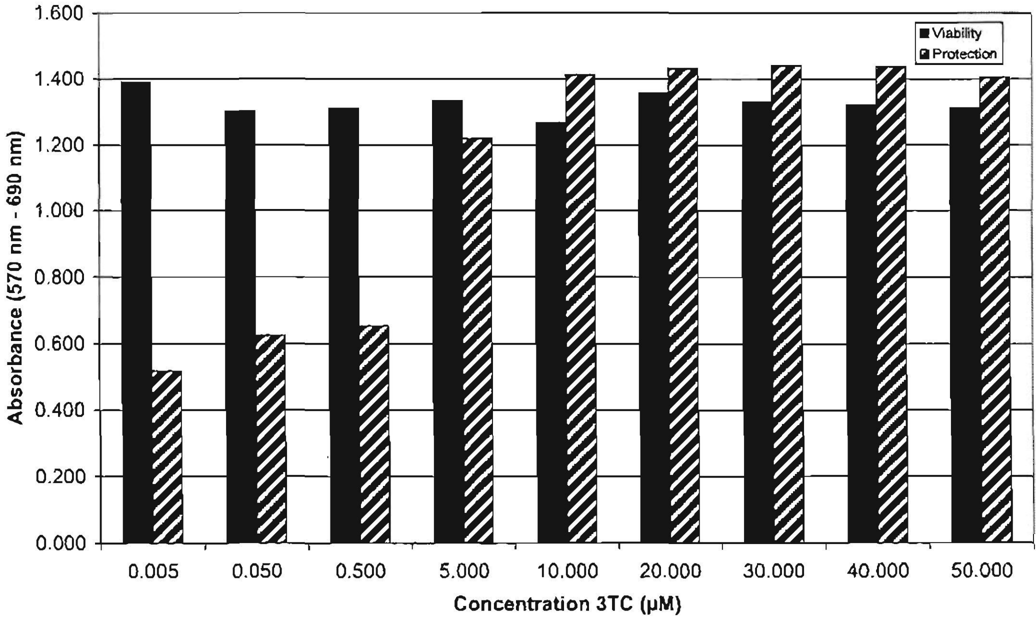

Figure G.4 Absorbance reflecting cell growth plotted against an extended concentration series, indicating viability and protection.

1.2.4 Discussion

Results were better than expected. The viability never reached below 90%, even with a concentration of 500 µM, showing that very high concentrations 3TC can be used without cytotoxicity.

Protection was observed from a drug concentration of 0.160 µM to a maximum of 120% at 20 µM. A viability percentage of 120% is probably due to the fact that cell growth could progress as normal, without an inhibitory effect by either infection or toxicity. This result could indicate that lamivudine inhibited all infection at that specific concentration.

Lamivudine required a higher concentration to produce the same protective properties than stavudine. The maximum protective effect of stavudine was at a concentration of 2 µM in contrast to the 20 µM of lamivudine.

1.3 The viability and protective properties of different concentrations of NVP

1.3.1 Introduction

Nevirapine is known to have solubility problems, which were also experienced in this experiment. A cell stock was prepared to have a cell concentration of 1 x 10^4 cells/well with a final virus dilution of 1250 (diluted form the stock solution).

1.3.2 Method

To enhance the solubility of NVP, it was incubated with medium at 37°C and occasionally agitated. It was then filtered through a 0.2 µm filter and added to the wells in pre-determined dilutions. The MTT assay was used as described above. 125 µl was extracted from the wells, on day 3, and replaced with the same amount of medium containing the appropriate amount of nevirapine. 150 µl supernatant was removed from the well on day 7, 10 µl MTT was added, the plate was incubated for 5 hours, crystals were dissolved with SDS/DMF and the plate was read.

1.3.3 Results

Absorbance obtained is given in table G.5 and plotted in figure G.5.

