# Evaluation and validation of *in vitro* assays to determine cell viability for HIV/AIDS experimentation with Pheroid™ technology

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Dissertation submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE (PHARMACEUTICS)

at the
POTCHEFSTROOM CAMPUS OF THE NORTH-WEST UNIVERSITY

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# Acknowledgements

I would like to express my sincerest appreciation to all the following groups and people, without whom this study would not have been possible:

- **The Innovation Fund** for their financial assistance.
- **The Phertech group** for giving me the opportunity to participate in this amazing project.
- **The NICD** for kindly providing me with their expertise, laboratory facilities, equipment and reagents.
- **Me. Anne Grobler** for guiding me with knowledge and logic; and inspiring me with her love for research.
- **Dr. Adriaan Basson** for his support, assistance and guidance in the laboratory at the NICD and his friendship.
- **Dr. Lissinda du Plessis** for her never-ending flow of ideas and unique ability to relate to any topic and give appropriate advice.
- **Me. Liezl-Marié Nieuwoudt and Silverani Padayachee** for providing me with Pheroids even on short notice and for great friendship.
- **Prof. Awie Kotzé and Prof. Wilna Liebenberg** for listening and supervising me while Anne was ill.

I would also like to thank the following people for enriching my personal life:

- **Righard Lemmer**, my best friend and the love of my life. Thank you for truly understanding and supporting me.
- My parents: **Johan and Hela van der Merwe** for believing in me and providing me with their love and the necessary encouragement.
- My brother **Nicohan** for his friendship, support and entertainment.
- **My friends** and fellow students for their invaluable participation in my everyday life and continuous support and understanding during my studies.
- **The breakfast club:** thank you for enlightening my day with caffeine and gossip.

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# Abstract

The Southern parts of Africa have the highest prevalence of HIV-infected people and South Africa is the country with the highest number of infections in the world. There is still no cure for AIDS, but anti-HIV medicine can prolong and enhance the quality of life of an HIV infected person. Patient adherence with antiretroviral therapy is extremely low due to difficult dosing intervals, problematic dosage forms, instability of the antiretrovirals (ARVs) and the severe side-effects caused by these drugs; this leads to resistance of HIV to these drugs.

Pheroid™ technology is a patented delivery system. Pheroid™ vesicles were used during this study. The entrapment of an active within the Pheroid™ would generally provide a safer, more effective formulation than the active alone. This could mean that the amount of drug needed for treatment of HIV can be decreased while producing fewer adverse effects and reducing the price of treatment.

The main objectives of this study were to optimise and validate the cell viability and viral replication assays that can be used in an *in vitro* viral infection model. The MTT assay was used to asses the viability of the cells and to determine the toxicity of the antiretroviral drugs and Pheroid™ on the cells. HIV-1 assays were evaluated and used to determine the viral replication in the cells.

Two different continuous cell lines were chosen for this study, an anchorage dependent GHOST cell line and suspended M7-Luc cells. Both these cell lines were best infected with the SW7 virus. SW7 is a subtype C, CXCR4 utilising virus. Subtype C is responsible for 60 % of the HIV infections worldwide and is the prevalent subtype in Sub-Saharan Africa. Infection enhancers were not added to the cells to improve viral infection since it was observed that the Pheroid™ in combination with DEAE-dextran or Polybrene caused cytotoxicity probably by disrupting the cell's membrane. Antioxidants were added to the Pheroid™ formulation since it was observed that the viability of the cells incubated with the Pheroid™ decreased as the Pheroid™ matured. The added antioxidants had no significant effect on the cells.

Abacavir (ABC) was chosen as the test substance for this study since it showed low cytotoxicity in cell cultures and is water soluble and would not present solubility issues in the media. It was entrapped within the Pheroid™ and its *in vitro* efficacy and toxicity was tested on HIV-infected and uninfected cell cultures.

One direct/HIV-specific (p24 antigen ELISA assay) and one indirect (Luciferase) assays were used to asses the inhibition of HIV replication caused by ABC. The p24 antigen ELISA (Enzyme-Linked ImmunoSorbent Assay) assay required a lot of washing steps and were rather expensive to use. The Luciferase assay was only used on the M7-Luc cells; this assay was sensitive, inexpensive and easy to use.

The MTT (3-(4,5-demethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) viability assay was used to measure the toxicity caused by the Pheroid™ and/or ABC on the cells. MTT is a widely used quantitative colorimetric assay to measure the viability of cells. The vitamin E and antioxidants contained in the Pheroid™ reduced the MTT and produced results that were misinterpreted as enhanced viability when the Pheroid™ was present during MTT analysis. To prevent this problem an additional washing step should be introduced prior to analysis to reduce the interference of the Pheroid™ with analytical methods.

In conclusion, the efficacy of ABC entrapped within the Pheroid™ is still inconclusive and further studies will have to be done. MTT should be used with care for viability analysis of cells incubated in the presence of Pheroid™.

**Keywords:**

Abacavir (ABC), HIV and AIDS, Luciferase assay, MTT, p24 antigen ELISA assay, Pheroid™, viability.

# Uittreksel

Suiderlike Afrika is die streek met die hoogste prevalensie op aarde van mense besmet met die menslike immuniteit gebrek virus (MIV).. Daarby is Suid-Afrika die land met die meeste MIV-besmette persone. Daar is steeds geen kuur teen die verworwe immuniteitsgebrek sindroom (VIGS) wat deur MIV veroorsaak word nie. MIV-besmette persone se lewens kan verleng word en hul lewenskwaliteit verbeter word deur aan hul antiretrovirale behandeling te gee. Pasiënt meewerkendheid is ongelukkig baie laag met hierdie medikasie, as gevolg van moeilike dosering, onaangename doseervorms en die slegte newe-effekte wat hierdie medikasie veroorsaak. Die swak pasiënt meewerkendheid is een van die oorsake vir weerstandbiedendheid van MIV teenoor hierdie geneesmiddels.

Pheroid™ tegnologie is 'n gepatenteerde geneesmiddel aflewerings sisteem. Farmakologies-aktiewe middels kan binne die Pheroid™ vasgevang of verpak word. Hierdie produk is gewoonlik meer effektief en 'n veiliger doseervorm as die oorspronklike produk. 'n Verminderde hoeveelheid geneesmiddel kan gebruik word as hierdie geneesmiddels in die Pheroid™ aflewerings sisteem vasgevang word. Dit sal nie net lei tot 'n verlaging in die koste van behandeling nie, maar die geneesmiddel sal ook minder newe-effekte veroorsaak.

Die hoofdoel van hierdie studie was die optimalisering en validering van 'n analitiese metode wat gebruik kan word om die lewensvatbaarheid van selle te bepaal. Daarby is verskillende virus replisering analise metodes beproef om die moontlike verbetering van die effektiwiteit van 'n antiretrovirale geneesmiddel vasgevang in die Pheroid™ te bepaal. Die bekende MTT (3-(4,5-dimetielthiasol-2-yl)-2,5-dipheniel tetrazolium bromied) analise metode is gebruik om die lewensvatbaarheid van die selle en die toksisiteit van die antiretrovirale geneesmiddels en/of Pheroid™ te bepaal.

Twee geneties-gemanipuleerde sellyne is gebruik. Die M7-Luc sellyn groei gesuspendeerd in groei medium terwyl die selle van die GHOST sellyn aan die oppervlakte van die houer waarin dit groei moet vasheg. Beide hierdie sellyne is geïnfekteer met die SW7 virus. Hierdie is 'n subtipe C virus. Sestig persent van die MIV-infeksies wêreldwyd word deur die MIV subtipe C veroorsaak.

Die byvoeging van Polybrene of DEAE-dextraan kan normaalweg infeksie van die selle deur MIV in *in vitro* sisteme verhoog. Toe hierdie stowwe egter in kombinasie met die Pheroid™ by die selle gevoeg is, het dit seldood veroorsaak. 'n Moontlike rede hiervoor is dat hierdie kombinasie die selmembrane versteur. Verhoogde seldood is ook waargeneem by selle wat met verouderde Pheroid™ geïnkubeer is. Anti-oksidant is om hierdie rede by die Pheroid™ gevoeg. Die byvoeging van anti-oksidante het geen waarneembare effek op die selle gehad nie.

Abacavir (ABC) is as proefstof tydens hierdie studie gebruik. ABC is goed wateroplosbaar en het daarom maklik in die verkillende groeimedia opgelos. Dit was ook nie toksies vir die selle gewees by die konsentrasie wat gebruik is nie. Die effektiwiteit en toksisiteit van ABC en Pheroid™ is afsonderlik op geïnfekteerde en ongeïnfekteerde selle bepaal. Laastens is ABC verpak in die Pheroid™ en die effektiwiteit en toksisiteit van hierdie kombinasie is eksperimenteel bepaal.

Die p24 antigeen analise metode is 'n direkte MIV-spesifieke analise metode. Hierdie analise metode is duur, tydsaam en arbeid intensief. Die Luciferase analise metode is sensitief, goedkoper as die p24 antigeen metode en maklik om te gebruik. Hierdie analise metode kon egter slegs vir die M7-Luc sellyn gebruik word.

MTT is gebruik om die toksisiteit van ABC en/of Pheroid™ te bepaal. MTT is 'n populêre analise metode om selle se lewensvatbaarheid te bepaal. Vitamiene E of ander anti-oksidante meng met hierdie analise metode se effektiwiteit in. Laasgenoemde stowwe kan die MTT reduseer in die afwesigheid van selle; die resultate word dan misinterpreteer as verhoogde lewensvatbaarheid van die selle. In 'n poging om hierdie verskynsel te vermy, kan die selle gewas word alvorens die analise gedoen word.

Ten slotte, daar is steeds nie sekerheid rakende die effektiwiteit van ABC verpak in die Pheroid™ nie en verdere studies sal gedoen moet word om dit te bepaal. Die gebruik van die MTT analise metode moet noukeurig oorweeg word wanneer die lewensvatbaarheid van selle in die teenwoordigheid van die Pheroid™ bepaal word.

**Sleutel woorde:**
Abacavir (ABC), MIV en VIGS, lewensvatbaarheid, Luciferase, MTT, p24 antigeen, Pheroid™.

# Introduction and Aim of this Study

The human immunodeficiency virus (HIV) is the primary cause of an acquired immunodeficiency syndrome (AIDS). South Africa is the country with the largest number of infections in the world (UNAIDS, 2008b). There is still no cure for AIDS, but anti-HIV medicine can prolong and enhance the quality of life of an HIV infected person. Highly active antiretroviral therapy (HAART) has transformed the treatment and management of HIV/AIDS. The main problems with HAART are the severe side-effects caused by these drugs, the problematic patient adherence and the increased resistance to these drugs. In 2007, only 2.99 million (31%) of the 9.7 million people who were in dire need of anti-HIV medicines received it (Avert, 2008b).

Pheroid™ technology is a patented delivery system. When using the term Pheroid it will refer to Pheroid™ vesicles. The entrapment of an active within the Pheroid would generally provide a safer, more effective formulation than the active alone (Grobler, 2004).

The main objectives of this study were:

- To conduct a literature overview of HIV/AIDS and the treatment thereof.
- Deciding on a new cell line and virus type
- Optimisation of the *in vitro* incubation conditions.
- Optimisations of the MTT assay to asses the viability of the cells and to determine the toxicity of the antiretroviral drugs and Pheroid™ on the cells.
- Evaluating different methods to determine the viral replication in the cells.
- Experimenting with different Pheroid formulations, with and without anti-oxidation agents.
- Evaluating the *in vitro* efficacy of abacavir (ABC) and lamivudine (3TC) against HIV-1.
- Using Pheroid™ technology in order to enhance the *in vitro* efficacy of ABC.

Chapter 1 and 2 gives a literature introduction to HIV/AIDS and Pheroid™ technology. Chapter 3 converse on the materials and methods used for this study, but also include the necessary literature background of the methods used. Chapter 4 describes the results and findings generated. The final summary and conclusion will be explained in chapter 5.

# Abbreviations and Definitions

| | |
|---|---|
| **3TC** | Lamivudine |
| **ABC** | Abacavir |
| **ACC** | Average cells counted (haemocytometer) |
| **AIDS** | Acquired immune deficiency syndrome |
| **ART** | Antiretroviral therapy |
| **ARV** | AIDS-associated retrovirus |
| **AZT** | Zidovudine |
| **BHA** | Butylated hydroxyanisole |
| **BHT** | Butylated hydroxytoluene |
| **CA** | Capsid or p24 |
| **$CC_{50}$** | 50 % Cytotoxic concentration |
| **CCR5** | A chemokine receptor (co-receptor for HIV entry into the cell) found upon macrophages |
| **CD4** | HIV's target receptor found upon CD4-bearing lymphocytes. This is the primary mechanism for viral entry into cells via viral docking mechanism with gp120 |
| **CDC** | US centre for disease control |
| **CRF** | Circulating recombinant forms |
| **CSF** | Cerebrospinal fluid |
| **CXCR4** | A co-receptor for HIV entry found upon T-lymphocytes |
| **d4T** | Stavudine |
| **ddC** | Zalcitabine |
| **ddI** | Didanosine |
| **DLV** | Delavirdine |
| **DMEM** | Dulbecco's modified minimum essential media |
| **DMF** | Dimethylformamide |
| **DMSO** | Dimethylsulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EFV** | Efavirenz |
| **FCS** | Foetal calf serum |
| **FTC** | Emtricitabine |

| | |
|---|---|
| **G418** | Geneticin (antibiotic) |
| **gp** | Glycoprotein. Protein that is modified after translation from messenger RNA form by the by the addition of one or many sugar residues to specific amino acids within the protein |
| **Gp160** | HIV coat glycoprotein composed of separate gp41 and gp120 |
| **GFP** | Green fluorescent protein |
| **HAART** | Highly-active antitroviral therapy |
| **HIV** | Human immunodeficiency virus |
| **$IC_{50}$** | 50 % Inhibitory concentration |
| **IN** | Integrase enzyme |
| **l** | Litre |
| **LTR** | Long terminal repeat |
| **Luc** | Luciferase |
| **M** | Molar (mol/l) |
| **MA** | Matrix or p17 |
| **mg** | Milligram |
| **ml** | Millilitre |
| **mRNA** | Messenger ribonucleic acid |
| **MTCT** | Mother to child transmission |
| **MTT** | (3-(4,5-demethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Cell viability assay |
| **MVC** | Maraviroc |
| **$N_2O$** | Nitrous oxide |
| **NICD** | National Institute for Communicable Disease |
| **NIH** | National Institute of Health |
| **NNRTI** | Non-nucleoside analogue reverse transcriptase inhibitor |
| **NRTI** | Nucleoside analogue reverse transcriptase inhibitor |
| **NVP** | Nevirapine |
| **PEG** | Polyethylene glycol |
| **PBMC** | Peripheral blood mononuclear cells |
| **PBS** | Phosphate-buffered saline |
| **PI** | Protease inhibitors |
| **PR** | Protease enzyme. Viral enzyme that cleaves long precursor proteins into shorter functional ones |
| **Replication** | Process of copying of gentic information |
| **Retrovirus** | A virus whose genome is stored in RNA rather than in the DNA |

| | |
|---|---|
| **RGV** | Raltegravir |
| **RNA** | Ribonucleid acid |
| **RLU** | Relative light units |
| **Rpm** | Revolutions per minute |
| **RPMI 1640** | Roswell Park Memorial Institute (designed media) |
| **RT** | Reverse transcriptase. Enzyme within HIV viral particle; copies and translates HIV's viral RNA into DNA |
| **SDS** | Sodium dodecyl sulphate |
| **SOP** | Standard operating procedure |
| **SU** | Surface glycoprotein or gp120 |
| **T-20** | Enfuvirtide |
| **TB** | Tuberculosis |
| **TLC** | Thin-layer chromatography |
| **TM** | Transmembrane glycoprotein or gp41 |
| **TMB** | Tetramethylbenzidine (substrate for p24 elisa) |
| **TNF** | Tenofovir |
| **Translation** | Action of converting messenger RNA code to equivalent protein sequence of amino acids within ribosome's of endoplasmic reticulum |
| **µg** | Microgram |
| **µl** | Microlitre |
| **URF** | Unique recombinant forms |
| **WHO** | World Health Organization |

# Chapter 1

## HIV/AIDS: An Introduction

This section will converse on the statistics of HIV-infected people around the world, properties of the virus, viral replication, the course of the HIV disease, and the therapeutic agents used to combat HIV/AIDS.

## 1.1 Introduction to HIV/AIDS

In 1983 the human immunodeficiency virus type I (HIV-1) was defined as the primary cause of an acquired immunodeficiency syndrome (AIDS) (Gallo & Montagnier, 2003). This was two years after the first immunodeficiency syndrome case was observed in homosexual males (Vanley *et al.*, 1982). HIV causes AIDS by damaging the immune system and thus making the body susceptible to infections and tumours that would not have harmed the human body otherwise. The Centre for Disease Control and Prevention (CDC) defined AIDS as being HIV positive, developing an opportunistic infection and having a $CD4^+$ lymphocyte count of <200 $CD4^+$ lymphocytes/µl of blood or a total CD4 T lymphocyte count of <14 % (Castro *et al.*, 1992). The primary routes of infections are through unprotected sexual intercourse with an infected partner, injection or transfusion of contaminated blood and mother-to-child transmission (MTCT) (CDC, 1999b).

Since 1981, 25 million people have died because of AIDS-related illnesses. Approximately 3 million people died because of AIDS in 2007 alone. In the same year, there were 33 million people living with AIDS worldwide (see Figure 1.1); children under the age of 15 represent 2 million of them.

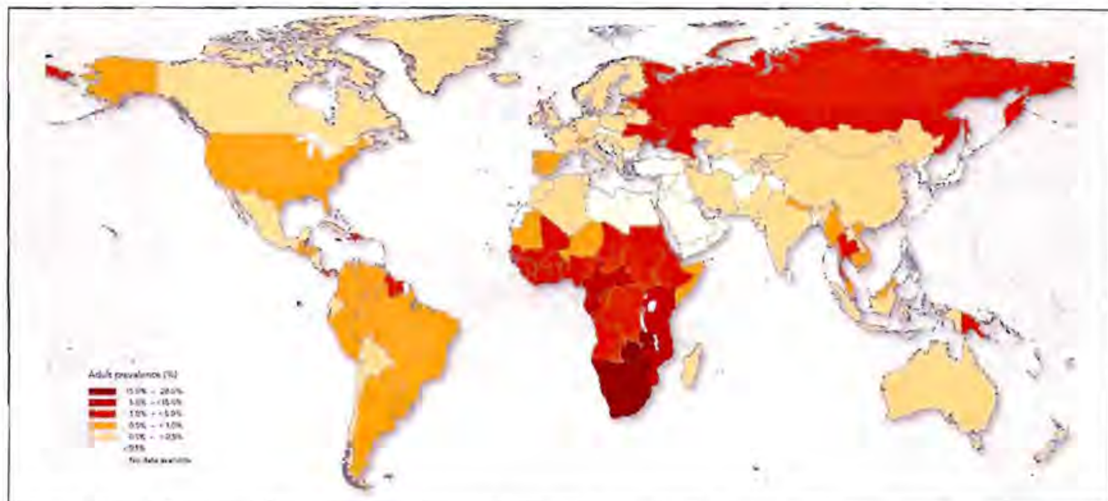


**Figure 1.1. Global prevalence of HIV infection at the end of 2007, ranging between < 0.1 % (light yellow) and 15 – 28 % (dark red)** (UNAIDS, 2008a).

Sub-Saharan Africa is home to 22 million known HIV infected people of whom 1.8 million are children (Avert, 2008a-c and UNAIDS, 2008b). Figure 1.2 shows the increase in prevalence of HIV infected adults with time within Sub-Saharan Africa. South Africa (with a prevalence of 18 %) is the country with the largest number of infections in the world and Swaziland has the highest prevalence (26 %) of HIV infected adults in the world (UNAIDS, 2008b). Ninety five percent of newly infected children are babies born to HIV-positive women (Kamps and Hoffmann, 2007), despite a less than 2 % transmission rate if the mother is treated with anti-HIV medicine prior to birth (CDC, 2007a). There is still no cure for AIDS, but anti-HIV medicine can prolong and enhance the quality of life of an HIV infected person. In 2007, only 2.99 million (31 %) of the 9.7 million people who are in dire need of anti-HIV medicines received it (Avert, 2008c).

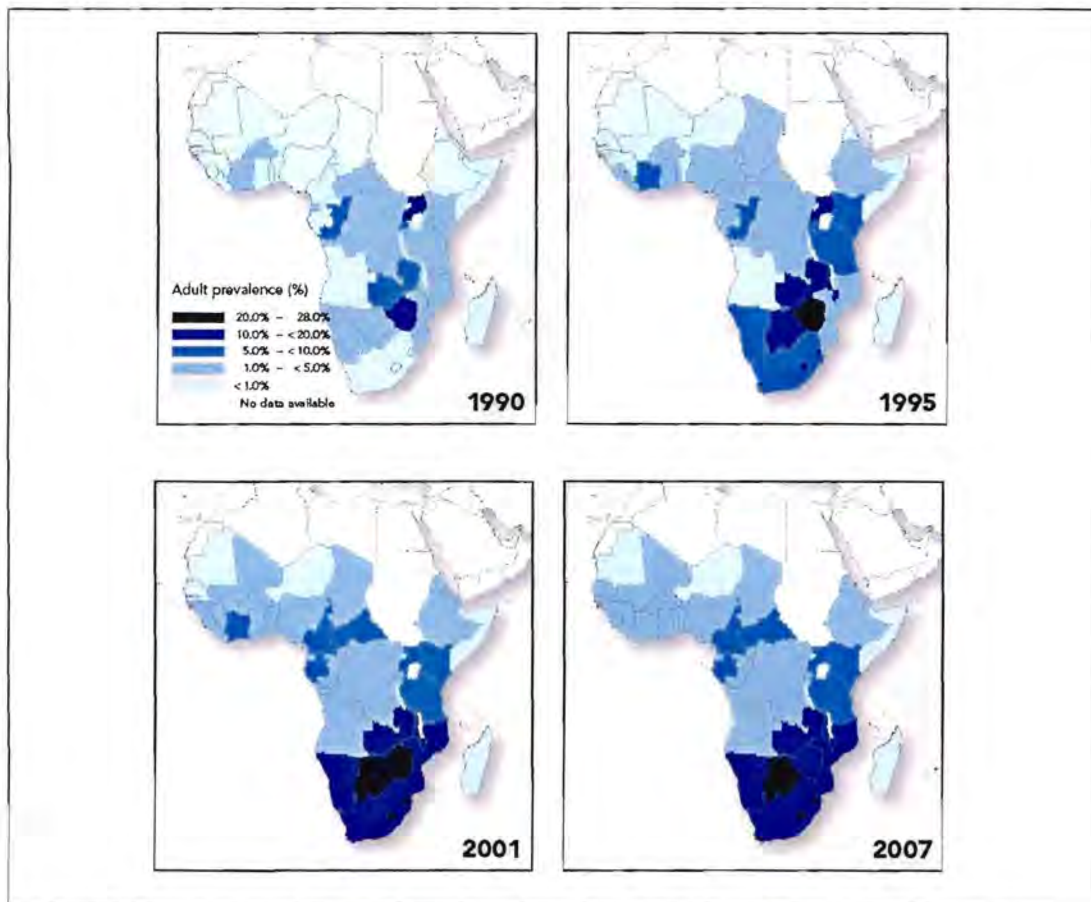



**Figure 1.2. Increased prevalence of HIV infected adults in Africa over time** (UNAIDS, 2008a).

## 1.2 The Human Immunodeficiency Virus

### 1.2.1 Structure and Genome

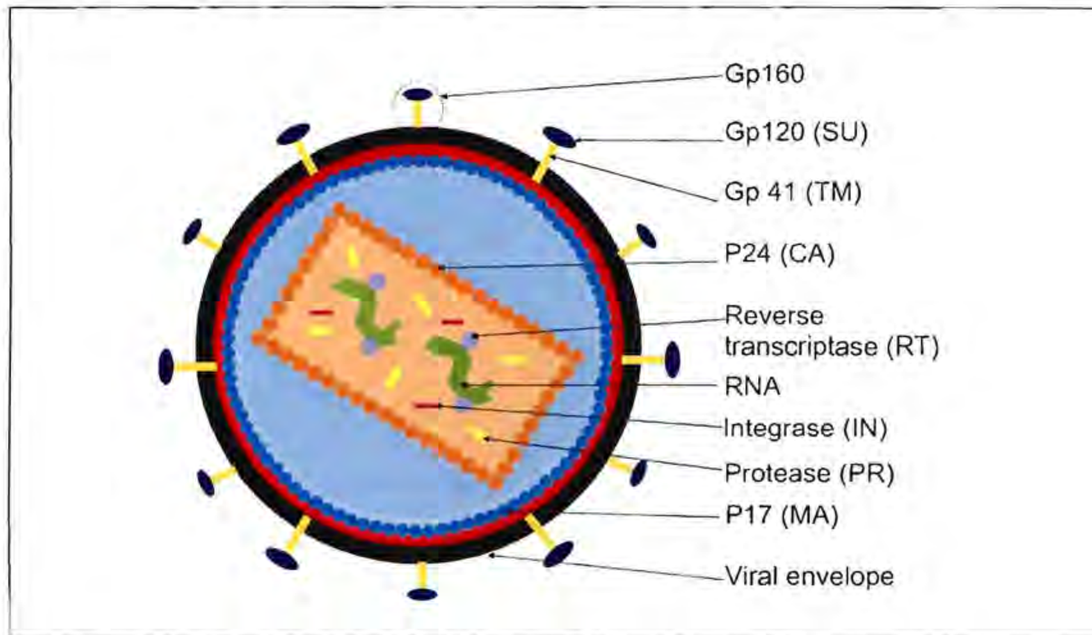



**Figure 1.3. Schematic structure of an HIV-1 virion** (adapted with permission from Costin, 2007).

HIV belongs to the retroviridae family and the genus lentivirus (Gallo and Montagnier, 2003). The genomes of lentiviruses are characterized by the structural genes *gag*, *pol*, and *env*. Like all viruses, HIV can not replicate on its own. For these functions, it hijacks the machinery of the human body (Requejo, 2006). Each virion contains two complete RNA genomic strands (Burke, 1997). The HIV particle (see Figure 1.2) is spherical and has a diameter of 120 nm. It is surrounded by a spiky viral envelope. The 72 spikes consists of glycoprotein (gp) 120 (size in kDa), a surface glycoprotein (SU) and a transmembrane glycoprotein (TM/gp41), which protrude the viral envelope to form the polyprotein gp160. SU's (gp120) main function is to recognize HIV's primary receptor $CD4^+$ and co-receptors (e.g. CCR5, CXCR4) on the different target cells. It also determines the viral tropism, which is the cell type the virus can infect (Chan *et al*, 1997). HIV mainly targets T-lymphocytes (T-tropic), macrophages (M-tropic) and dendritic cells (Clapham and McKnight, 2001). T-tropic viruses replicate rapidly and form syncytia, while M-tropic viruses are slow replicators and do not form syncytia readily (Björndal *et al.*, 1997). M-tropic viruses can be found in all stages of HIV-infection, including asymptomatic HIV-infected patients. T-tropic viruses predominate in people progressing to AIDS (Schuitemaker *et al.*, 1992). TM

(gp41) mediates fusion with the cellular membrane (Chan *et al.*, 1997). The matrix (MA or p17) anchors the viral envelope and glycoproteins and also mediates nuclear transport of the viral core (Kuiken *et al.*, 2008). The viral capsid consists of CA or p24. P24 antibodies form the basis of the HIV ELISA test (Higgins *et al.*, 1986).

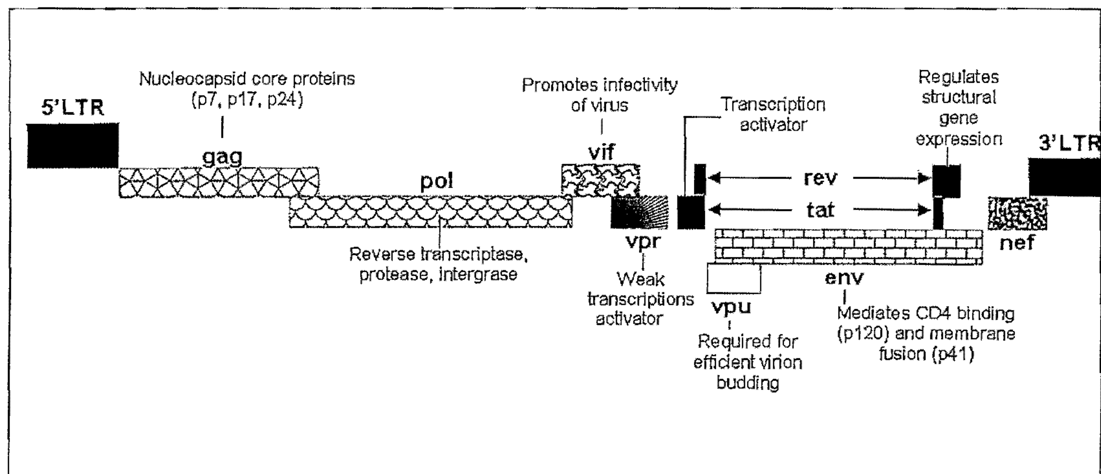



**Figure 1.4. The HIV genomes, their functions and the proteins they encode** (adapted with permission from Costin, 2007; Greene & Peterlin, 2002).

Each strand of HIV RNA contains an RNA sequence called the long terminal repeat (LTR). The LTR acts as a switchboard that controls the production of new viruses. The virus has just 9 genes (see Figure 1.4), of which only three are necessary for making new structural proteins. *Env* encodes the viral envelope (gp120 and gp41), *gag* encodes core proteins like p24, p17, p7 & p6; and *pol* is responsible for the enzymes: reverse transcriptase (RT), RNAse, integrase (IN) and protease (PR). The remaining 6 genes are *rev*, *tat*, *nef*, *vif*, *vpr* and *vpu*. They encode proteins that assist the virus with infection and production of new viruses. These genes are responsible for disease induction (Kuiken *et al.*, 2008). They can further be divided into two groups: *rev* and *tat* are regulatory genes, while *nef*, *vif*, *vpr* and *vpu* are accessory genes (Costin, 2007). Regulatory genes modulate transcriptional and post-transcriptional steps of viral gene expression and are responsible for viral propagation. The function of the accessory or auxiliary genes continues to be elucidated (Kuiken *et al.*, 2008).

### 1.2.2 Replication cycle

Features that are essential to the understanding of HIV replication are illustrated in Figure 1.5. Gp120, (1) uses the $CD4^+$ receptor and the chemokine co-receptor of the host cells as binding sites (Rang *et al.*, 2003). HIV has to bind to both a receptor ($CD4^+$) and a co-receptor. The main co-receptors used by subtypes A to E and G are CCR5 and CXCR4 (Bjőrndal *et al.*, 1997). More than a dozen other co-receptors have been identified *in vitro*, but do not seem to be important for *in vivo* infection (Clapham and McKnight, 2001). The viral glycoprotein-41 (gp41) is responsible for fusion of the virion with the cell membrane, which leads to the uncoating of the viral core (2) in the cytoplasm and the release of the RNA genome (Chan *et al.*, 1997). The viral RNA is reverse transcribed by the viral reverse transcriptase into DNA and transported to the nucleus (3). Within the nucleus, the viral DNA is integrated into the host DNA to form a provirus (4). During HIV-1 replication *tat*, *rev* and *nev* are the first genes to be transcribed (5), followed by the remaining 6 genes (Costin, 2007). After leaving the nucleus, the viral mRNA is translated into a viral protein (polypeptide) (6) that is then cut up by viral protease to form structural proteins and enzymes (7). A new virion is reconstructed and buds off at the plasma membrane (8).

The replication error rate of HIV is extremely high since HIV lacks enzymes for editing the freshly replicated nucleotide strands. The HIV-1 reverse trancriptase introduces point mutations, insertions and deletions during reverse transcription. HIV has a turnover of $10^{10}$ viral particles per day in an HIV-infected person (Quiňones-Mateu *et al.*, 2002). HIV averages one error per $10^4$ nucleotides, which is almost the size of its genome; this means that every provirus is a new mutant strain (Requejo, 2006). The advantages of evolution for the virus are to escape immune surveillance and to produce drug resistant variants (Zhuang *et al.*, 2002).

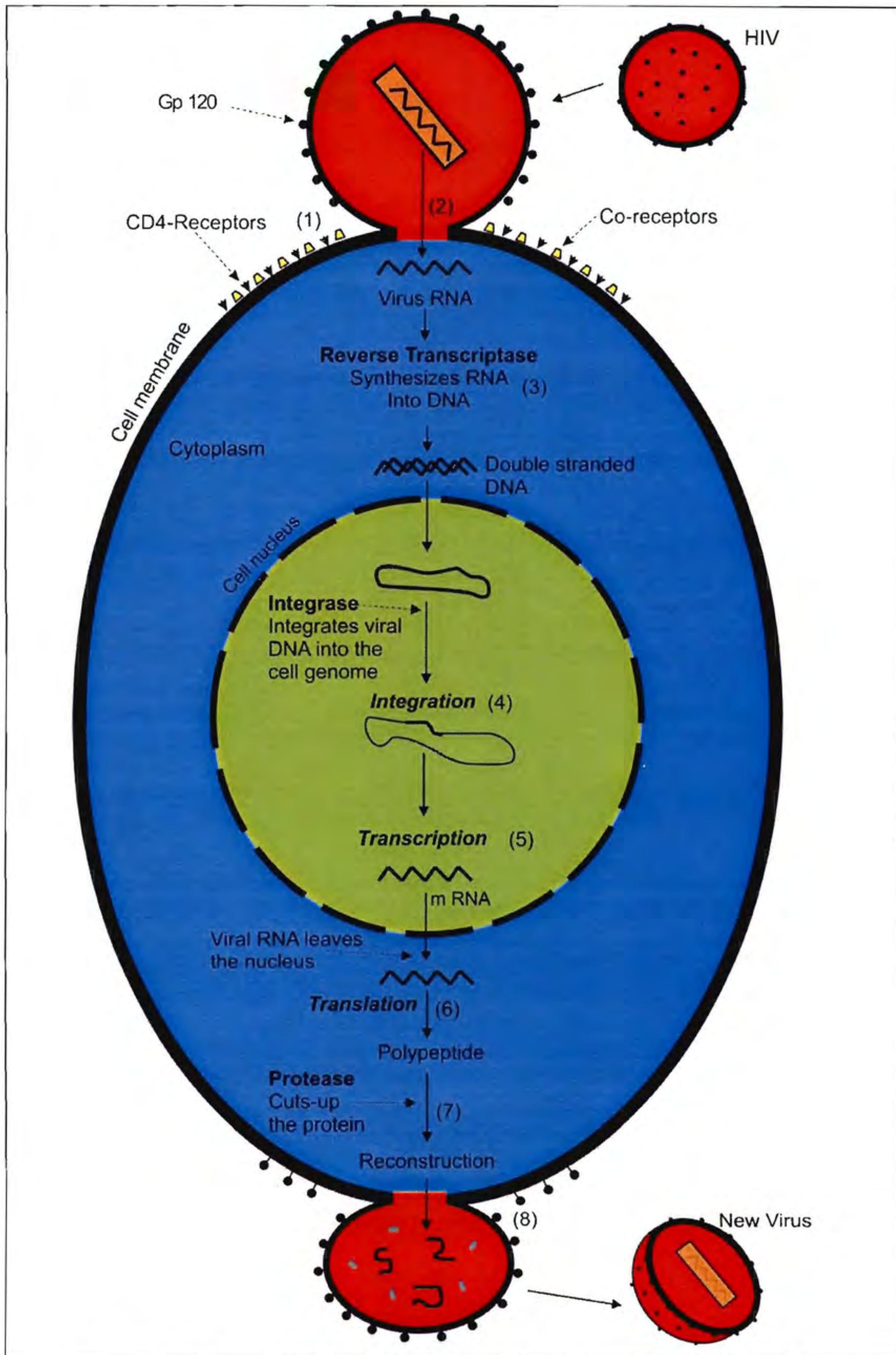



**Figure 1.5. Replication cycle of HIV in a T-cell** (adapted with permission from Costin, 2007 and Rang *et al.*, 2003).

### 1.2.3 HIV Subtypes and Recombination

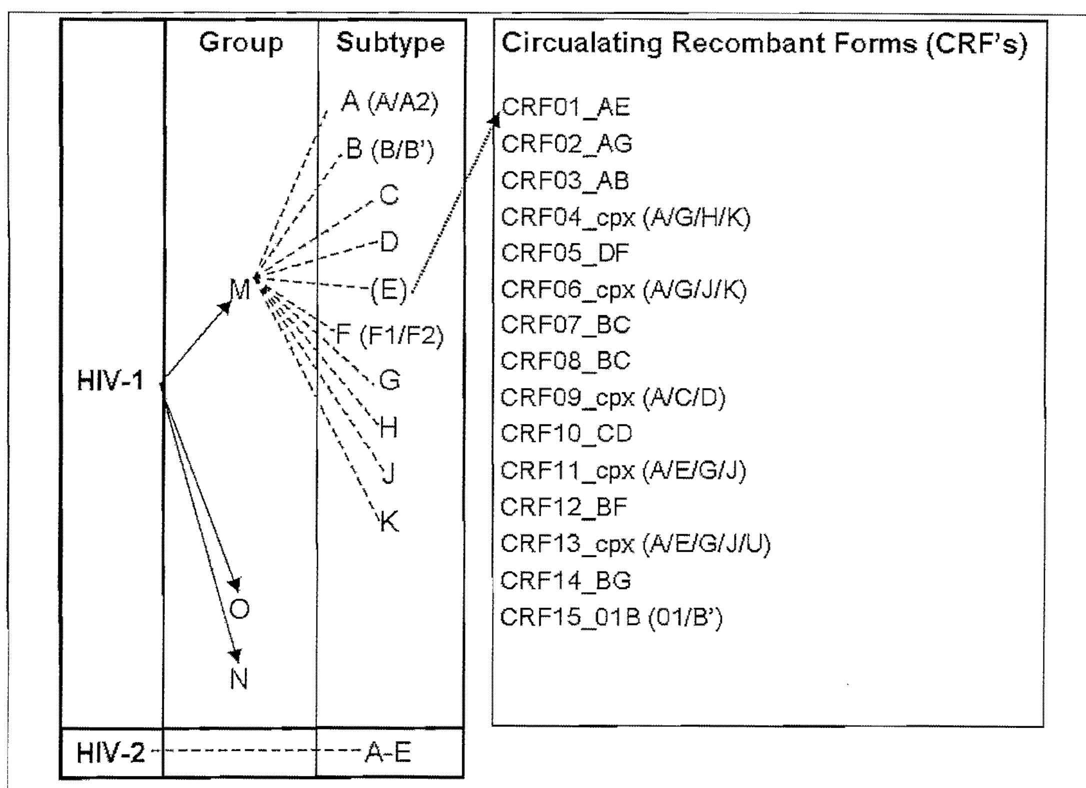



**Figure 1.6. Classification of HIV** (constructed from Requejo, 2006).

There are two types of HIV: HIV-1 and HIV-2. HIV-2 is uncommon and rarely found outside West and Central-Africa. HIV-1 can be subdivided into three groups: the "major" group M, the "outlier" group O and the "new" N group. The M group can further be divided into subtypes (clades) or circulating recombinant forms (CRF). The subtypes are: A, B, C, D, F, G, H, J and K. CRFs are recombinations of subtypes that are found in more than one person. Recombination is considered a characteristic feature of retroviruses (Quiñones-Mateu *et al.*, 2002). It takes place in an individual when one cell is co-infected with two different proviruses and form new virions with one RNA transcript from each provirus (Burke, 1997). Subtypes E and I were later found to be recombination of other subtypes. Figure 1.6 is a schematic representation of the classification of HIV. Figure 1.7 represents an overview of the distribution of HIV subtypes around the world, but does not report the full details of the different subtypes in each demographic area. Subtype C is responsible for 60 % of the HIV infections worldwide (Requejo, 2006).