Table G.5 Absorbance for NVP

NVP (dilution)	Uninfected cells				Infected cells			
	570nm-690nm							
	1	2	3	Average	1	2	3	Average
0	2.226	2.256	2.176	2.219	0.439	0.605	0.435	0.493
640.0	1.830	2.134	2.053	2.006	2.020	2.111	2.004	2.045
320.0	1.909	2.081	2.048	2.013	1.985	2.081	2.059	2.042
160.0	2.076	2.093	2.062	2.077	1.933	2.067	2.083	2.028
80.0	2.069	2.048	2.018	2.045	2.045	2.138	2.046	2.076
40.0	2.089	2.040	2.065	2.065	1.998	2.211	2.004	2.071
20.0	2.195	1.864	2.121	2.060	2.041	2.185	2.133	2.120
10.0	2.322	2.091	2.241	2.218	2.112	2.114	2.198	2.141
5.0	2.069	1.679	2.039	1.929	1.944	1.996	1.967	1.969
2.5	1.094	1.030	1.173	1.099	1.048	1.114	1.096	1.086

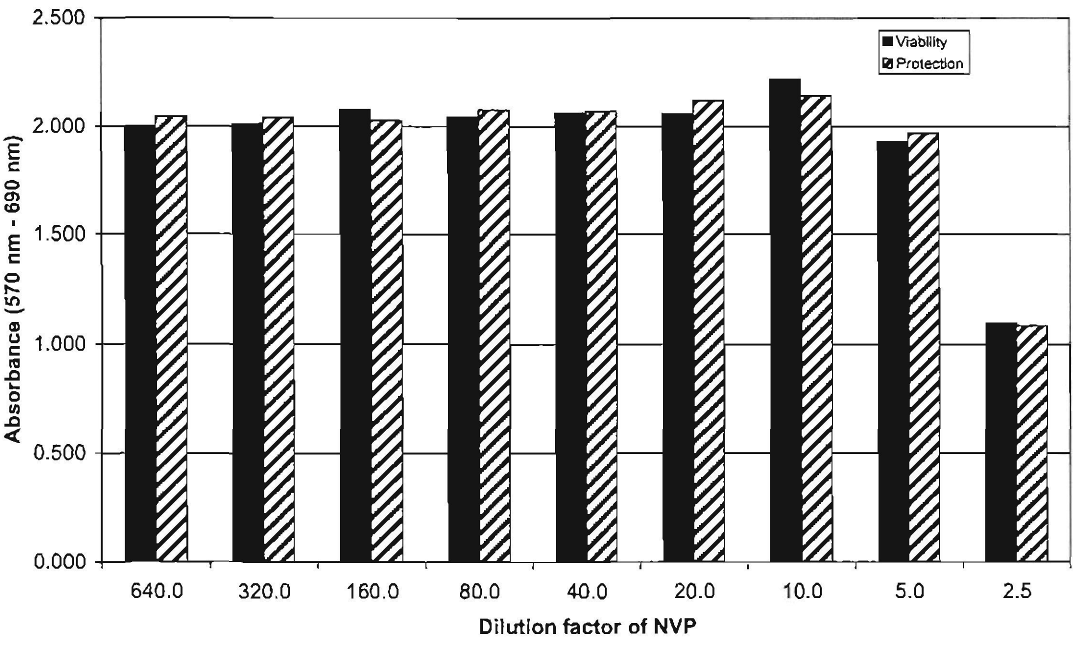

Figure G.4 Absorbance, reflecting cell viability and protection plotted against dilution factors of NVP.

1.3.4 Discussion

The observed toxicity and viability were almost identical. This result could be explained by the solubility problems of NVP or by product degradation. No differentiation between viability and protection was possible. The experiment indicated that NVP is not the best drug to use in this study, unless solubility could be enhanced and degradation prevented.

ANNEXURE H

1 Optimization of the cell concentration and virus dilution to be used with a three day incubation period.

1.1 Introduction

Due to the massive cell toxicity and cell death that occurred when the Pheroid™ drug delivery system was used (see chapter 5), the assay was changed to a three day incubation period. The changes included titration of different concentrations of viruses and cells. Once these factors were determined, further studies could be performed.