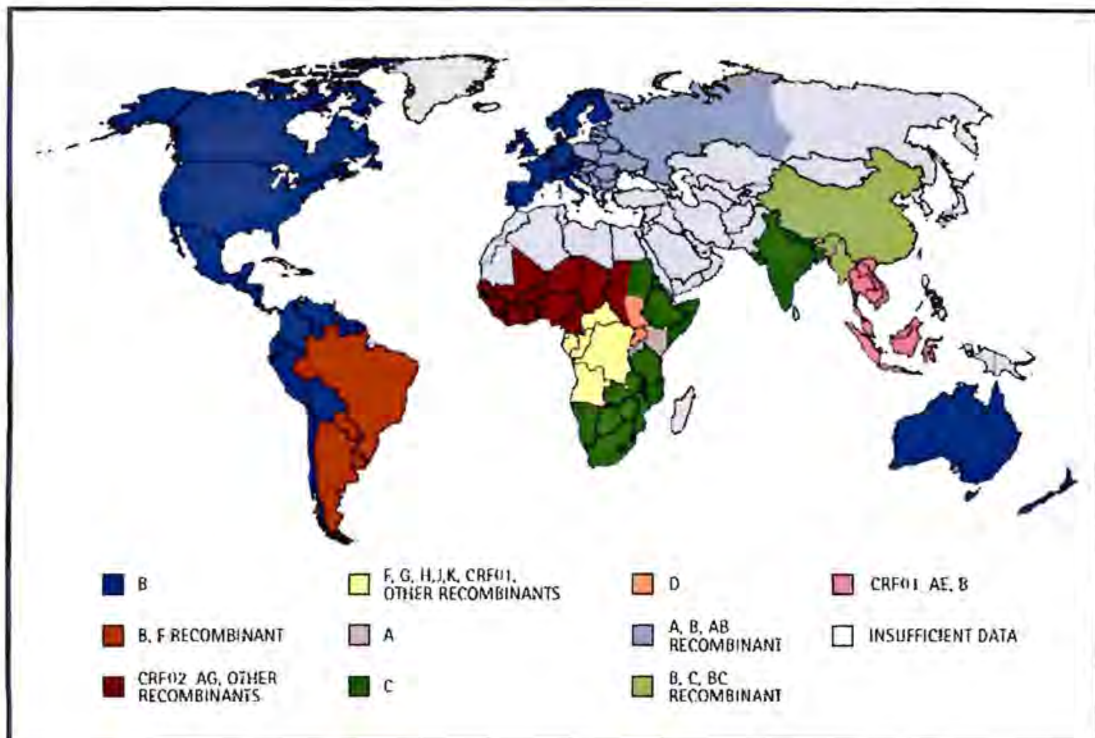



**Figure 1.7. Global distribution of the HIV-1 subtypes** (McCutchan, 2003).

## 1.3 Clinical disease

HIV infection can be categorised into three stages: acute infection, the latency stage and AIDS. An untreated person's stage of infection can be derived from measuring the $CD4^+$ cells and viral count in his/her blood. After infection, the incubation period lasts for two to four weeks during which the person may develop non-specific flu-like symptoms. At around three months after infection the acute infection stage occurs - this is a month-long period during which the virus is abundant in the person's blood. This causes a decrease in the $CD4^+$ cell count and is synonymous with fever, lymphadenopathy, myalgia and malaise. After acute infection, the viral level in the blood plummets to give rise to the latency stage, which is known for its absence of symptoms. The latency stages lasts for an average of ten years, during which time the viral load is low but starts rising eventually when the virus starts oppressing the immune system. AIDS is defined as the stage when the $CD4^+$ cell count is below 200 cells/mm$^3$ and the person has developed an opportunistic illness. HIV/AIDS is not directly responsible for the high morbidity and mortality rates – it is the result of

opportunistic infections, 90 % of which are caused by organisms that are common in one's environment (Wells *et al.*, 2003).

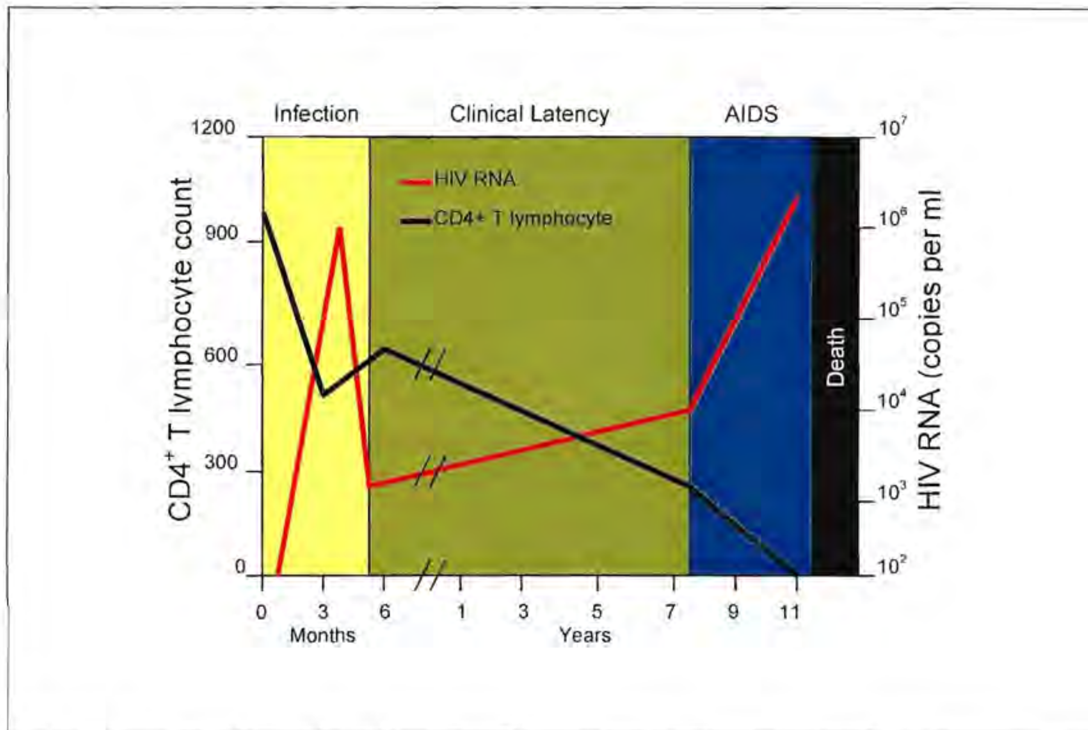



**Figure 1.8. Generalized relationships between HIV copies (viral load) and CD4 counts of an untreated individual** (adapted with permission from Costin, 2007).

### 1.3.1 Disease progression

The WHO (2005b) has categorized HIV infection into different clinical stages. The clinical stage classification below is useful when there is no access to laboratories to define $CD4^+$-levels.

- **Primary HIV infection, incubation time.** Shortly after infection some people will show little or no symptoms or signs, but it is a common occurrence for the person to develop flu-like symptoms, also called non-specific symptoms of infection. These signs and symptoms include fever, headache, a sore throat, rash and swollen lymph glands.

- **Clinical stage 1 (latency phase).** An HIV infected individual may live for more than eight years without any symptom, but as the virus multiplies and starts

oppressing the immune system, some symptoms develop. Swollen lymph nodes are one of the first signs of HIV infection.

- **Clinical stage 2.** Unexplained weight loss, recurrent respiratory tract infections, oral ulcerations, fungal nail infections and *Herpes zoster* are common symptoms of a developed HIV infection.

- **Clinical stage 3.** On average, ten years after infection the virus would have severely damaged the person's immune system, which would make this person very susceptible to opportunistic infections that would not have otherwise plagued the body. During stage three and four, defined clinical signs or simple investigations can be used as a presumptive diagnosis. The signs and investigations defining clinical stage 3 are: severe weight loss (> 10 % of body weight), chronic diarrhoea (unexplained and longer than one month), persistent fever, oral candidiasis and hairy lekoplakia, severe bacterial infections, diagnosed pulmonary tuberculosis (TB) in the past two years. Three quarters of HIV-infected persons are also infected with *Mycobacterium tuberculosis*. TB is responsible for almost half of the deaths of HIV-infected persons (CDC, 2008).

- **Clinical stage 4.** Defined clinical signs and investigations of stage 4: depletion of body cell mass, known as wasting syndrome (Kotler *et al.,* 1989), *Pneumocystis* pneumonia or recurrent bacterial pneumonia, chronic *Herpes simplex* infection, oesophageal *candidiases*, Karposi's sarcoma (skin tumour) and HIV encephalopathy. The most common opportunistic infections during 1990-1994 was *Pneumocystis carinii* pneumonia which occurred in 45 % of all the AIDS patients. It is followed by *Mycobacterium avium* complex, 25 %; wasting syndrome, 25 %; bacterial pneumonia, 24 %; cytomegalovirus disease, 23 %; and candidiasis, 22 % (Wells *et al.*, 2003).

CD4 testing is a useful tool to determine the degree of immunosuppression by HIV. Table 1.1 gives a summary of the CD4 levels used in CD4 testing and how it relates to immunosuppression.

**Table 1.1. Severity of immunosuppression in relation to CD4 levels** (adapted from WHO, 2005b)

| **Severity of immunosuppression** | **CD4 level** |
|---|---|
| Non-significant immunosuppression | > 500 CD4/$mm^3$ |
| Mild immunosuppression | 350 – 499 CD4/$mm^3$ |
| Advanced immunosuppression | 200 – 349 CD4/$mm^3$ |
| Severe immunosuppression | < 200 CD4/$mm^3$ |

### 1.3.2 Children with HIV/AIDS

Children have higher baseline viral loads and metabolize anti-HIV medication faster than adults. If left untreated, 20-30% of them will develop an AIDS-defining illness when one year old, and will die before age 2-3 (McFarland, 2005). Fifty percent of untreated HIV-infected children will die before the age of five (WHO, 2005a). New born babies who contracted HIV from the mother rarely demonstrate the non-specific symptoms that a child or an adult would after infection with HIV. The physical signs include lymphadenopathy, hepatomegaly, splenomegaly. The clinical signs of paediatrics infected with HIV correlate with the clinical stages of HIV-infected adults. Delayed growth can be seen as soon as four months after birth in some infants (McFarland, 2005). These infants have difficulty gaining weight and may present with delayed mental development. They present with diarrhoea, fevers and sweats of unknown origin, and severe opportunistic infections (Wells *et al.*, 2003).

## 1.4 Antiretroviral therapy (ART)

The following section will focus on the classification, mechanism and side effects of antiretroviral drugs.

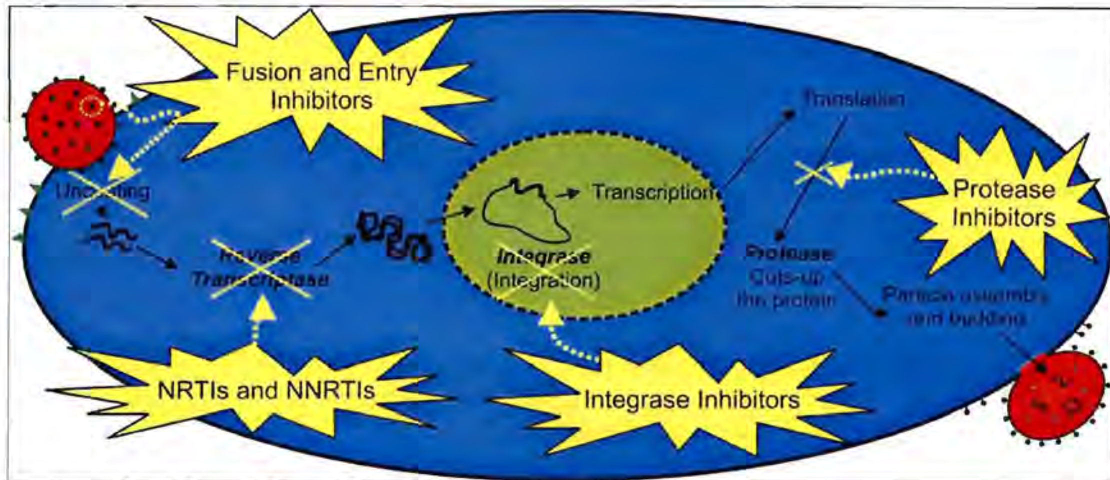



**Fig 1.9. Overview of HIV replication cycle and ART interventions** (adapted from Costin, 2007 with permission, Safrin, 2004; and Rang *et al.*, 2003).

There are currently four steps within the HIV replication cycle where ART can intervene. HIV needs both a receptor (CD4) and a co-receptor to bind to a cell (see section 1.2.2 for a more detailed explanation of the replication cycle of HIV). HIV's tropism, recognition of CD4 receptor and use of co-receptors (CCR5 and/or CXCR4) are dependant on gp120, a surface glycoprotein. Fusion of HIV with the cell membrane is mediated by gp41, a viral transmembrane glycoprotein. The first intervention step focuses on the prevention of entry and fusion of the virion with the cell (see section 1.4.5). Uncoating of the virion can not take place if fusion is repressed. Maraviroc, an entry inhibitor, inhibits the binding of HIV's gp120 to co-receptor CCR5. Enfuvirtide, a fusion inhibitor, binds to the gp41 subunit on the viral envelope, which prevents the conformational changes needed for fusion. The second intervention step takes place when uncoated viral RNA is transcribed into DNA by the enzyme reverse transcriptase. Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and Nucleotide Reverse Transcriptase Inhibitors like tenofovir act as false substrates for viral reverse transcriptase; this leads to the formation and termination of defective DNA strands (see section 1.4.1 and 1.4.2). Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) bind directly onto the reverse transcriptase enzyme itself; this prevents the enzyme from converting RNA to DNA (see section 1.4.3). DNA is then transported to the nucleus where it is integrated by

an integrase enzyme into the host's DNA to form a provirus. The integration step functions as the third step for ART intervention. Raltegravir, an integrase inhibitor or strand transfer inhibitor, inhibits the integration of the reverse transcribed DNA into the host's DNA (see section 1.4.5). The integrated DNA is then transcribed and translated into viral protein. This polypeptide is cut up by the enzyme protease to yield the structural proteins and enzymes needed for a new virion. Protease inhibitors (PIs) prevent the protease enzyme from cleaving the polypeptide by binding to the site where cleavage occurs (see section 1.4.4). This leads to the formation of immature and non-infectious virions.

### 1.4.1 Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

In order to understand the mechanism of the NRTIs, one must have a basic understanding of the building blocks of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). RNA is a polymer of ribonucleotides. DNA is a polymer made up from deoxyribonucleotides (Garret and Grisham, 1997). There are 4 nitrogenous bases in a DNA strand; adenine (A), cytosine (C), guanine (G) and thymine (T). Within RNA the bases are the same except that thymine is replaced by uracil. Nucleosides are named by adding *–idine* or *–osine* to the bases' name. This makes the nucleosides cytidine, uridine, thymidine, adenosine and guanosine (Garret and Grisham, 1997; Berg *et al.*, 2006).

NRTIs are all pro-drugs; they act as false substrates for viral reverse transcriptase to form defective viral DNA, which leads to chain termination (Rang *et al.*, 2003; Safrin, 2004). Chain termination is induced since bond formation can not occur when NRTIs are incorporated into the growing viral RNA strand. This happens since they have an $-N_3$ group attached to the 3' carbon chain, instead of an OH-group like thymine (Greenstein and Greenstein, 2007).

#### 1.4.1.1 Zidovudine (AZT)

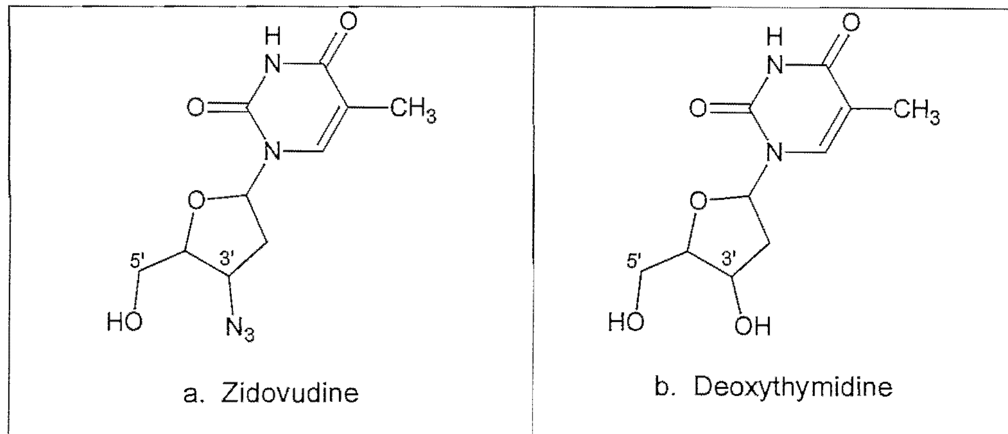



**Figure 1.10. Chemical structures of zidovudine and its nucleoside analogue, deoxythymidine** (adapted from Safrin, 2004).

AZT was the first licensed antiretroviral agent in 1987. It is a structural analogue of deoxythymidine (see figure 1.10 a & b), (Safrin, 2004). AZT can be used for treatment of HIV-1 infection in persons of all ages, including pregnant women where vertical HIV-1 transmission from mother to child is reduced (Safrin, 2004). Bioavailability (65%) is not influenced by food. AZT permeates the cerebrospinal fluid (CSF). The concentration of AZT within the CSF is approximately 65% of the concentration within the blood plasma (Gibbon, 2005). High level resistance to AZT can develop when three or more mutations develop (Safrin, 2004). AZT causes myelosuppression, which can be seen as anaemia or neutropenia. Taking stavudine or other myelosuppressive drugs (like ganciclovir, cotrimoxazole, dapsone and amphotericin B) in combination with AZT will worsen the myelosuppression. The most common side effects of taking AZT are anaemia, nausea and vomiting, abdominal discomfort and headache. Neutropenia and lactic acidosis are rare (Kamps and Hoffmann, 2007).

#### 1.4.1.2 Abacavir (ABC)

Abacavir is a guanosine analogue (Rang *et al.*, 2003). It is well absorbed after oral administration (80%) and food does not influence the uptake of the drug. The concentration within the CSF is about one third of the concentration in plasma. High level resistance to ABC tends to develop slowly since a minimum of two related mutations are needed (Safrin, 2004). Abacavir is generally well tolerated, but a life

threatening hypersensitivity reaction occurs in 2 to 8% of people treated with ABC. This reaction usually starts within the first six weeks after treatment commenced. The hypersensitivity reaction is characterized initially by a fever but malaise may develop. If a person has shown sensitivity towards ABC, the treatment should be discontinued. Re-exposure to ABC can be fatal (McNicholl, 2007).

#### 1.4.1.3 Didanosine (ddI)

Didanosine, a synthetic deoxyadenosine analogue (Safrin, 2004), is only used in emergencies for certain resistance situations because of its severe side effects (Kamps and Hoffmann, 2007). The intake of food one hour before or two hours after taking ddI or a low gastric pH can decrease the oral bioavailability. This leads to adherence and efficacy problems when taking the drug (Coffey and Peiperl, 2006a). CSF permeation is not good and only 20% of the plasma concentration can be found in the CSF (Gibbon, 2005). Pancreatitis, peripheral neuropathy, vomiting and diarrhoea are common (McNicholl, 2007). Taking stavudine with ddI is contra-indicated since this aggravates their side-effects. Pregnant women are also contra-indicated for ddI treatment because it causes lactic acidosis with pancreatitis or steatosis.

#### 1.4.1.4 Lamivudine (3TC)

Lamivudine is an analogue of cytosine (Rang *et al.*, 2003). Oral bioavailability is 83% and is not influenced by food consumption. 3TC permeates the CSF. High level resistance can develop rapidly since only one point mutation is needed. Resistance to 3TC also reduces susceptibility to ABC, ddI and zalcitabine (Safrin, 2004). Side effects are rare when 3TC is taken as an individual drug. Fatigue, nausea, vomiting, diarrhoea, headache and insomnia may present itself. Pancreatitis, lactic acidosis and anaemia are extremely rare (Kamps and Hoffmann, 2007).

**Stavudine (d4T)**

Stavudine is a thymidine analogue. Long-term treatment with d4T is no longer advised because of d4T's severe mitochondrial toxicity, which presents as lipoathrophy (loss of fat tissue), lactic acidosis and peripheral neuropathy. d4T causes more mitochondrial toxicity than any other NRTI. This can be aggravated by the use of ddI in combination with d4T (Kamps and Hoffmann, 2007). Another problem with d4T is that it is not stable in solution; degradation of 60% within a week at 37°C was seen by Kühn & Van der Merwe (2007) using a commercial product on the market (the poster is attached as annexure A.1).

#### 1.4.1.5 Emtricitabine (FTC)

Emtricitabine is a cytidine analogue. It is comparable to d4T both biochemically and to its resistance profile, but has a longer half-life than d4T. It is generally a well tolerated drug, but sometimes headache, nausea, diarrhoea, rash or hyper-pigmentation may occur (Kamps and Hoffmann, 2007).

#### 1.4.1.6 Zalcitabine (ddC)

Distribution of ddC was stopped in 2006, due to moderate efficacy, complicated dosing and problems with cross-resistance (Kamps and Hoffmann, 2007).

### 1.4.2 Nucleotide inhibitors

#### 1.4.2.1 Tenofovir (TNF)

Tenofovir is an analogue of adenosine. It is administered as its prodrug tenofovir disoproxilfumarate (TDF) which is converted *in vivo* to the active tenofovir (TNF). Oral bioavailability is poor if taken on an empty stomach; to enhance the bioavailability TNF has to be taken with a high-fat meal (Safrin, 2004). TNF can be taken once a day. Fixed dose tablets are available for TNF in combination with FTC or in combination with FTC and efavirenz (Coffey and Peiperl, 2006e). Cross resistance to 3TC and ABC has been shown to diminish the virologal response of TNF. Adverse effects of treatment are gastrointestinal-related such as nausea, diarrhoea and flatulence (Safrin, 2004).

### 1.4.3 Non-Nucleoside reverse transcriptase inhibitors (NNRTI)

These drugs bind directly to a binding site on reverse transcriptase, inhibiting the enzyme from converting RNA to DNA. This binding site of the NNRTIs is close to the binding site of the NRTIs but not distinct from that site. The NNRTIs are not pro-drugs like the NRTIs (Safrin, 2004).

NNRTIs can be inducers, substrates or inhibitors of the cytochrome P450 liver enzyme to a varying degree (Rang *et al.*, 2003). High level resistance can develop easily; therefore it has to be used in combination with drugs of the other classes. Cross-resistance between the NNRTIs occurs (Gibbon, 2005).

#### 1.4.3.1 Delavirdine (DLV)

Delavirdine is rarely used and not licensed in Europe because of adherence problems caused by its dosing requirements and drug interactions. Delavirdine has to be taken four times a day (Kamps and Hoffmann, 2007).

#### 1.4.3.2 Efavirenz

Efavirenz has a very long half-life of 40-55 hours, which makes a once daily dosing possible. Absorption after oral administration is moderate (45%), but bioavailability can be increased by taking a fatty meal prior to administration. CSF permeation (0.3% – 1.2%) is almost three times higher than the percentage free drug in the blood since it binds almost completely (99%) to the plasma proteins (Safrin, 2004). The most common adverse effects involve the central nervous system (CNS). Side effects present as dizziness, drowsiness, insomnia, headache, delusions, nightmares, depression and euphoria. The appearance of a mild rash is also possible during the first weeks. Both the CNS effects and the rash resolve with time. Other adverse effects include elevated liver functions, dyslipidemia and occasionally painful gynecomastia. Efavirenz is a substrate, an inhibitor and a moderate inducer of CYP3A4 (Safrin, 2004). This means that it induces its own metabolism and accelerates the metabolism of the protease inhibitors but inhibits the metabolism of other medicines like cisapride and benzodiazepines. The high percentage of plasma protein binding and the severe CNS side effects lead to a lot of drug interactions and adherence problems (Kamps and Hoffmann, 2007). The use of efavirenz is contra-

indicated for pregnant women or women of child bearing age because of its potential teratogenic effects. Efavirenz is not approved for use in children under the age of three. Resistance to efavirenz is associated with resistance to delavirdine and nevirapine (Coffey and Peiperl, 2007b).

#### 1.4.3.3 Nevirapine

Oral bioavailability is excellent (> 90%) and not food dependent after oral administration. Nevirapine permeates the CSF; about 45% of the concentration in the plasma can be found in the CSF. Nevirapine can be given to pregnant women to prevent transmission of HIV from mother to child. The FDA advised that nevirapine should not be given to healthy patients with a good immune status, due to the increased risk of hepatotoxicity. Hepatotoxicity and a life threatening skin rash are the severe side-effects of nevirapine and both present itself during the first few weeks of treatment. Frequent liver function tests are necessary to detect the hepatotoxicity and treatment should be stopped in case of a severe rash. Nevirapine is both a substrate and inducer of CYP3A which leads to problematic drug interactions (Safrin, 2004).

### 1.4.4 Protease Inhibitors (PIs)

When the mRNA leaves the nucleus of the host cells, it is translated to form biochemically inert polypeptides (see Figure 1.5, step 6 and 7 of the replication cycle of HIV). These polypeptides are cleaved into the various structural and functional proteins at the appropriate positions by the protease enzyme. The cleaved proteins can then be packaged to form the new virion core. The PIs are HIV-specific; they bind only to the site where cleavage occurs. By preventing cleavage, they result in the production of immature, non-infectious virions (Rang *et al.*, 2003 and Safrin, 2004).

Resistance occurs readily when these drugs are used in monotherapy and predicting cross-resistance between the PIs is intricate. The PIs are substrates of the isoenzyme CYP3A4. Some (amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir) are inhibitors of CYP3A4, while ritonavir is a CYP3A4 inducer as well. This leads to a lot of drug interactions (Rang *et al.*, 2003 and Safrin, 2004).

#### 1.4.4.1 Indinavir

Indinavir was one of the first PIs, but is rarely used today because of its side-effects, especially skin and renal problems. Treatment can be boosted with ritonavir to produce a twice daily dosing. Without ritonavir boosting, indinavir has to be taken three times a day (Kamps and Hoffmann, 2007). Absorption can be optimized by taking indinavir on an empty stomach. Oral bioavailability is about 65% and indinavir has the highest CSF permeation of all the PIs. Resistance to indinavir is associated with multiple mutations. Cross-resistance to the other PIs is less predictable (Safrin, 2004). A specific mutation is associated with cross-resistance to a specific other PI (Coffey & Peiperl, 2006b). Indinavir has interactions with other antiretroviral medications like the NNRTIs and the other PIs. Adequate hydration (at least 1.5 litres a day) is very important when treated with indinavir to prevent the crystallization of the drug, which leads to nephrolithiasis (Coffey and Peiperl, 2006b), better known as kidney stones. Nephrolithiasis and indirect hyper-bilirubinemia are common side-effects of treatment, while tromobocytopenia, nausea, diarrhoea and irritability are rare (Safrin, 2004).

#### 1.4.4.2 Amprenavir/Fosamprenavir

Production and sale of amprenavir was discontinued in 2007 by GlaxoSmithKline (Coffey and Peiperl, 2007a), but oral formulations are still available (Kamps and Hoffmann, 2007). Amprenavir was replaced by fosamprenavir, a prodrug of amprenavir which is more soluble and better absorbed. Amprenavir is rapidly absorbed after oral administration and can be taken with or without food, although fatty foods decrease the absorption (Safrin, 2004). The side effects of amprenavir are rash, headache, diarrhoea, nausea and vomiting. Fosamprenavir have fewer gastrointestinal side effects than amprenavir, but may increase triglycerides in the blood. Oral formulations of amprenavir contain propylene glycol, which is contraindicated for young children, pregnant women and people using metronidazole or disulfiram (Coffey and Peiperl, 2007a). Fosamprenavir is a sulfa drug; people who are allergic to sulfa drugs should avoid taking this drug. Drug interactions can be potentially fatal (Kamps & Hoffmann, 2007).

#### 1.4.4.3 Atazanavir

Atazanavir is indicated for treatment-experienced adults with therapy failure. The drug is taken once a day in combination with ritonavir, and has to be taken with meals. Frequent side effects are hyperbilirubinemia with jaundice, diarrhoea, nausea and rash. High level resistance develops with the accumulation of five or more key mutations (Coffey, 2008a). Atazanavir does not cause dyslipidemia like the other PIs (Kamps and Hoffmann, 2007). Interactions with other medicines frequently occur and can be fatal.

#### 1.4.4.4 Lopinavir

This drug is used for the treatment of treatment-naïve and treatment-experienced patients usually in combination with ritonavir as a booster. Ritonavir inhibits the metabolism of lopinavir, thus increasing its plasma concentration (Gibbon, 2005). The bioavailability can be enhanced by taking food with administration of the drug. Lopinavir is metabolized by CYP3A isozyme and hepatic cytochrome P450. Because of this, drug interactions have to be observed. Diarrhoea is a frequent adverse effect of lopinavir treatment. Lopinavir causes the worst dyslipidemia of all the PIs.

#### 1.4.4.5 Ritonavir

Ritonavir has a good bioavailability of 75 %, which can still be increased when taken with food. In contrast to the other PIs, this is a CYP3A4 substrate (Safrin, 2004), a potent inhibitor and also a slight inducer; this makes ritonavir an ideal drug to boost the plasma levels of the other PIs (Gibbon, 2005). This means that ritonavir at low doses can boost the performance of another drug to reduce the amount of drug needed and the dosing frequency (Coffey and Peiperl, 2006c). Patients should be advised to expect nausea, vomiting and abdominal pain during the first few weeks of treatment. This drug also causes altered taste and hypertriglyceridemia (Safrin, 2004).

#### 1.4.4.6 Nelfinavir

Nelfinavir can be used for previously untreated and treatment-experienced patients (Coffey and Peiperl, 2008b). It is important to take food with nelfinavir to increase

absorption. It is an inhibitor of CYP3A. The most frequent side-effects of treatment are diarrhoea and flatulence (Safrin, 2004).

#### 1.4.4.7 Saquinavir

Saquinavir is poorly absorbed and has to be taken within two hours after a fatty meal to improve its bioavailability. It should be used in combination with a low dose of ritonavir as a booster (Coffey and Peiperl, 2006d). Permeation of the CSF by saquinavir is insignificant. This drug is a CYP3A4 substrate and inhibitor; drug interactions with saquinavir are possible (Safrin, 2004). The adverse effects of saquinavir treatment are diarrhoea, abdominal discomfort and dyslipidemia, but the drug is often well tolerated (Coffey and Peiperl, 2006d).

### 1.4.5 Fusion and entry inhibitors

Fusion inhibitors block the entry of the HIV virion into the cell. It binds to the gp41 subunit of the viral envelope, which prevents the conformational changes needed for fusion of the virion with the cell's membranes (Safrin, 2004).

#### 1.4.5.1 Enfuvirtide (T-20)

This drug is used as a salvage drug for treatment-experienced patients with a history of viral replication despite ongoing highly active antiretroviral therapy (HAART) treatment. This drug has to be injected subcutaneously twice a day, which causes a local skin irritation, but otherwise it is well tolerated (Kamps and Hoffmann, 2007).

#### 1.4.5.2 Maraviroc (MVC)

MVC is the first selective CCR5-antagonist. It inhibits the binding of HIV-1's gp120 to the CCR5 co-receptor, thus preventing fusion with the cell (Dorr, 2005). The receptor tropism of the HIV-infected person should be determined prior to treatment since MVC only inhibits R5-trope viruses. MVC is indicated for treatment experienced adult patients with CCR5-trope HIV strains. Treatment with MVC has shown excellent tolerability; headaches, dizziness, loss of appetite and muscle pains were rarely experienced (Kamps and Hoffmann, 2007).

### 1.4.6 Integrase inhibitor

An integrase inhibitor is also know as a strand transfer inhibitor. Integrase is an enzyme which is essential for the integration of HIV DNA into the genome of the host cell. This enzyme is HIV specific since humans do not posses this enzyme (Kassahun, 2007).

#### 1.4.6.1 Raltegravir (RGV or MK-0518)

The United States food and drug administration (FDA) approved RGV for anti-HIV treatment in October 2007 (Coffey, 2007c). RGV is indicated for treatment-naïve or HIV-infected persons with resistance to at least one of the drug classes: NRTIs, NNRTIs or PIs (Grinsztejn, 2007). Treatment with RGV is a well tolerated; nausea, dizziness and headaches were not commonly experienced (Kamps and Hoffmann, 2007).

### 1.4.7 Current regimes

The WHO's clinical stages can be used to determine when to initiate ART. Treatment is definite for a person who is at clinical stage four of HIV-infection. Treatment during stage three, two and one will only be initiated when an HIV-infected persons $CD4^+ < 200$ $CD4^+/mm^3$. Since virological testing is not possible in children under 18 months, there are a number of characteristics that can be used to identify stage four in these children. To presume an HIV-infected child is at stage four, the child has to test positive for HIV-antibodies and must be symptomatic for two or more of the following problems: oral thrush, severe pneumonia, severe wasting/malnutrition or severe sepsis WHO, 2005b). HAART is a combination of three or more ARTs. It is usually comprised of two NRTIs or two PIs together with an NNRTI. Monotherapy with a drug is not advised, since optimal viral suppression can not be achieved and resistance develops rapidly. First line therapy is the initial ART combination a person receives. The choice of ARTs for a first-line regime should be well thought through. Adherence, tolerability and convenience are some of the factors that have to be taken into account when deciding on an ART regime (Safrin, 2004). Despite these factors, the drugs or classes that remain after taking the first-line regime should be taken into consideration, since a second-line regime should consist of at least two new drugs that have no cross-resistance to the previous drugs,

another class of drugs should be introduced. The patient may discontinue treatment due to resistance, non-adherence or intolerance to adverse effects; when this happens, a second line regime has to be given (Wells *et al.*, 2003).

**Table 1.2. South African National Department of Health regimes for antiretroviral therapy** (Gibbon, 2005).

| Regime | Drugs | Indications (WHO stage 4 or CD4 < 200 cells/mm$^3$) |
|---|---|---|
| **Regime 1a** | Stavudine + lamivudine + efavirenz | HIV-infected persons over the age of three years old, who are not of childbearing potential or who are using injectable contraception. |
| **Regime 1b** | Stavudine + lamivudine + nevirapine | HIV-infected persons of all ages, including infants and women who are unable to guarantee reliable contraception. |
| **Regime 2** | Zidovudine + didanosine + lopinaivr-ritonavir | Second-line regime for patients who failed the first-line regime, despite good adherence to treatment. |

Zidovudine in monotherapy (300 mg, twice daily) or a combination of zidovudine and lamivudine is given to an HIV-infected mother for the last twelve weeks of pregnancy to prevent mother to child transmission (MTCT). The baby should receive zidovudine (2mg/kg, six hourly) after birth. Nevirapine in a single dose can also be given to the mother within 72 hours of birth and the baby should also receive nevirapine (2 mg/kg) within 72 hours after birth. Efavirenz is prohibited during pregnancy since it induces teratogenicity (Gibbon, 2005).

**Table 1.3. Summary of the antiretroviral drugs** (Drugbank, 2008; Kamps and Hoffmann, 2007; Rang *et al.*, 2003; Safrin, 2003; Gibbon, 2005).

| **Nucleoside Reverse Transcriptase Inhibitors (NRTIs)** | | | | | |
|---|---|---|---|---|---|
| **Drug** | **Metabolism** | **Excretion** | **Half life** | **Protein binding** | **Predicted solubility in water** |
| **Abacavir** | Liver | Urine 82%<br>Faeces 16% | 1.5 hours | 50% | 77 mg/ml |
| **Lamivudine** | Minimal | 70% of dose is excreted unchanged in urine | 5-7 hours | 36% | 70 mg/ml |
| **Zidovudine** | Liver | Metabolites excreted renally, 65-75% | 1 hour | 30% | 16.3 mg/ml |
| **Stavudine** | Intracellularly to active triphosphate | Forty percent of dose is excreted in the urine | 1-1.5 hours | - | 40 mg/ml |
| **Didanosine** | Metabolized intracellularly to active form and then in liver to metabolites | Excreted by kidneys and urine | 30 minutes in plasma, > 12 hours in intracellular environment | Low, less than 5% | 6.58 mg/ml |
| **Emtricitabine** | Minimal | 86% of dose excreted unchanged in urine | 10 hours | Low, less than 4% | 2 mg/ml |

| **Nucleoside Reverse Transcriptase Inhibitors (NRTIs)** | | | | | |
|---|---|---|---|---|---|
| **Drug** | **Metabolism** | **Excretion** | **Half life** | **Protein binding** | **Predicted solubility in water** |
| **Efavirenz** | Liver | Faeces 16-61%<br>Urine 14-34% | 40-55 hours | 99% | Not soluble in water,<br>Log -4.57 g/ml |
| **Delavirdine** | Liver | | 5.8 hours | 98% | Not soluble in water,<br>8.6 e-02 mg/ml |
| **Nevirapine** | Liver | Metabolites are excreted renally | 45 hours | 60% | Not soluble in water,<br>1.05e-01 mg/ml |
| **Nucleotide Reverse Transcriptase Inhibitors** | | | | | |
| **Tenofovir** | Possible hepatic metabolism | Urine 70-80% | 17 hours | Very low, < 0.7% | 1.87 mg/ml |
| **Protease Inhibitors (PIs)** | | | | | |
| **Indinavir** | Liver | Primarily in the faeces | 1.8 hours | 60% | Not soluble in water,<br>0.82e-02 mg/ml |
| **Amprenavir** | Liver | Metabolite excreted in urine and faeces | 7-10 hours | 90% | Not soluble in water,<br>4.19 e-02 mg/ml |
| **Fosamprenavir** | Gastro intestinal tract | Faecal excretion of metabolites | 7.7 hours | 90 % | - |
| **Atanzanavir** | Liver | Faecal and renal | 6.5 hours | 60-68% | - |
| **Lopinavir** | Liver | | 5-6 hours | 98-99% | - |

| Drug | Metabolism | Excretion | Half life | Protein binding | Predicted solubility in water |
|---|---|---|---|---|---|
| **Ritonavir** | Liver | Elimination via the hepatobiliary systems. Metabolite excretion via faeces | 3-5 hours | 98-99% | Not soluble in water 1.26e-03 mg/ml |
| **Nelfinavir** | Liver | Primarily in the faeces | 3.5-5 hours | > 98% | Slightly soluble in water |
| **Saquinavir** | Liver | | N/A | 98 % | Insoluble in water |
| **Fusion Inhibitor** | | | | | |
| **Enfuvirtide** | Liver | Unknown | 3.8 hours | 92% | - |
| **Maraviroc** | Liver | | 14 – 18 hours | 76 % | 1.06e-02 mg/mL |
| **Integrase inhibitors** | | | | | |
| **Raltegravir** | Liver | Urine and faeces | 3.8 hours | 92% | - |

# Chapter 2

## Pheroid™ Technology

This section will converse on the Pheroid™; the types available, formulation, the advantages, and clinical applications of this delivery system.