1.2 Methods

The method used was an adapted version of the previous assays used, changing only the incubation period from 7 days to 3 days. An extra day (day 4) was incorporated into this study to determine the optimal incubation time.

The plate layout is presented in figure H.1.

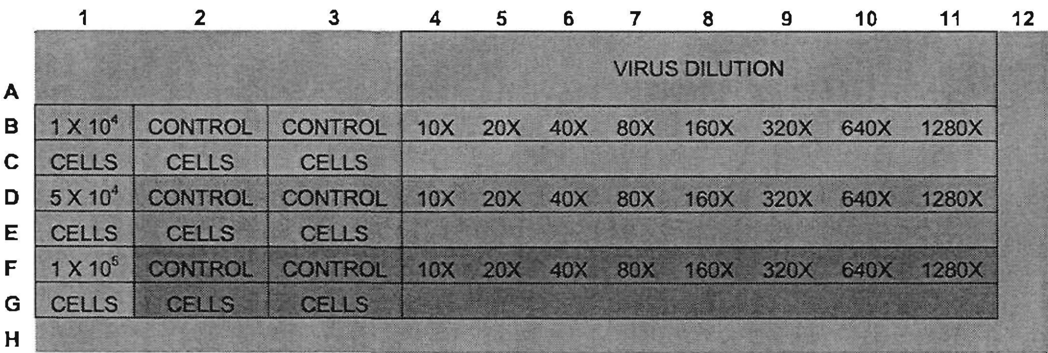

Figure. H.1 Plate layout.

1.3 Results

The results presented below show cell growth, as reflected by absorbance, plotted against virus dilutions for each cell concentration. Eight virus concentrations were used to infect the cells.

Figures H.2, H.3, H.4 and H.5 illustrate the daily results obtained.