## 2.1 Introduction

Pheroid™ technology is a patented delivery system. It is a unique submicron emulsion formulation and should not be confused with lipid-based delivery systems. To simplify reading, the trademark of the Pheroid™ will be left out. The Pheroid has a stable structure which can be manipulated in terms of morphology, structure, size and function to entrap active drugs for quick and effective delivery to various types of tissue.

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 (Grobler, 2004).

## 2.2 Pheroid™ classification and structural characteristics

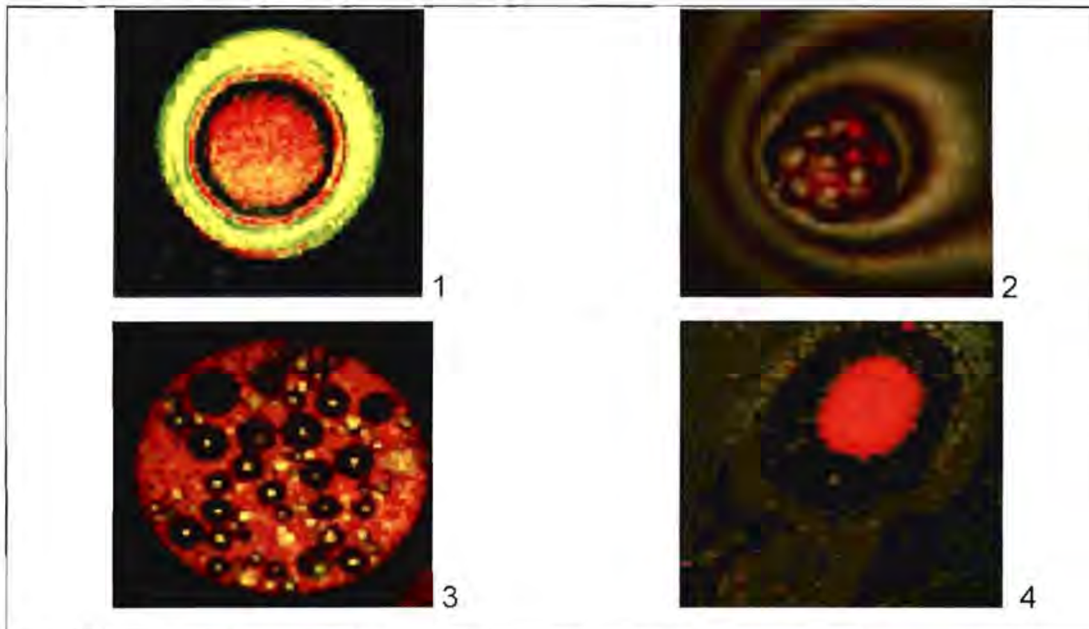



**Figure 2.1. The micrographs show some of the basic Pheroid types. 1. A bilayer membrane vesicle containing Rifampicin. 2. The formation of small pro-Pheroids from a pro-Pheroid formulation used for oral administration. 3. A reservoir containing multiple particles of coal tar. 4. A depot containing pro-Pheroids within a hydrophobic core, which is surrounded by a hydrophilic zone and an outer vesicle-containing zone** (with permission from Grobler, 2004).

A variety of Pheroid types can be formulated, depending on the composition and method of manufacturing. The three main types of Pheroids, each with a distinctive composition, are:

- **Pheroid vesicles**, lipid-bilayers generally ranging between 0.5 µm and 1.5 µm;
- **Pheroid microsponges** (1.5 µm to 5 µm) (Steyn, 2006) and
- **Pro-Pheroids** contained within depots or reservoirs. The sizes of the depot vesicles or reservoirs are determined by the amount of pro-Pheroids contained within the reservoirs (Uys, 2006) and the manner in which they will be released.

The microsponges and depots can support prolonged release according to the concentration gradient (Steyn, 2006; Uys, 2006)

### 2.2.1 Ingredients of the Pheroid delivery system

The Pheroid consists mainly of plant and essential fatty acids which are emulsified in sterile water and saturated with nitrous oxide. The Pheroid can be divided into three phases:

- An aqueous phase;
- An oil phase and
- Nitrous oxide

The oil phase is a mixture of fatty acids and vitamin E. The fatty acids are incorporated as ethylated and pegylated poly unsaturated fatty acids, including the essential omega-3 and -6 fatty acids but excluding arachidonic acid. Essential fatty acids are vital for various cell functions but can not be manufactured by human cells; it has to be ingested. The functions of the fatty acids within the Pheroid are the maintenance of cell-membrane integrity, energy homeostasis, modulation of the immune system through prostaglandins and regulation of programmed cell death. All Pheroid formulations contain a tocopherol or tocophopherol-based molecule, also known as vitamin E or derivates of vitamin E. The function of vitamin E within the Pheroid is to act as an anti-oxidation agent. It is an efficient inhibitor of lipid peroxidation *in vivo* (Burton & Ingold, 1981), thus preventing the oxidation of unsaturated fatty acyl residues of membrane lipids. An additional function of vitamin E is to act as a membrane stabiliser by forming a complex with the products of membrane lipid hydrolysis such as free fatty acids and lysophospholipids, thereby counteracting their disruptive effects (Grobler *et al.*, 2007).

The nitrous oxide ($N_2O$) is a volatile anaesthetic compound with both water and fat soluble properties. The addition of $N_2O$ has three functions: it contributes to the solubility of the fatty acids in the dispersed medium (Grobler *et al*, 2007), it assists with the self-assembly process of the Pheroids (Uys, 2006) and enhances the stability of the formed Pheroids at high and low pH (Grobler *et al*, 2007).

Molecular modelling indicates that there is some interaction between the fatty acids and the nitrous oxide, resulting in stable vesicular Pheroid structures. The formed nitrous oxide essential fatty acid matrix functions as a transport model for hydrophobic and hydrophilic drugs. The efficacy and stability of the formulation decreased dramatically if either the $N_2O$ or the essential fatty acids were left out of the formulation (Grobler *et al.*, 2007). The components of this matrix can be manipulated in a specific manner to ensure its high entrapment capabilities The entrapment of an active within the Pheroid would generally provide a safer, more effective formulation than the active alone (Grobler, 2004).

All Pheroids also contain a small amount of polyethylene glycol (PEG). PEG is a relatively non-reactive and non-toxic polymer that is frequently used in food and pharmaceutical products. The pro-Pheroid system has significant advantages over other delivery systems. It has been shown that PEG contributes to the following aspects of drug administration: increased bioavailability, increased drug stability and extended circulating life, lower toxicity and enhanced drug solubility

Oral administration of the Pheroid and pro-Pheroid is completely safe. Urinary analysis of Sprague-Dawley rats administered with Pheroid and pro-Pheroid showed no toxic effects. Mutagenic testing was done with Pheroid and pro-Pheroid on mutated *Salmonella typhimurium* bacteria strains. The conclusion was that both the Pheroid and pro-Pheroid showed no mutagenic activity (Elgar, 2008).

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

- Changing the fatty acid composition or concentrations;
- Addition of non-fatty acids or phospholipids such as cholesterol;
- Addition of cryoprotectants and charge-inducing agents;
- Changing the hydration medium (ionic strength, pH);
- Changing the method of preparation;

- Changing the character and concentration of the active compound (Grobler *et al.*, 2007).

## 2.3 Pheroid technology versus other lipid based delivery systems

Commercialised colloidal systems can be divided into liposomes, emulsions and microemulsions, polymeric microspheres and macromolecular microspheres. Pheroids combine some of the characteristics of these lipid-based colloidal delivery systems (Grobler *et al.*, 2007).

**Table 2.1 Comparison of some of the advantages and differences of the Pheroid in contrast to other lipid-based drug delivery systems** (Grobler, 2004).

| Pheroid delivery system | Lipid-based delivery systems |
|---|---|
| The Pheroid causes no immune response or cytotoxicity and assists with the maintenance of the cell's membrane. The Pheroid consists mainly of essential acids, which is a natural and essential part of the body's ingredients. Essential fatty acids form part of the natural biochemical pathways. | Cytotoxicity, immunological responses and impaired cell integrity are common problems with foreign substances that enter the body. Lipid delivery systems contain a proportion of these substances such as artificial polymers. |
| Repeatability for the production of the desired type can be ensured (Grobler, 2004). Filtration of the Pheroid will be discussed in Chapter 4. | Repeatability is a problem with lipid based delivery systems, especially in large batches. The variation in size of the lipid systems have to be limited using filtration. |

| | |
|---|---|
| This system can be manipulated in terms of size, charge, lipid composition and membrane packaging to accommodate the active compound (lipophilic or hydrophilic) and the indication of the drug. This is done by using different combinations of fatty acids and/or other added molecules (Grobler *et al.*, 2007). The Pheroid forms by self-assemblance (Uys, 2006) in contrast with liposomes which uses specific methods for preparation. | Most delivery systems are either lipophilic or hydrophilic. There are three ways to prepare liposomes to adapt to the nature of the drug and the lipids used: (1) phase separation; (2) spray or shear method through orifices; and (3) by coacervation (Shargel & Yu, 1999). |
| The Pheroid is sterically stable in colloidal suspensions, although it contains no cholesterol to maintain the interior volume, as is the case with liposomes. The Pheroid also shows a degree of elasticity and fluidity, with a high phase transition temperature. | Most lipid-based delivery systems contain phospholipids and cholesterol, the cholesterol is responsible for the maintenance of the interior of the vesicle. They also have a higher transition temperature than the fatty acids in the Pheroid. Therefore the lipid-based particles have less fluidity and elasticity. |
| The Pheroid penetrates the cell via fatty acid membrane binding proteins within lipid rafts in the cell membranes. The system passively targets the retriculo-endothelial system and it is metabolised within the mitochondria or the peroxisomes of the cell. | Phospholipids are metabolised in the cell membrane but similarly target the reticulo-endothelial system. |

## 2.4 Drug entrapment, delivery and uptake of Pheroid vesicles

The efficiency of entrapment within the Pheroid is generally determined by confocal laser scanning microscopy and both the Pheroid and the active compound are visualized through fluorescence labelling or autofluorescence. Figure 2.3.A. shows

the number of active molecules (auto fluorescent molecules) within the stained Pheroid The number of molecules of the active compound that are entrapped within one Pheroid depends mainly on the size, charge and solubility of the active compound (Grobler *et al.*, 2007).

The mean particle size of the Pheroid vesicles decrease over a period of three months as a result of larger droplets showing faster creaming than the smaller droplets (Uys, 2006). According to Lubbe (2007) the other hypothesis to describe this phenomenon is to assume that the Pheroid system contains a form of equilibrium. This would mean that the older Pheroid vesicles constantly break up to form newer and smaller vesicles up to a point. This phenomenon would cause enhanced entrapment efficiency over time. Lubbe also indicated that the development of a method for the determination of drug loading and drug release from the Pheroid is necessary for adequate testing of the Pheroid-drug combination.

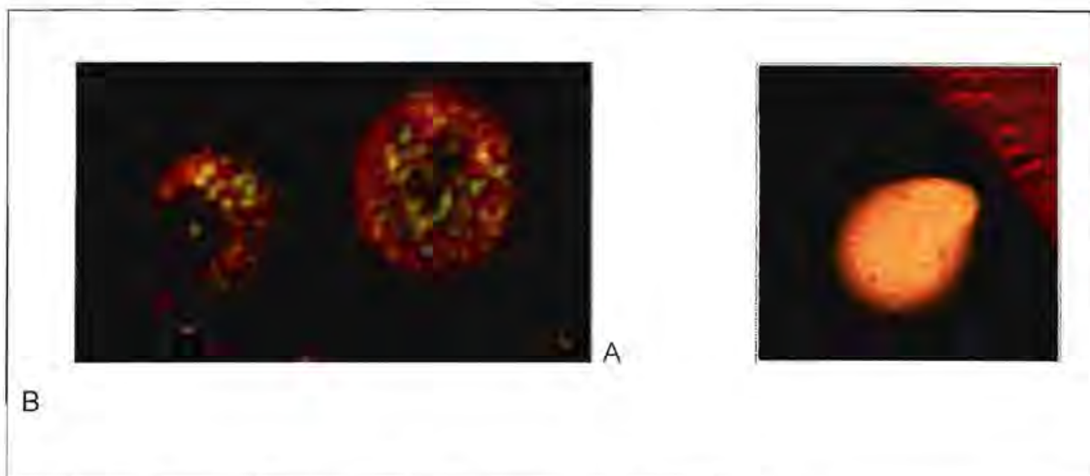



**Figure 2.2. A. Pheroids (red) containing on average 13 auto fluorescent active molecules. B. This micrograph shows the attraction between a primary fibroblast and a Pheroid vesicle.** (with permission from Anne Grobler, NWU, Potchefstroom).

An affinity exists between the fatty acids within the Pheroids and the cell membranes as seen in figure 2.3.B; this ensures penetration and delivery. The fatty acid membrane binding proteins within lipid rafts in the cell membranes are responsible for the binding and uptake of essential fatty acids (Grobler *et al.*, 2007). The Pheroid interacts with the cell membrane and then follows the endosome sorting mechanism, which results in the penetration and delivery of the active (Grobler, 2004). These

fatty acid-binding proteins are not equally expressed in all tissue. This feature may lead to specific cell targeting by the Pheroid in the future (Grobler *et al.*, 2007).

The ability of Pheroids to permeate cells is influenced by the following factors:

- The size of the Pheroids;
- The morphology of the Pheroids;
- The molecular geometry of the fatty acids themselves;
- The concentration and ratios of the various fatty acids;
- The hydration medium (ionic strength etc);
- The pH of the preparation;
- The presence of charge-changing molecules;
- The presence of molecules that influence the electrostatic milieu;
- The character and concentration of the active or drug;
- The state of the Pheroid, i.e. either gel state or fluid state or in between (Grobler *et al.*, 2007).

Pheroids are metabolised in either the mitochondria or the peroxisomes of the cell; this causes the release of the active compound (Grobler *et al.*, 2007). Mitochondrial toxicity caused by the NRTI drugs like stavudine might be amplified (Vigouroux *et al.*, 1999) because of this mechanism of the Pheroid.

## 2.5 Advantages of Pheroid delivery system

A major advantage of using the Pheroid delivery system is the various routes (oral, nasal and topical formulations) by which an active can be delivered with only minor adjustments to the formulation itself (Lubbe, 2007). Other key advantages of the Pheroid include increased delivery of active compounds, decreased time to onset of action, decreased cytotoxicity, penetration of most cellular barriers, ability to target specific treatment areas, lack of immunological response, ability to transfer genes to cell nuclei and reduction of drug resistance. An added advantage of using the Pheroid delivery system is that it has inherent therapeutic qualities and nutritional value that give it significant advantages over other delivery systems (Grobler, 2004). Malnutrition especially in children, caused by a deficiency of vitamins, trace minerals, essential amino acids and fatty acids, is the underlying reason for increased susceptibility to infections (Ambrus & Ambrus, 2004). AIDS is also responsible for

causing malnutrition. This results in a vicious circle for malnutritioned HIV-infected patients, particularly in Sub-Saharan Africa where quality food resources are scarce. Providing these patients with Pheroids containing the necessary antiretroviral drugs would enhance the lives of these patients significantly.

## 2.6 Therapeutical uses and characteristics of the Pheroid system

Intense research has been done to better understand the Pheroid delivery system as well as its possible applications in the treatment of infectious diseases. A few key characteristics which will contribute to the therapeutic application of Pheroid technology has been identified (Grobler, 2004). Highlights of selected clinical applications will be included for clarification of these characteristics.

### 2.6.1 Decreased time of onset

Research showed that an active delivered across most physiological barriers using the Pheroid acted significantly faster compared to the same active delivered via a conventional approach. This would mean that patients would experience faster relief of symptoms when they receive pharmaceutical actives entrapped within the Pheroid (Grobler, 2004). This could mean that the viral load of HIV-infected patients can be suppressed in a shorter time leading to less AIDS-related symptoms.

### 2.6.2 Increased delivery of active compounds

Studies done *in vitro* and *in vivo* have shown that the percentage active compound delivered was enhanced by entrapment within the Pheroid (Grobler, 2004). Lubbe (2007) saw that the nasal absorption of salmon calcitonin, administered to Sprague Dawley rats was significantly enhanced when entrapped within the Pheroid.

### 2.6.3 Reduction of minimum inhibitory concentration (MIC)

Research has shown that the use of 1/40$^{th}$ of the indicated amount of certain actives entrapped within the Pheroid, resulted in an effective Pheroid formulation (Grobler, 2004). This could mean that the amount of drug needed for the treatment of HIV can

be decreased while producing lower adverse effects and reducing the price of treatment.

### 2.6.4 Increased therapeutic efficacy

The efficacy of most active compounds was increased when entrapped within the Pheroid. TB drugs entrapped within Pheroid were very effective *in vitro* compared to the commercial product on the market (Grobler, 2004). Matthee (2007) did an *in vivo* study on black CD57 inbred mice using 60 % of the prescribed dosage regime entrapped within pro-Pheroid and comparing it to the commercial product on the market. He saw a 205 % increase in the efficacy of the 60 % pro-Pheroids entrapped rifampicin group as opposed to the commercial product. The 60 % pro-Pheroid formulation containing isoniazid or pyrazinamide rendered an increased efficacy of 20 % and 19 % respectively.

### 2.6.5 Reduction in cytotoxicity

The Pheroid system has the potential to enhance normal cell integrity and minimize cellular damage that occurs as a result of exposure to harmful effects of active ingredients. Side effects of active ingredients are, in most instances, the result of cellular damage (Grobler, 2004).

### 2.6.6 Pro-Pheroid concept

All Pheroids contain a small amount PEG; the use of increased concentrations and larger polymers has led to the development of the pro-Pheroid. PEG has been shown to render a protein therapeutically effective, where the unmodified form had not been effective (Grobler, 2004).

### 2.6.7 Immunological responses

Immunological responses caused by proteins or peptide drugs can be masked using the Pheroid. The Pheroid reduces recognition by the patient's immune response, since essential fatty acids are immunologically friendly. This can decrease the

frequency of dosing, or increase the amount of drug that can be given, to achieve a more powerful therapeutic impact (Grobler, 2004)

### 2.6.8 Transdermal delivery

Although Pheroids can be used in other routes of administration, topical administration of drugs holds certain advantages over oral dosage forms including localised delivery of anti-inflammatory or antimicrobial drugs. Topical dosage forms also have the added advantage of bypassing the digestive system which can negate many of the unwanted side effects of oral dosage forms (Grobler, 2004).