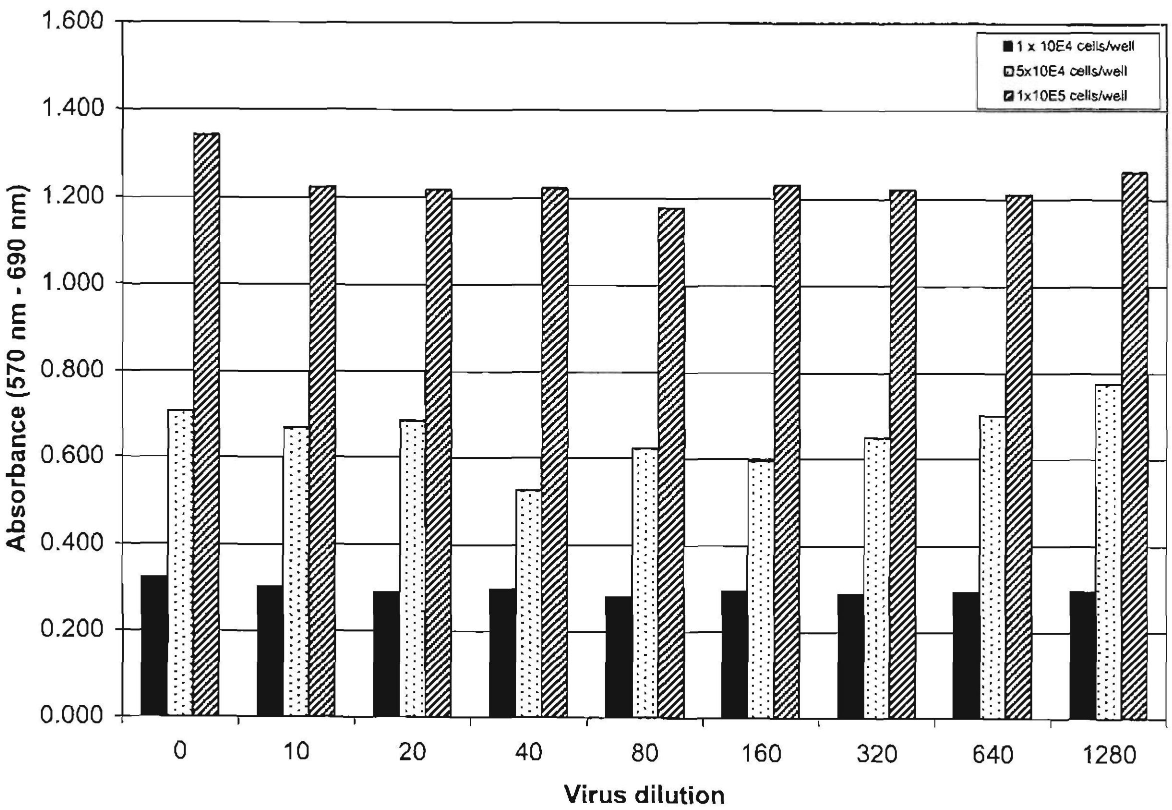

Figure H.2 Day 1.

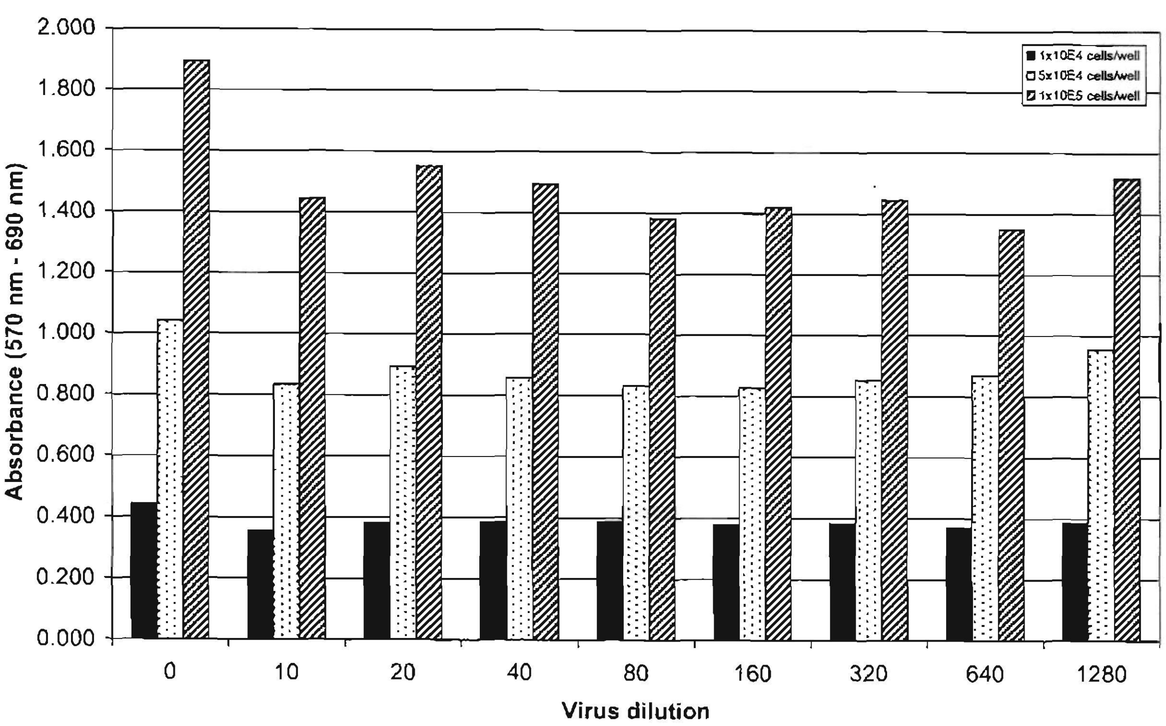

Figure H.3 Day 2.