Holmes (2006) experimented with transdermal delivery of stavudine and its derivatives in PBS and Pheroid. The Pheroid did not enhance transdermal delivery of stavudine, but delivery of the Pheroid entrapped propionyl- and the buteryl derivatives were significantly enhanced across the skin.

### 2.6.9 Ability to entrap and transfer genes to cell nuclei and expression of proteins

It was shown previously that Pheroids are capable of facilitating gene transfection, opening many new avenues for DNA vaccines and gene therapy. *In vitro* studies have shown that entrapment of human and viral DNA of various lengths into the Pheroid led to reproducible expression of the appropriate proteins after tranfecting the cells with the Pheroid-entrapped genes (Grobler 2004).

### 2.6.10 Reduction and suggested elimination of drug resistance

*In vitro* studies demonstrated that drug resistance can be reduced or eliminated by incorporating a drug in the Pheroid delivery system. Analysis of multidrug resistant TB showed that formulations containing the standard antimicrobial, rifampicin, entrapped in Pheroids alleviated pre-existing drug resistance (Grobler, 2004). Entrapment of chloroquine within a Pheroid formulation showed a 15 times increase in efficacy against a chloroquine resistant strain of malaria. Pheroid entrapped mefloquine, artesunate and artemether gave a 3.1, 2.38 and 2.54 times enhanced efficacy respectively compared to the drug formulated in water (Langley, 2007). The Pheroids' ability to enhance the effectiveness and reverse the resistance of

antibiotics such as anti-malarials, antiretrovirals, and anti-tuberculoses drugs can have widespread implications for the healthcare industry.

## 2.7 Conclusion

Pheroid technology is a patended delivery system. The entrapment of an active within the Pheroid would generally provide a safer, more effective formulation than the active compound (Grobler, 2004). This could mean that the amount of drug needed for treatment of HIV can be decreased while producing lower adverse effects and reducing the price of treatment.

# Chapter 3:

## Cell cultures, Materials and Methods

This section will converse on the cell lines and virus types used, the conditions needed for cell culturing and also the analytical methods used.

## 3.1 Introduction

The Centre for Disease Control (1999a) requires that manipulation of concentrated HIV stocks should be conducted in a Biosafety Level 3 (BSL 3) laboratory. For this reason, the study could not be performed at the NWU, Potchefstroom campus. This study was conducted at the National Institute of Communicable Diseases (NICD), Sandringham. They kindly provided the use of their expertise, assays, systems, laboratory facilities, equipment and reagents. The provided AIDS unit safety manual was followed strictly (Annexure B).

A drug generally has to be analysed *in vitro* for effectiveness and toxicity in order to evaluate the *in vivo* effects of the drug. The first step of *in vitro* experimenting is to choose an appropriate cell line. Primary cells, such as peripheral blood mononuclear cells (PBMC) are obtained from HIV sero-positive and -negative humans. They have a close resemblance to the cells found *in vivo* since they contain all the receptors necessary for HIV-infection. These cells are short lived, expensive to obtain and difficult to maintain. Therefore continuous cell cultures were used in this study. Continuous cell cultures are easy and inexpensive to obtain and maintain, but a disadvantage of these cells is that they do not represent the original cell it was obtained from. These cells were transfected to stably express certain co-receptors or genes to mimic *in vivo* conditions. The second step is to choose an appropriate virus type according to the biological phenotype and the genetic subtype of the virus. It would be futile to choose a virus with a different subtype than the virus that is prevalent in the indicated area. Proper infection will also not take place if the cell's expressed receptors and the virus's receptor usage do not match. Not all cultured cells are infected as readily as primary cells; therefore infection has to be enhanced by spinoculation or addition of cationic polymers. Finally, to asses the toxicity and/or efficacy of the drug or Pheroid entrapped drug a cell viability assay was performed. One direct/HIV-specific and one indirect assays were performed to determine the inhibition of the virus by the drug. The following sections will give a detailed discussion of the cultivation, experimental design and assays used in this study.

## 3.2 Selection of an appropriate cell line

Primary cells were not used in this study since they are difficult maintain and expensive to obtain. The continuous cell lines used are mostly transformed immortalized cancer cells. Cancer cells in comparison to primary cells are immortal since it can multiply infinitely (Barker, 2005). They do not represent the true *in vivo* situation and they may react differently than the original cells they were derived from. These transfected immortalised cells are not ideal for assessing the effects of antiretroviral drugs and/or novel delivery systems on the individual cells, but they are uncomplicated and inexpensive to maintain. It can be divided into two groups based on their growth characteristics: suspension cells and anchorage-dependent cells.

The suspension cells that were used in this study are T-lymphoblastoids that naturally express the CD4 receptor and the CXCR4 chemokine receptor (Brandt, 2002). Suspension cells are sphere-shaped and grow suspended in the growth media. The MT2 cell line is an immortalized cell line derived from the peripheral blood of a Japanese woman who had adult T-cell leukaemia. Her lymphocytes were co-cultured with male infant umbilical cord leucocytes to give the MT2-lymphoblasts (Miyoshi *et al.*, 1981). Other suspension cell lines used in this study are CEM and M7-Luc cells; they were derived from cancerous lymphocytes in the peripheral blood. CEM.NK$^R$.CCR5 is a CD4$^+$ human T-lymphoblastoid cell line, transduced to stably express CCR5 chemokine receptors (see section 1.2.1) by a retroviral vector (Trkola *et al.*, 1999). M7-Luc is a CEMx174 cell clone. It was transduced by a retroviral vector to express CCR5 and transfected to contain *Tat*-responsive reporter genes. These genes encode the firefly luciferase (Luc) enzyme and green fluorescence protein (GFP) which are expressed once HIV-1 replication occurs (Brandt, 2002).

Table 3.1 gives a summary of the characteristics of the suspension and anchorage-dependent cells used in this study.

Coreceptor-transfected cell lines were obtained from the NIH Reference and Reagent Repository.

**Table 3.1. Characteristics of the different cell lines used during this study** (Bjőrndal *et al.*, 1997; Brandt, 2002; Miyoshi *et al.*, 1981; Trokola *et al.*, 1999; Vődrős *et al.*, 2001).

| **Cell line** | **Type of cell** | **Suspension of adherent cells** | **Infection enhancer** | **Special modifications** |
|---|---|---|---|---|
| MT2 | Lymphoblasts | Suspension | None | - |
| CEM.NK$^R$.CCR5 | Lymphoblasts | Suspension | Polybrene | Transduced to express CCR5 (CD4 and CXCR4 are naturally expressed. |
| M7-Luc (CXCR4 & CCR5) | Lymphoblasts CEMx174 clone | Suspension | DEAE-Dextran | Transduced to express CCR5 (CD4 and CXCR4 are naturally expressed. Transfected to contain Tat-responsive reporter genes that express Luciferase and GFP. |
| U87-CCR5 | Glioblastoma/ astrocytoma (epithelial tissue) | Adherent | DEAE-Dextran | Transduced to express CCR5 (CD4 and CXCR4 are naturally expressed. |
| GHOST cells.CXCR4.CCR5 | Human osteo sarcoma cells (osteoblasts) | Adherent | Polybrene | Transduced to express CCR5 (CD4 and CXCR4 are naturally expressed. Transfected to express GFP. |

Anchorage-dependent cells require a physical surface for attachment and growth. These cells have a flattened shape when observed under a microscope. The GHOST cell line was derived from a person with human osteosarcoma (HOS) (Vődrős *et al.*, 2001), which is the most common type of bone cancer. The U87 cell line was derived from glioblastoma/astrocytoma (Bjőrndal *et al.*, 1997), which is the most common type of brain cancer in adults (Machado *et al.*, 2005). GHOST.CXCR4.CCR5 cells are transduced with an MV7-T4 (CD4) retroviral vector to stably express CCR5. This cell line also contains the gene that is responsible for the expression of GFP. U87-CCR5 cells were transduced to express CCR5 (Bjőrndal *et al.*, 1997). These cells were grown in a 48-well plate to provide adequate surface for the cells to adhere to.

## 3.3 Selection of an appropriate virus type

Chemokine receptor (co-receptor) usage by gp120 of HIV-1 isolates is defined by the biological phenotype and not the genetic subtype of the virus. HIV isolates are categorized into two distinct groups according to its biological phenotypes. T-tropic viruses (adapted to T-lymphocytes) utilize CXCR4 and non-T-cell-line adapted (M-tropic; adapted to macrophages) viruses utilize CCR5 co-receptors. T-Tropic or CXCR4 viruses can replicate rapidly and form syncytia *in vitro*. They also possess the ability to infect and replicate in a broad spectrum of T-lymphoid or monocytoid cell lines. CCR5 using or M-tropic viruses replicate slowly and induce few if any syncytia. Syncytias are giant multinucleated cells and are rarely found *in vivo* except during autopsies within the brains of patients who had AIDS encephalitis (Epstein, 1985). All primary viral isolates are able to form syncytia *in vitro*, provided that the cells express the particular co-receptor used by the virus. The relative level of expression of co-receptors may differ between PBMCs and transformed continuous cell lines, but there would be no intrinsic difference between the receptors (Vődrős *et al.*, 2001; Bjőrndal *et al.*, 1997). CCR5 utilizing viruses are present at all stages of the HIV infection, while CXCR4 viruses can be observed in patients developing AIDS (Schuitemaker *et al.*, 1992). Subtype C is responsible for 60 % of the HIV infections worldwide (see section 1.1) and it is the prevalent clade in Sub-Saharan Africa (Requejo, 2006). Subtype C isolates were therefore chosen for this study. All virus isolates were obtained from reference samples grown at the NICD.

**Table 3.2. Characteristics of the different virus types used for this study** (Cave *et al.*, 2006; Cilliers *et al.*, 2005a; (Cilliers *et al.*, 2005b).

| **Virus type** | **Sub-type** | **Coreceptor used for entry** |
|---|---|---|
| SW7.TCLA | C | CXCR4 |
| CM9 | C | CXCR4 & CCR5 |
| DU151 | C | CCR5 |
| DU179 | C | CCR5 |
| DU422 | C | CCR5 |
| SM1 | B | CCR5 |

The short half-lives of retroviruses limit the distance that they can travel in solution by way of Brownian motion (Chuck *et al.*, 1996). Sufficient amounts of virus are produced and transported by the circulation in the human body to surpass this problem. Some viruses do not readily infect certain cultured cells. The following techniques were used in an effort to facilitate infection: spinoculation (centrifuging) or the use of cationic polymers. Spinoculation stimulates *in vitro* viral adsorption of cells. It firstly gives direction to cell-free viruses, by decreasing the distance the virus has to travel to an uninfected cell. Secondly it forces direct contact between infected cells and uninfected cells, which reduces the time between budding of new virus particles from one cell and binding of the virus particle to another cell (O'Doherty *et al.*, 2000). Polybrene (used to enhance infectivity of GHOST and CEM cells) and DEAE-dextran (used for M7-Luc and U87 cells) are cationic polymers. The infection facilitative mechanism for polymers such as polybrene and DEAE-dextran are therefore the neutralization of the charges of the negatively charged cell and virus surfaces. It inhibits the repulsion between the cell and the virus resulting in enhanced infection (Davis *et al.*, 2004; Fan *et al.*, 1992). Polybrene and DEAE-dextran enhance the formation of syncytia (Konopka *et al.*, 1991).

## 3.4 Experimental procedures

### 3.4.1 Materials

DEAE-dextran, Geneticin (G418) (50 mg/ml), Gentamicin (10 mg/ml), Puromycin (10 mg/ml) and Polybrene were obtained from Sigma (St. Luis, USA); DMEM basal media, FCS, 1M HEPES buffer, PBS, RPMI 1640 and 0.25 % Trypsin were obtained from Gibco Invitrogen (Carlsbad, USA) and the Penicillin/Streptomycin/Fungizone (100x) was obtained from Highveld Biological (Sandringham, RSA). A Nikon TMS-F microscope was used to count the cells and a Rotanta 46 Centrifuge (Hettich Zentrifugen) was used to collect the cells.

### 3.4.2 Cultivation of cells

In order to successfully grow and maintain mammalian cells *in vitro*, it is necessary to mimic the *in vivo* conditions. These conditions include the optimal temperature, oxygen and carbon dioxide concentrations, pH, osmolality, relative humidity and nutrition. The culture medium is the most important component for *in vitro* culturing, since the incubator is only responsible for providing the specific temperature, oxygen and carbon dioxide concentrations. The function of culture media are to provide the cells with the required substances which it can not produce itself and it also provides the environment for cell growth by maintaining the correct pH and osmolality levels. A complete cell culture medium consists of two distinct components: (a) basal medium that can provide nutrients, salts and a way to control pH and (b) supplements to permit cell growth, such as serum (Cartwright and Shah, 2002).

The suspension cells used for this study were grown in Roswell Park Memorial Institute (RPMI) designed media. Dulbecco's modified minimum essential medium (DMEM) was used for the anchorage dependent cells. DMEM is a modification of Eagle's medium, which was designed to support monolayer growth. Suspension cells require culture medium with low levels of calcium ($Ca^{2+}$) to minimize attachment of the cells to the surface of the culture vessel and to one-another. Culture media for suspension cells also have advanced buffering capability to minimize acidification caused by dense

cultures. Anchorage dependent cells require substantially more divalent cations such as $Ca^{2+}$ (Ham & McKeeham, 1979).

Foetal calf serum (FCS) is the most frequently used type of serum. Serum contains growth factors, plasma proteins and hormones that are needed for cell growth and maintenance (Schantz & Ng, 2004). Serum can also buffer the cell cultures against pH changes, endotoxins and the presence of heavy metals (Cartwright & Shah, 2002).

Several antibiotics were used during the *in vitro* cell culturing in this study: Geneticin (G418), gentamycin sulphate, hygromycin, penicillin/streptomycin/fungizone (Pen/Strep/Fungizone) and puromycin. Pen/Strep/Fungizone consists of Penicillin, Streptomycin and amphotericin B. This combination can be used as an antibacterial (both gram-positive and gram-negative), anti-fungal, anti-yeast and anti-mould agent. G418 has a similar structure to gentamycin B1. It is used to select genetically engineered cells; for instance to stably express GFP (Gubin *et al.*, 1997) or to express luciferase. Gentamicin is used to treat gram-positive and -negative bacteria, and also mycoplasma (Schantz & Ng, 2004). Hygromycin kills bacteria, fungi and higher eukaryotic cells. Puromycin is non-selective, but kills both prokaryotic (cells without a nucleus, like bacteria) and eukaryotic cells (cells with a nucleus, such as fungi, amoebozoa and animal cells). G418, hygromycin and puromycin are used to select and maintain stable eukaryotic cell lines that have been tranfected with vectors containing its resistance gene.

Complete growth media is not indicated during analysis; only minimal use of antibiotics is permitted. Gentamicin was the only antibiotic used during experimentation with M7-Luc cells.

**Table 3.3. Complete growth media for suspension cells.**

| | MT2 | CEM | M7-Luc |
|---|---|---|---|
| RPMI 1640 | 45 ml | 44 ml | 44 ml |
| FCS | 5 ml | 5 ml | 5 ml |
| Geneticin (G418) | - | 800 µl | 300 µl |
| Hygromycin B | - | - | 166 µl |
| Puromycin | - | - | 2.5 µl |
| Pen/Strep/Fungizone | - | 500 µl | - |
| Gentamicin | - | - | 250 µl |
| HEPES buffer | - | - | 1.25 ml |

**Table 3.4. Complete growth media for anchorage dependent cells.**

| | GHOST | U87 |
|---|---|---|
| DMEM | 44 ml | 42.5 ml |
| FCS | 5 ml | 7.5 ml |
| Geneticin (G418) | 500 µl | 300 µl |
| Hygromycin B | 85 µl | - |
| Puromycin | 5 µl | 5 µl |
| Pen/Strep/Fungizone | 500 µl | - |
| Gentamicin | - | - |
| HEPES buffer | - | - |

The suspension cells were analysed in a 96-well plate. The anchorage-dependent cells were analysed in a 48-well plate to provide adequate surface for the cells to adhere to. All the different cell types were incubated at 37°C and 5 % $CO_2$, in a humidified incubator (Forma Scientific $CO_2$ water jacketed Incubator, Forma Scientific).

### 3.4.3 Procedure for preparation and infection of the cells

The number of wells with infected and uninfected cells that were used for each experiment was calculated to prepare the correct number of cells. Anchorage dependent cells were seeded for all experiments at a density of 5 x $10^4$ cells/well, a day before infection. Suspension cells were seeded at a density of 7.5 x $10^4$ cells per well on the day of infection, which is one day before the experiment commenced. Cells were collected using a centrifuge at 1200 rpm for 10 minutes. The supernatant was discarded and the cells were re-suspended in growth medium. Trypan blue was used to stain the non-viable cells blue. The charged trypan blue molecules can not enter viable cells because of the integrity of the membrane. The cells were counted according to the instructions given by Schantz & Ng (2004).

Suspension cells were counted and infected with the appropriate number of viruses a day before the addition of the drug or drug plus Pheroid and left to incubate overnight. Anchorage dependent cells were seeded into the wells two days before the assay and incubated overnight to give them proper time to adhere to the surface of the well. The virus was added a day later (one day before the assay) to the adhered cells and again left overnight to incubate at 37°C (5 % $CO_2$). Polybrene or DEAE-dextran can be added to enhance infectivity, but was prohibited when experimenting with the Pheroid; the reason will be given in chapter 4.

### 3.4.4 Incubation with the Pheroid/ABC/3TC

The cells were washed and collected at 1200 rpm for 10 minutes in the centrifuge prior to the assay to remove any used media or virus that did not penetrate the cells. Abacavir sulphate (obtained from Sri Sai Nikitha Pharma PVT. LTD., India, Batch nr. CAB0770029) was predominantly used for this study; it will be referred to as ABC for simplicity reasons hence forth. Lamivudine (3TC) was obtained from Sri Sai Nikitha

Pharma PVT. LTD., India, Batch nr. 002022007. The concentration range of the ABC and (3TC) was in the region of the $IC_{50}$ value obtained by Daluge (1997) and Soudeyns (1991) respectively; and the concentration of the stock solution was calculated accordingly. On the day of the assay, a fresh stock solution of ABC, 3TC or Pheroid was prepared in media and the serial dilutions were done in tubes to minimize well-to-well variation. The well plate was marked and laid out as planned. The total volume of each well was 250 µl in the case of suspension cells and 500 µl for anchorage dependent cells. The drug solution constituted 40 % of the total volume of each well and the cell-suspension constituted 60 % of the total volume. The drug solution was added first to each well, followed by the cell suspension to minimize the time the cells spend outside the incubator.

## 3.5 Analytical methods

A cell viability assay, namely the MTT-cell viability assay was used to determine the toxic effects of the drugs and/or Pheroid on the cells. To measure the survival or inhibition of the virus, an enzyme-linked immunosorbent assay (ELISA) (using p24-antigen) as well as a luminescense assay (luciferase) was used. Figure 3.1 gives a summary of the volumes used in the analytical methods. On the day of assessment the plates were carefully taken out of the incubator and 25 µl of the undisturbed supernatant was transferred to another plate for p24-antigen analysis. This was done for both suspension and anchorage dependent cells. An additional 275 µl (or 300 µl if p24-antigen was not done) was removed from the wells of the anchorage dependent cells. The suspension cells were then thoroughly mixed and an additional 100 µl was removed (or 125 µl if p24 was not done) and discarded. But in the case of the M7-luc cells, 100 µl was deposited into a black 96-well plate for luciferase analysis. The remaining volume of cells was used for the MTT–viability assay.