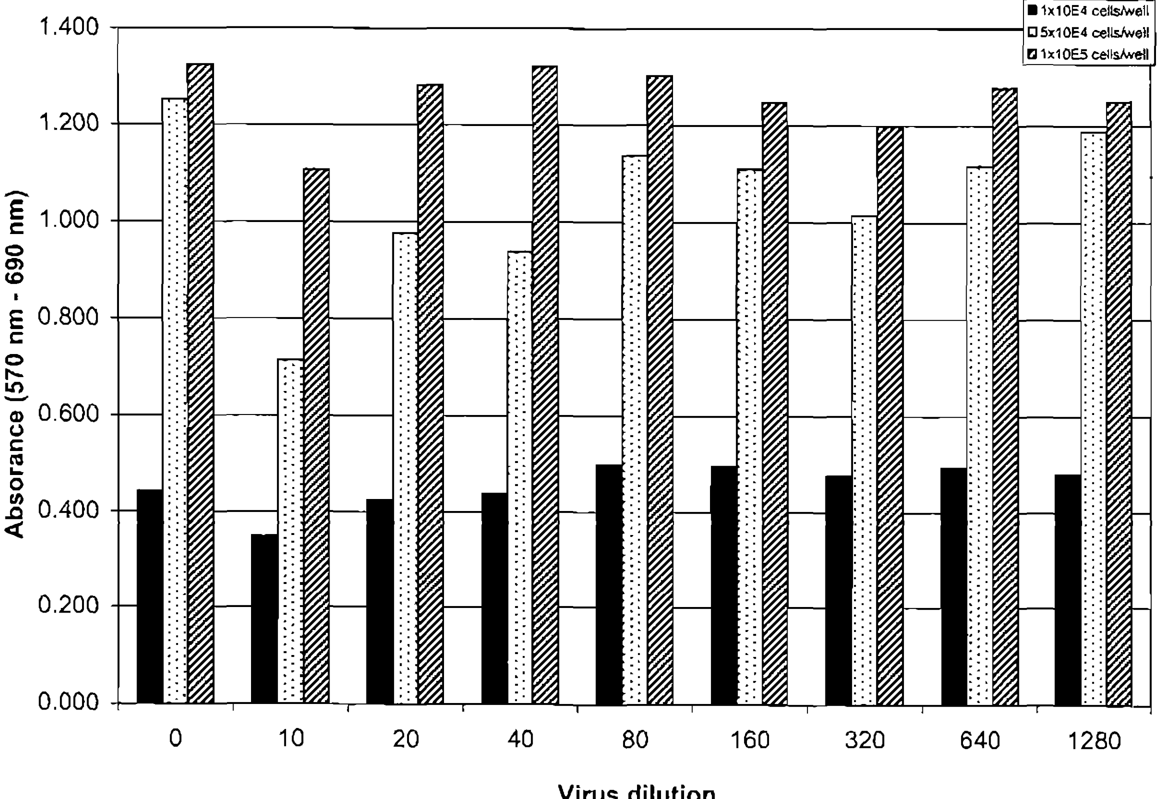

Figure H.4 Day 3.