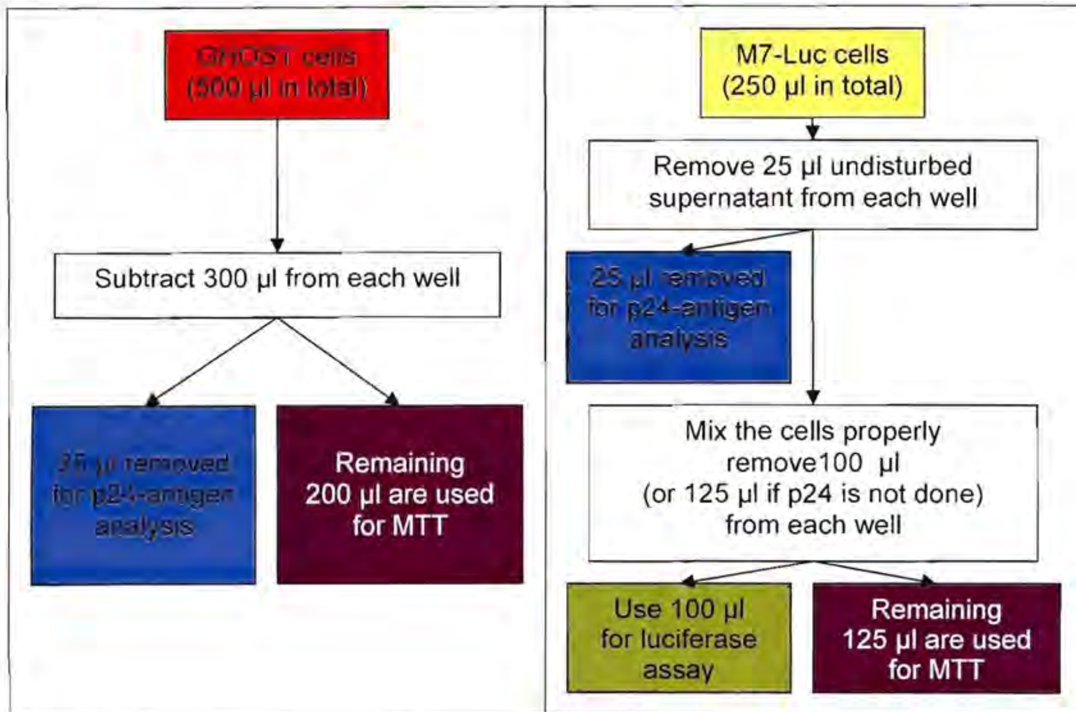



**Figure 3.1. Explanation of the different volumes removed from each well and the difference between the anchorage dependent (GHOST cells) and the suspension cells (M7-Luc).**

### 3.5.1 MTT-cell viability assay

This is a quantitative colorimetric assay to measure the number/viability of cells and reflects on the cytotoxicity of the drugs or other compounds. MTT (3-(4,5-demethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a yellow tetrazolium salt which is converted by the enzyme succinate-dehydrogenase (found in the mitochondria of living cells) to water-insoluble, dark blue formazan crystals. A mixture of 20% SDS (sodium dodecyl sulphate) and 50% DMF (dimethylformamide) in distilled water was added to lyse the cells and solubilise the formazan crystals. The dissolved formazan crystals give a purple colour, which can be measured using a spectrophotometer (VERSAmax tunable microplate reader, Molecular Diagnostics) at wavelengths of 570nm and 690nm (Mosmann, 1983; Holst-Hansen & Brünner, 1998).

#### 3.5.1.1 Materials

MTT was obtained from Sigma (St. Louis, USA); SDS was obtained from USB (Cleveland, USA) and the DMF was obtained from BDH Laboratory Supplies (UK). A Julabo EC Water bath (Julabo Labortechnik GMBH, Seelbach, Germany) was used to thaw the MTT and dissolve the SDS/DMF solution. A VERSAmax tunable microplate reader (Molecular Diagnostics, Roche, Pleasanton, USA) was used for analysis.

#### 3.5.1.2 Assay procedure

MTT (5 mg/ml in PBS) was added in a 10 % (v/v) ratio to the remaining supernatant in each well after the indicated volume was removed. The plate was then incubated within an incubator at 37°C (5 % $CO_2$) for exactly two hours for the purpose of repeatability. MTT is a known carcinogen and the specific standard operating procedure (SOP) was followed in terms of health and safety. A mixture of 20% (w/v) SDS and 50% (v/v) DMF in distilled water were added in a 100 % ratio to the supernatant left in each well. This means that 125 µl were added to the wells of suspension cells and 200 µl to the anchorage dependent cells. This mixture was added to lyse the cells and solubilise the formazan crystals that formed within the cells. The SDS/DMF solution was stored at room temperature and heated to 45°C prior to use to eliminate precipitations that may have formed during storage. The well plates were left overnight in the laminar flow cabinet to ensure that the crystals have dissolved completely. The coloured reaction was measured using a spectrophotometer.

### 3.5.2 P24-antigen Enzyme-linked Immunosorbent Assay

This Enzyme-Linked ImmunoSorbent Assay (ELISA) is based on a "sandwich" principle. The assay is a qualitative and quantitative *in vitro* biochemical technique that detects the presence of an antibody or an antigen. It makes use of two different antibodies. The first antibody is specific to the antigen involved; p24 in this case. The antibody is coupled to an enzyme (horseradish-peroxidase), which reacts with the antigen-antibody complex (Higgins *et al.*, 1986).

#### 3.5.2.1 Materials

The p24 ELISA kits were obtained from Biomérieux (Marcy l'Etoile, France). A Sanofi PW40 Plate washer (Sanofi Pasteur Diagnostics, France) and a VERSAmax tunable microplate reader (Molecular Diagnostics, USA) was used for anlysis.

#### 3.5.2.2 Assay procedure

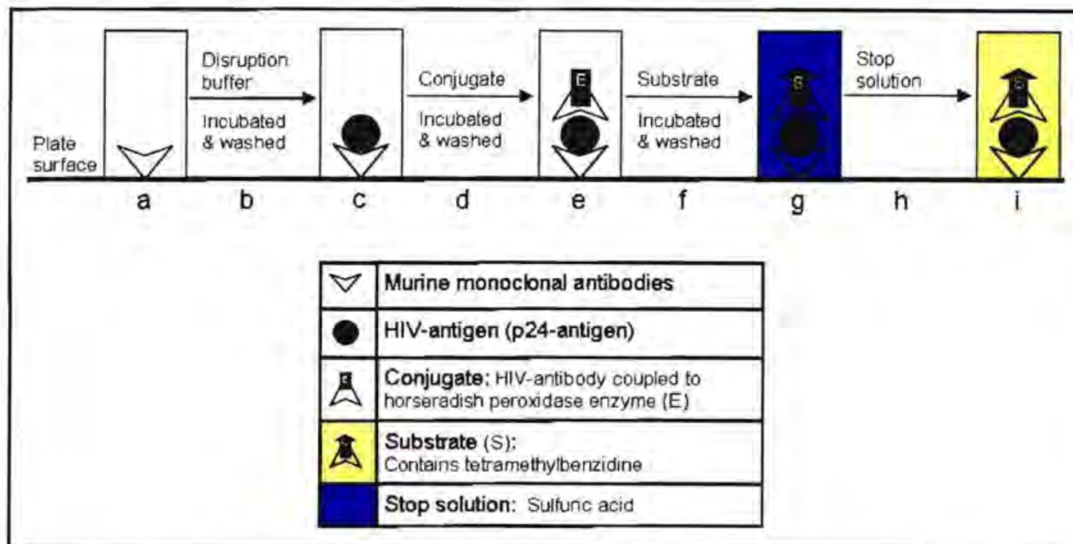



**Figure 3.2. Demonstration of the p24-antigen ELISA "Sandwich method"** (adapted from Goldsby *et al.*, 2000).

A calculated volume of the supernatant from each well was placed in an empty plate and topped up to 200 µl with growth media. This volume had to be precise for the expected concentration to lie within the range of the calibration curve. Disruption buffer (25 µl) was added to this suspension to disrupt the HIV virion (Fig 3.2 (b)). This exposes the HIV-antigen within the virion. Hundred micro litres of this suspension were placed in the wells assigned by the manufacturer. The HIV-antigen forms a complex with the solid phase antibodies (a) on the plate (c). The wells are then incubated for an hour and washed four times with phosphate buffer to remove anything that did not form a complex with the solid phase antibodies. Hundred micro litres conjugate (d), consisting of HIV-antibodies coupled to an enzyme (E), was added to the wells. It was followed by incubation for an hour and another washing step. The conjugate binds to the solid

phase antigen-antibody complex (e). Substrate (100 µl), was then added to the wells (f), the plate was incubated for an hour and a washing step followed. During this incubation time, the enzyme (E) from the conjugate converted the substrate (S) to a blue coloured substance (g). To stop the reaction, 100 µl sulphuric acid was added (h). This turns the blue colour to yellow (i). The colour was determined within fifteen minutes with a spectrophotometer at 450 nm (adapted from Biomerieux's Vironostika HIV-1 antigen instruction manual and Goldsby *et al.*, 2000).

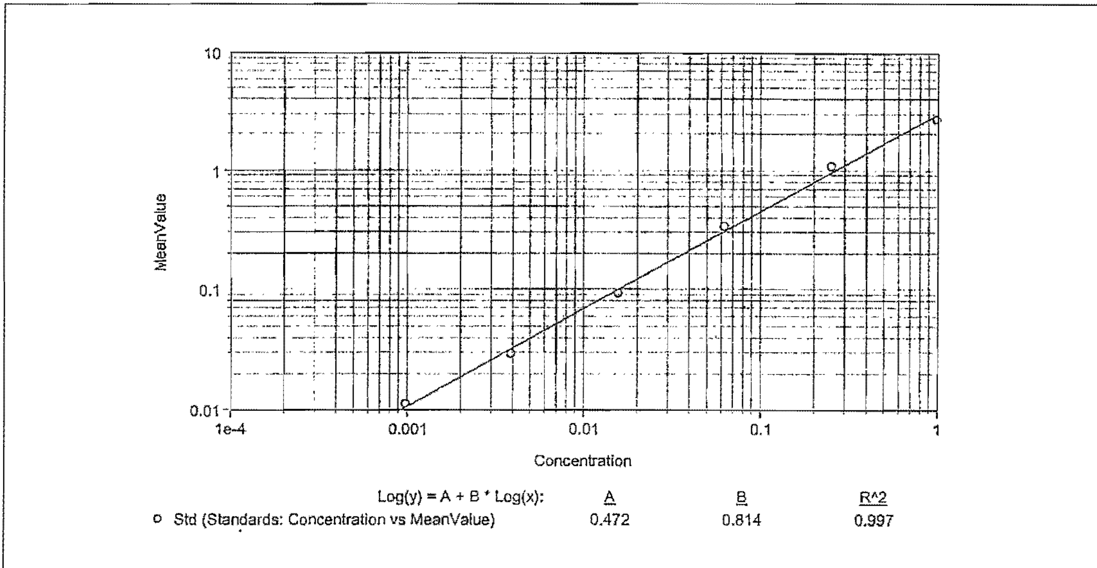



**Figure 3.3. Example of a typical HIV p24-antigen calibration curve.**

The mean absorbance of each serial dilution of the positive control was used to set up a quantitative calibration curve (Figure 3.3). This graph represents the mean absorbance values of the positive control dilutions (Y-axis) against their relative p24 antigen concentrations (X-axis). The values were plotted on a log-log axis to improve the regression of the curve. The absorbance of each specimen was interpolated from the calibration curve to give the HIV p24 concentration (pg/ml) and the regression value will be given for each experiment.

### 3.5.3 Luciferase

This assay is rapid, reliable and easy to perform compared to the p24 ELISA. The luciferase end-point simplifies the performance of inhibitor-screening assays compared to the use of more conventional end-points such as the detection of extra-cellular p24 antigen.

The principle of the assay is illustrated in Figure 3.4. Firefly luciferase catalyzes the mono-oxygenation of beetle luciferin. Beetle luciferin is found in fireflies. The energy needed for photon production comes from molecular oxygen with ATP as a co-factor (Beutler & Mathai, 1967). This assay is exclusive for cells engineered to express Luc upon translation of the viral RNA, such as the M7-Luc cell line. M7-Luc cells were transfected to contain a Tat-responsive reporter gene. This gene is responsible for the expression of luciferase (Luc) and green fluorescence protein (GFP). The luciferase enzyme is produced upon activation of transcription of the HIV-1 genome. The amount of luciferase produced or rather the light units measured gives an indication of the viral replication that has taken place. The emitted light packages or photons were measured in relative light units (RLU) using a luminometer.

The Bright-Glo™ substrate contains dithiothreitol that can be potentially hazardous; therefore protective eyewear, gloves and a lab coat was worn when working with this assay system.

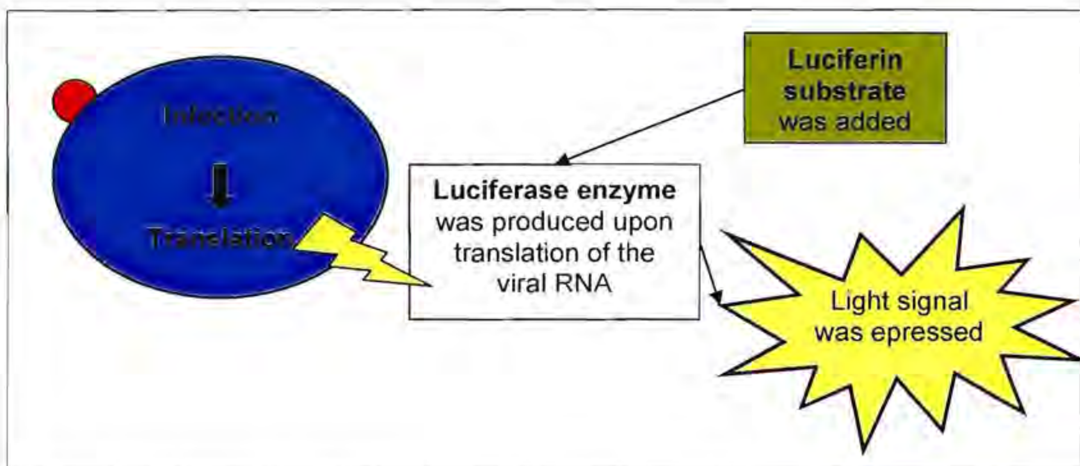



**Figure 3.4. Visualization of luciferase production, the enzymatic conversion of the luciferin substrate and the emission of light.**

#### 3.5.3.1 Materials

The BrightGlo™ reagents were obtained from Promega (Madison, USA), a Victor$^3$ 1420 Multilable Counter (Perkin Elmer, Waltham, USA) luminometer and a Zeiss Axiovert 25 microscope was used.

#### 3.5.3.2 Assay procedure

The 100 µl properly mixed cell suspension from each well was deposited into a black 96-well plate. The black well plate prevented one well's luminescence to be read from within another well. An equal volume of reagent (100 µl) was added to each well containing the cells. A timer was set for two minutes to allow adequate time for the cell lyses to complete. The luminescence was measured immediately with a luminometer in relative light units (RLU). For maximum light intensity, the measurement was completed within 15 minutes.

### 3.5.4 Statistical analysis

All statistical calculations were done using GraphPad® Instat 3 and the graphs were plotted using GraphPad® Prism 4. The following statistical calculations were performed:

**Average:** Calculated mean of the different values.
**Standard error of mean (SEM):** is a measure of how far your sample mean is likely to be from the true population mean. SEM is the standard deviation of the samples compared to the mean divided by the square root of the number of repeats.
**P-value:** The p-value is a probability and can indicate the significance of the observed result. If the p-value is small ($\leq$ 0.05); the differences are significant since it can be assumed that the difference observed between samples can not be explained by random sampling. This value was calculated using GraphPad® Instat.
**50 % Inhibitory concentration ($IC_{50}$):** This value indicates the ability of the antiretroviral drug to inhibit replication of HIV. This is a quantitative measurement that indicates how much drug is necessary to inhibit the viral replication by 50 %. This was calculated using GraphPad® Prism 4.

**50 % Cytotoxic concentration ($CC_{50}$):** This value indicates the cytotoxicity of the antiretroviral drug or Pheroid on the cells. This is a quantitative measurement that indicates how much drug or other added compound is necessary to kill 50 % of the cell population. This was calculated using GraphPad® Prism 4

## 4.1 Investigation with the Pheroid technology in combination with antiretroviral drugs:

Botha (2007) experimented on the effect of Pheroid in combination with lamivudine or stavudine on MT2 cells. He experienced difficulties with the reproducibility of the MTT assay and the action of sterilising the Pheroid with gamma rays. According to him the fluctuations in the MTT results were caused by the possible conversion of MTT to formazan crystals by the syncytia formed after infection of the MT2 cell line with HIV. He established the following factors as guidelines for future investigations:

- Design and development of accurate viability assays.
- 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 dugs in the cells.
- The use of the p24 antigen or reverse transcriptase assays, since these assays are HIV specific and would show better repeatability and reproducibility of results.
- Comprehensive stability and solubility studies on the drugs used.
- The reproducibility of the MTT assay, which should be evaluated within the context of each *in vitro* infection model used (Botha, 2007).

## 4.2 General design of study

When using the term Pheroid during the course of this chapter, it will refer to trade-marked Pheroid™ vesicles. To simplify the Pheroid formulation used for *in vitro* experimentation, the anti-oxidants were left out, until further notice. The following experiments were done to comply with the guidelines set by Botha in section 4.1 and the objectives and aims set for this study:

- A new cell line and virus type was chosen (section 4.4).
- Botha advised that the use of the MTT assay should be discontinued or used with caution for future experiments due to the repeatability problems experienced during his studies. This is still the most inexpensive and well known viability

assay and expertise exists at the NICD with this assay; therefore the use of this assay was continued.

- HIV-specific assays like the p24 antigen ELISA assay and the luciferase assay were implemented to asses the inhibition of HIV replication by the drug and drug/Pheroid combination. Repeatability and correlation between these two methods were addressed.
- The total incubation time was optimised during this study (section 4.4.3) from the three day incubation proposed by Botha (2007) to incubation for a total of four days. A three hour incubation time was also introduced.
- The effect of Pheroid in combination with infection enhancers like polybrene and DEAE-dextran on the viability of the cell was studied (section 4.4.2 and 4.4.4).
- The optimal Pheroid concentration was determined numerous times because of inconsistency of the results obtained when using the MTT assay.
- The stability of the Pheroid was addressed. The influence of anti-oxidants in the Pheroid formulation on the viability of the cells was determined (section 4.5.5). It was noticed that the viability of the cells decreased as the Pheroid batch matured. Anti-oxidants were added from this point on to Pheroid batches.
- The $IC_{50}$ of ABC and 3TC was established with M7-Luc cells infected with SW7 (section 4.6).
- ABC was entrapped in the Pheroid and the effect of this combination was investigated in terms of enhanced HIV replication inhibition (section 4.7). Several problems presented itself during this experiment and were repeated several times for this reason.
- Kűhn (2008) reported that the stability of ABC in pro-Pheroids was not finalised. My studies were done using Pheroid vesicles. This implied that Botha's fifth guideline was addressed, but are still open for further studies.