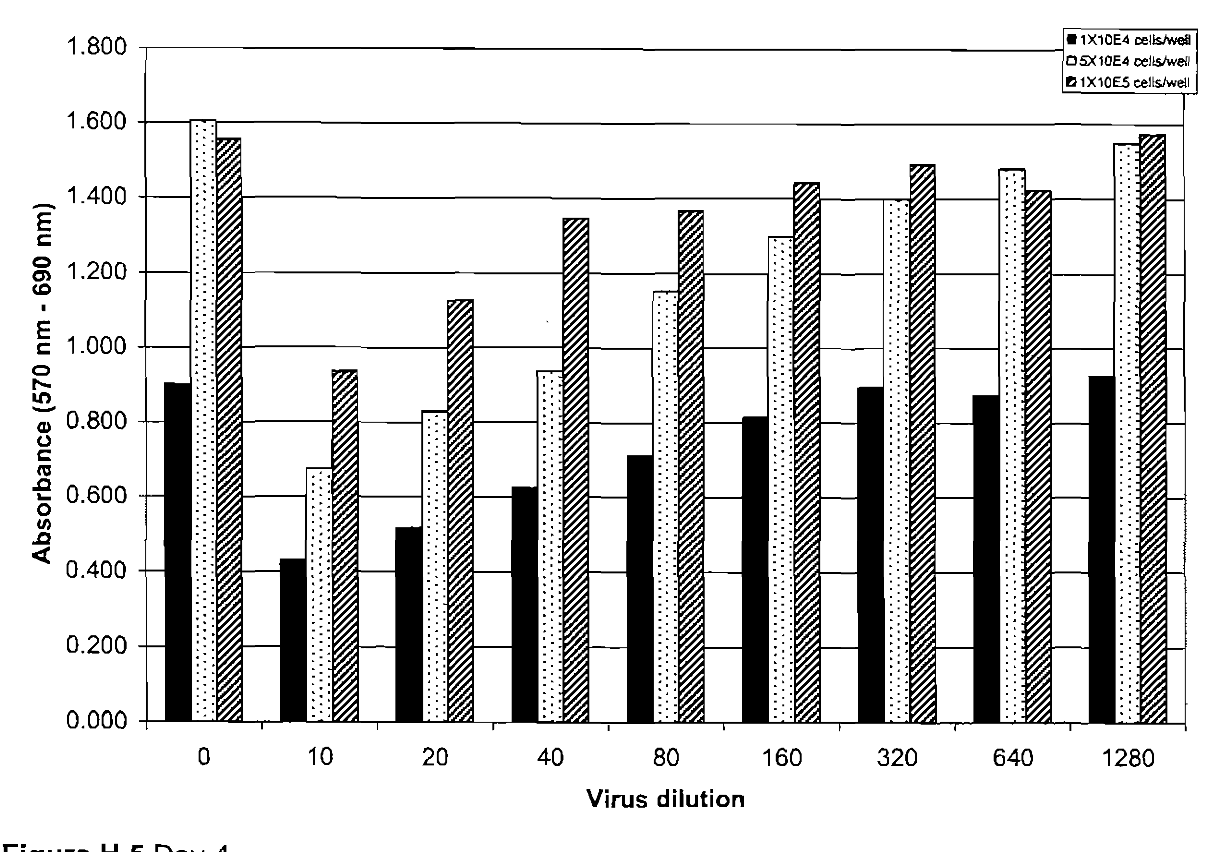

Figure H.5 Day 4.

1.4 Conclusion

The cell concentration of 1 x 10^4 cells/well gave the lowest absorbance, which increased with ascending cell concentration. The same effect was also observed with the virus dilution, where the 10 times virus dilution infected and killed the largest amount of cells. The optimal cell concentration and virus dilution are those which showed the lowest absorbance. A greater effect will then be observed between the cell control (where cells have not been infected) and the virus control. The cell control will give absorbance values which are high, whereas the virus control will give low absorbance values. The best results will be those with the highest contrast.

A cell concentration of 1 x 10^4 cells/well and a virus dilution of 10 times were decided on as the respective concentration and dilution to be used in future studies.

ANNEXURE I

The following annexure includes the Guide for Authors for the article written in Chapter 4.

4.1 Guide for authors

Scope

Cell Death and Differentiation is published on behalf of CFIC (IDI-IRCCS) Istituto Dermopatico dell'Immacolata by Nature Publishing Group, a division of Macmillan Publishers Limited.

Cell Death and Differentiation is a journal devoted to the cell biology, molecular biology and biochemistry of cell death and differentiation, both in normal tissue regulation and in disease. To this end, *Cell Death and Differentiation* provides a unified forum for scientists as well as clinicians. It is committed to the rapid publication of high-quality original papers that relate to these subjects, together with topical, usually solicited, reviews, meeting reports, editorial correspondence and occasional commentaries on controversial and scientifically informative issues.

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Editor-in-Chief:

Gerry Melino, Department of Experimental Medicine, University of Rome. (CDD has offices in Rome, San Diego and Tokyo.)

Authors whose first language is not English are requested to have their manuscripts checked carefully for linguistic correctness before submission.

4.1.1 Format of papers

Preparation of manuscripts

Cell Death and Differentiation publishes:

- Full papers
- Reviews
- Meeting Reports
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- News and Commentary

Full papers should be as comprehensive as possible, between 5 and 12 published pages (about 15-40 double spaced A4 pages, excluding tables and figures). The manuscript should be organised as follows:

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Please include:

- The name, institution and e-mail address of all contributing authors;
- Suggestions for potential referees, including contact information if known;
- Referees to exclude;
- Total number of words.

Title page:

- Title;
- Running title of less than 50 characters;
- Authors;
- Affiliations;
- Address/telephone/fax/e-mail of corresponding author.

Abstract

One single paragraph of no more than 210 words.

Keywords and Abbreviations

Main text:

The main text (excluding abstract, Materials and Methods, references and figure legends) must not exceed 3,500 words:

- Introduction;
- Results;
- Discussion - The discussion may be subdivided by further subheadings or may be combined.

Materials and methods

- This section should contain sufficient detail so that all procedures can be repeated, in conjunction with cited references;
- Where genetically manipulated (transgenic or knockout) mice are used, the Materials and Methods should state the name and genotype of the founder ES cells or oocytes;
- The genotype and breeding of experimental mice and controls should be described.

Display items

Original papers typically contain 6–8 display items (figures and/or tables).

References

Original papers may not exceed 40 references. Only articles that have been published or are in press may be included in the references. References follow the Vancouver format (for using End-Note, please follow the Leukemia journal reference style). References are numbered starting at 1 and listed sequentially at the end of the paper, according to the numbers and order of citations in the text. The first six authors are to be listed, after which, list only the first six authors and 'et al'. Abbreviations of journal names should conform to the Index Medicus style. The first and last page numbers for each reference should be provided. The following are examples of the reference style:

1. Gottschalk AR, Boise LH, Oltvai ZN, Accavitti MA, Korsmeyer SJ, Quintans et al The ability of Bcl-XL and Bcl-2 to prevent apoptosis can be differentially regulated. Cell Death Differ. 1996; 3: 113-118
2. Feramisco JR and Welch WJ, Modulation of cellular activities via microinjection into living cells. In: Celis JE, Graessmann A and Coyter A, (eds).*Microinjection and organelle transplantation techniques*, (London: Academic Press), 1986 pp.40-58

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- Image resolution of at least 300 dpi at publication size;
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- During software manipulation of images, care should be taken that resolution is maintained;
- Images may be rotated or scaled, but this must be the same in the x and y dimensions;
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- If black borders are drawn around the image, the lines should correspond to all edges where the image was cut;
- Protein molecular weights or DNA fragment sizes should be indicated for all figure panels showing gel electrophoresis.

Graphs, Histograms and Statistics

- Error bars must be described in the figure legend;
- Axes on graphs should extend to zero, except for log axes;
- Statistical analyses (including error bars and p values) should only be shown for independently repeated experiments, and must not be shown for replicates of a single experiment;
- The number of times an experiment was repeated (N) must be stated in the legend.

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These should be labeled sequentially as Table 1, Table 2, etc. Each table should be typed on a separate page, numbered and titled, and cited in the text. Reference to table footnotes should be made by means of Arabic numerals. Tables should not duplicate the content of the text. They should consist of at least two columns; columns should always have headings.

Authors should ensure that the data in the tables are consistent with those cited in the relevant places in the text, totals add up correctly, and percentages have been calculated correctly. Unlike figures or images, tables may be embedded into the word processing software if necessary, or supplied as separate electronic files.

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5. Use Si-units throughout;
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8. Text should be double spacing with a wide margin;
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