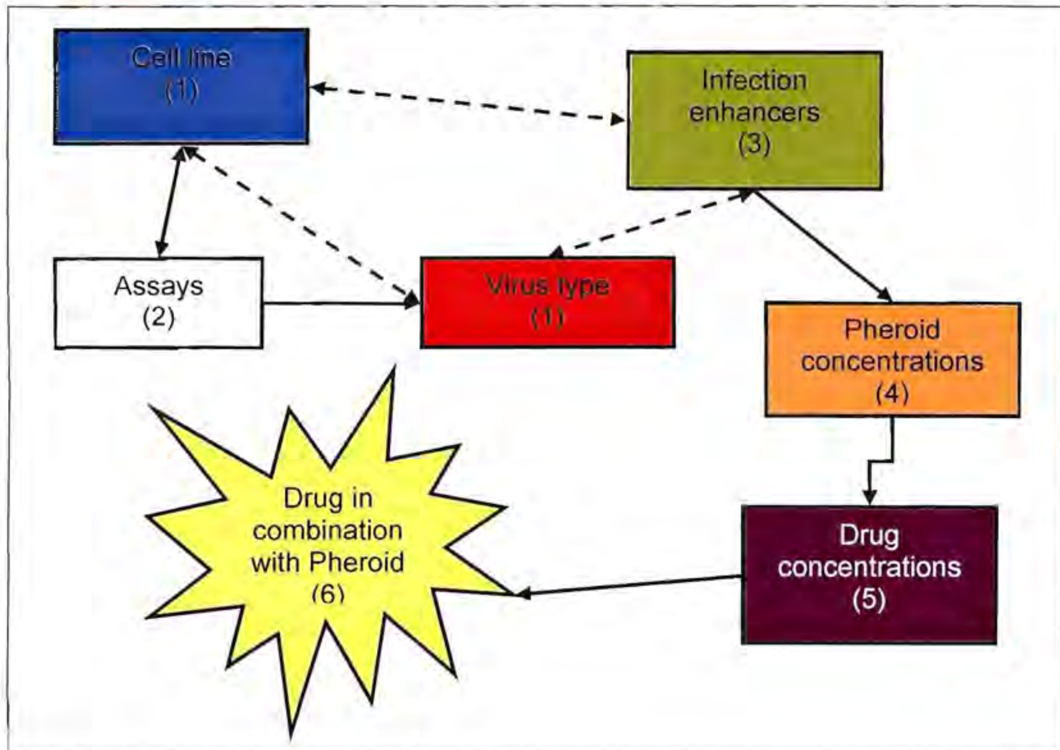



**Figure 4.1. Flowchart indicating the general procedure for this study. The cell line and virus were chosen first. A vicious circle was started when another cell line and virus type had to be chosen and the use of the infection enhancers was assessed. The next step was to determine the optimal ABC, 3TC and Pheroid concentration. Finally the ABC was entrapped in the Pheroid and the efficacy of this combination was determined.**

## 4.3 The effect of the Pheroid on p24-antigen ELISA

The anchorage-dependent GHOST.CXCR4.CCR5 cell line and CM9 virus (see section 3.2.2 and 3.2.3) were initially chosen to simplify experimentation. These adherent cells were easy to handle and would simplify methods for the shortened contact time with the Pheroid and drug. The expression of GFP made it easy to assess whether viral replication has taken place, without the need for intricate and expensive assays. If viral replication took place, the cells showed fluorescence under a fluorescence microscope. The fluorescence caused by the viral replication was not used as a quantitative analysis method to assess viral replication, but rather as an indication to assess whether adequate replication has taken place.

It was very important to establish the influence of the Pheroid™ on the analytical methods used to determine the amount of virus. The p24-antigen ELISA was used to assess the amount of virus.

### 4.3.1 Experimental design

GHOST cells (5 x $10^4$ cells/well) infected with CM9 virus was used. This experiment was done in triplicate on in a flat-bottom 48-well plate. The total volume of each well was 500 µl. Two hundred micro litres of the supernatant was removed and replaced by 200 µl of the diluted Pheroid. The Pheroid was diluted with complete growth medium. Incubation of the cells with the Pheroid continued for three days (at 37°C, 10 % $CO_2$). The viral infection was observed using a fluorescence microscope. The p24 antigen assay was performed as described in section 3.5.2 although poor infection was seen. Since the ELISA assay is very expensive; the replicates were pooled and 125 µl of this combination was assayed.

### 4.3.2 Results and Discussion

**Table 4.1. Summary of results to establish the influence of Pheroid on the p24-antigen assay (p24 antigen correlation curve $R^2$ = 0.999; n = 1).**

| Pheroid concentration | HIV p24-antigen values of infected GHOST cells incubated with Pheroid (pg/ml) | P24-antigen readings caused by cell-free Pheroid (pg/ml) |
|---|---|---|
| 0.0335 % | 65.891 | 0.907 |
| 0.0694 % | 49.617 | 0.851 |
| 0.1440 % | 15.384 | 0.944 |
| Average: | 43.631 | 0.901 |

Table 4.1 is a summary of the results obtained. The results obtained involving the cell-free Pheroid™ was significantly different (p = 0.027) and small enough to have no influence on the assay's ability to detect viral antigen. This indicates that the p24-antigen ELISA can be used for analysis with the Pheroid.

This was the first solo experiment. A possible reason for minimal viral replication levels was that the cells were manhandled; the cells were either damaged or washed out during the washing step. Still the common trend was that a higher Pheroid concentration rendered a lower virus-level as seen in Table 4.1.

## 4.4 Selection of the appropriate cell lines and virus types

### 4.4.1 Viral replication within the GHOST cells

The CM9 virus showed low replication rates in the GHOST cells as seen in Table 4.1. A p24-antigen value above 1 ng/ml was regarded as most favourable.

#### 4.4.1.1 Experimental design

In search of the optimal virus, three random viruses were incubated with the GHOST cell line for a total of three days (see Table 3.2 for the characteristics of the different viruses). The p24 antigen assay was performed according to section 3.5.2. Table 4.2 is a representation of the outcome of this experiment.

#### 4.4.1.2 Results and Discussion

Although SM1 showed the best viral replication within the GHOST cells, this is a clade B virus; therefore further experimenting with this virus was discontinued. Clade C is responsible for 60 % of the world's HIV infections (Requejo, 2006). SW7.TCLA, a clade C virus, was chosen instead but replication was still below optimal.

**Table 4.2. Viral infection of GHOST cells with the different virus types ($R^2$ = 0.996; n = 2).**

| **Virus types** | **SW7** | **SM1** | **DU179** |
|---|---|---|---|
| Viral infection (pg/ml) | 413 ± 32 | 535 ± 130 | 284 ± 4 |

### 4.4.2 The toxic effect of Polybrene in combination with the Pheroid

Genetically modified cells like the GHOST cell line are not readily infected with HIV. It is common protocol at the NICD to enhance infection by adding an infection enhancer such as polybrene (section 3.3).

#### 4.4.2.1 Experimental design

This experiment was done by plating 500 µl GHOST cells per well. The cells were infected overnight with the SW7.TCLA virus (with and without the addition of polybrene (30 µl in 6 ml cell-virus suspension). Two hundred microlitre supernatant was removed from each well and replaced with 200 µl of the diluted Pheroid on the day of the experiment. The cells were incubated for two hours with the diluted Pheroid. All the media were removed with a pipette and replaced with 500 µl new growth media. The cells were incubated for a total of three days after the Pheroid was removed. MTT analysis was done on 125 µl cell suspension according to the method described in section 3.5.1.

#### 4.4.2.2 Results and Discussion

Figure 4.2 is an indication of the effect of polybrene in combination with Pheroid on the viability of the GHOST cells.

Viability data is qualitative and presented as the percentage of viable cells in the experimental groups, compared to the viability of the negative control group. In other words: viability is an indication of the state of the living cells within the experimental group compared to the negative control group.

The viability of the cells incubated with the polybrene-Pheroid combination was significantly lower than in the presence of the Pheroid alone ($p < 0.01$). Polybrene enhances viral infection by neutralizing the charges of the negatively charged cell and virus surfaces (Fan *et al.*, 1992). One of the many functions of the Pheroid is to maintain the integrity of the cell's membrane (Grobler, 2004). The polybrene-Pheroid combination showed increased signs of cytotoxicity; a possible explanation could be that this combination severely disrupted the cell's membrane (see section 3.3). Despite the addition of polybrene to the cells, the viral replication was not notably enhanced for this experiment (from 412 pg/ml to 426 pg/ml).

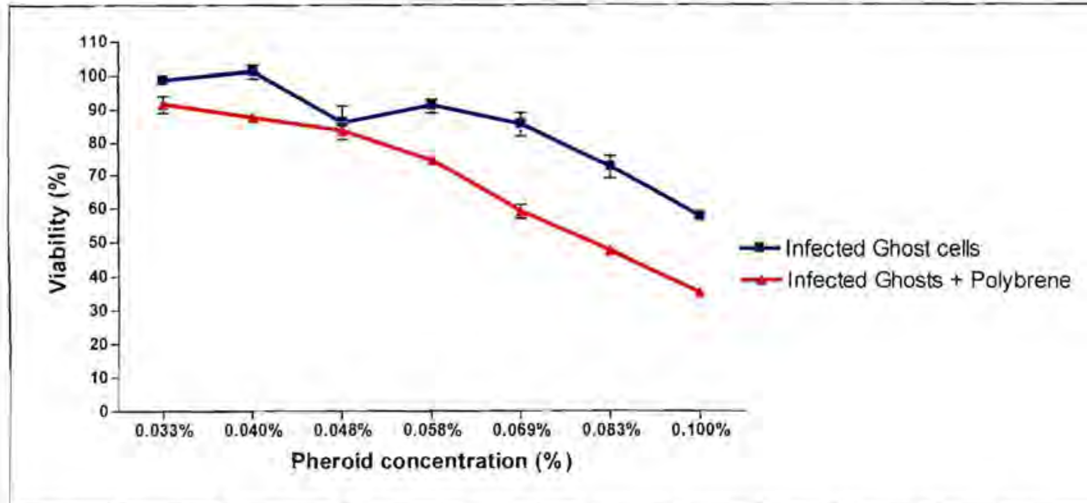



**Figure 4.2. The toxic effect of polybrene in combination with the Pheroid on the cells (n = 3).**

### 4.4.3 Selecting a new cell line and virus type

Selecting a new cell line and virus type were the only option to overcome the viral infection obstacle. Five cell lines and four different viruses were experimented with. Three of the cell lines (MT2, CEM and M7-Luc) were suspension cells and the other two (GHOST and U87 cells) were anchorage dependent cells (see Table 3.1). The viruses were all from clade C and utilized CCR5 co-receptors except SW7 which used the CXCR4 co-receptor for binding (see section 3.3). Different virus dilutions were experimented with.

#### 4.4.3.1 Experimental design

This experiment was done in 48-well plates to provide adequate surface for the anchorage-dependent cells to adhere. The suspension cells rendered a cell concentration of 7.5 x $10^4$ cells in each well, while the anchorage dependent cells rendered 5 x $10^4$ cells per well. The total volume of the wells was 500 μl. This volume comprised of 250 μl of the diluted virus. This experiment was not done in duplicate because of a virus stock shortage. The cells were incubated with the virus dilutions within a humidified incubatore at 37°C and 5 % $CO_2$ for three days. The p24 antigen assay was performed on all the cells at a virus dilution of one in twenty on day three. Two microlitres from the supernatant of each sample was diluted to 200 μl for p24 antigen analysis. A Zeiss Axiovert 25 fluorescence microscope was

used to observe the viral infection in the M7-Luc cells on day three. The cells were observed under a fluorescence microscope to inspect the number of "green" fluorescing cells. The GFP expression of the M7-Luc cells was used as a measure to determine the optimal time point at which luciferase should be measured. The luciferase assay was performed when approximately 10 % of the cells showed fluorescence.

#### 4.4.3.2 Results and Discussion

The M7-Luc cells incubated with the SW7.TCLA virus showed the best replication compared to the other cell lines and virus types (as seen in Figure 4.3). SW7 virus outrivaled the other virus types, since SW7 uses the CXCR4 chemokine co-receptor for binding. CXCR4-utilising viruses have the ability to infect and replicate rapidly within various cell lines compared to the other viruses which utilizes the CCR5 co-receptor (Vödrös *et al.*, 2001 and Björndal *et al.*, 1997) (see section 3.3).

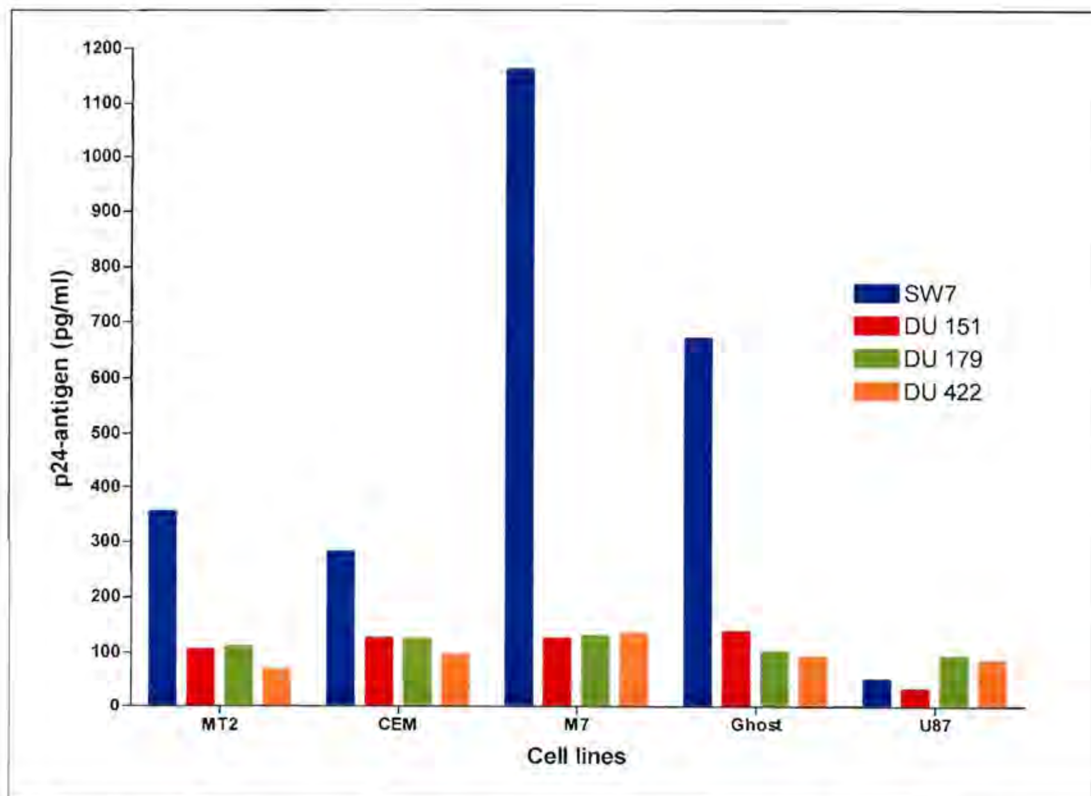



**Figure 4.3. Graph presenting the HIV p24-antigen values of the different virus types (dilution factor 20 x) incubated with the different cell lines for three days ($R^2$ = 0.997).**

Viral replication in the M7-Luc cells was also analysed using the luciferase assay. The ability to asses the viral replication in the M7-Luc cells with the luciferase assay was an added advantage of this cell line. The luciferase assay is less expensive and labour intensive than the p24 antigen assay.

The luciferase assay was done on day five (according to the method described in section 3.5.3) instead of day three when the p24 assay was done. There was not adequate viral infection on day three to produce a satisfactory GFP signal. It was decided to incubate all cells for a total of four days hence forth.

**Table 4.3. Viral replication in the M7-Luc cells (measured in RLU) at different virus dilutions for the different virus types incubated for five days (n = 1).**

| | **Virus types** | | | |
|---|---|---|---|---|
| **Dilution factor** | SW7 | DU151 | DU179 | DU422 |
| 2 x | 9176 | 223 | 345 | 21 |
| 10 x | 1327 | 36 | 21 | 34 |
| 20 x | 1130 | 30 | 26 | 32 |
| 100 x | 503 | 18 | 12 | 37 |
| 200 x | 126 | 26 | 21 | 27 |
| 1000 x | 101 | 40 | 26 | 26 |

Since this experiment was not done in duplicate, it would be unreliable to base the HIV infection levels on only one analysis method. For this reason the SW7 infection of the M7-Luc cell line was assessed with both the luciferase and the p24 antigen assay to determine the optimal virus dilution factor on day five (figure 4.4). The two methods showed acceptable correlation, but variations would probably have been reduced with increased sample sizes. Since the p24 antigen assay was very expensive and labour intensive the luciferase assay was used as the sole method to assess the HIV replication in the M7-Luc cells, except when confirmation of the

results was necessary. The final dilution factor chosen for the SW7.TCLA was two times.

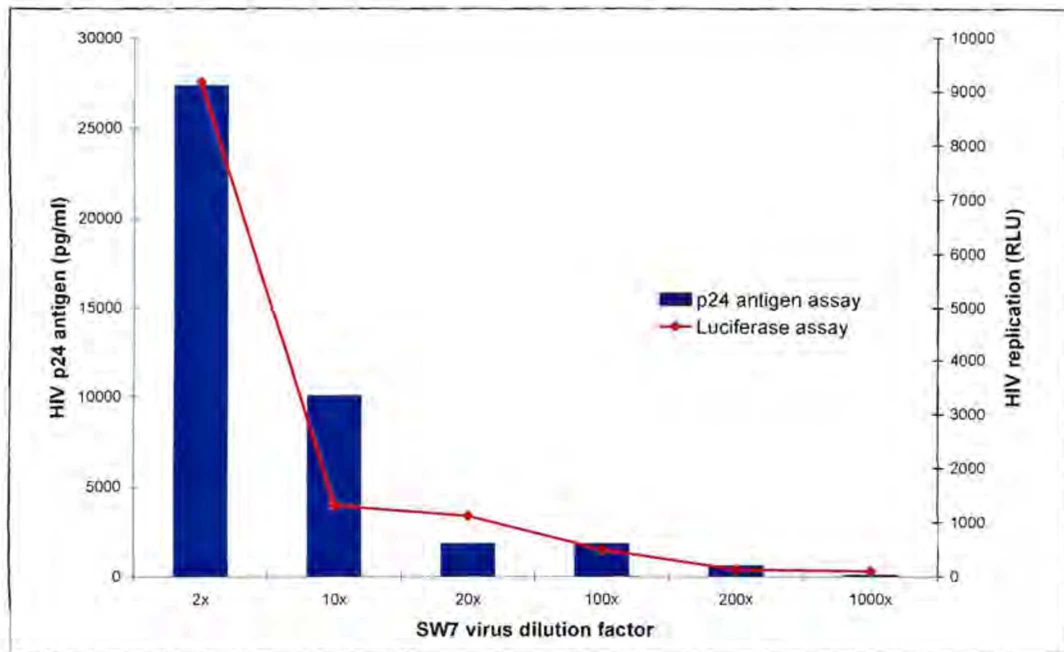



**Figure 4.4. Graph comparing the HIV p24-antigen to the luciferase values obtained for SW7 infection at different dilutions in M7-Luc cells, incubated for five days (n = 1).**

## 4.4.4 The toxic effect of DEAE-Dextran in combination with the Pheroid

The same issue suggested itself with the M7-Luc cells than with the GHOST cells: could DEAE-Dextran (another infection enhancer) be used in combination with the Pheroid? Acceptable viral replication was seen when M7-Luc cells was incubated with the SW7 virus, but it would be most favourable if the infection could be enhanced even more.

### 4.4.4.1 Experimental design

Uninfected M7-Luc cells ($7.5 \times 10^4$ cells in 250 µl per well) were provided with DEAE-dextran (1.32 µl/ml) and/or Pheroid (0.04 % (w/v)) and incubated for one, two, four and twenty-four hours. The cells were centrifuged (at 1500 rpm for ten minutes at room temperature) and washed three times with PBS to remove all DEAE-dextran and/or Pheroid present. The cell viability was analysed using MTT after twenty-four hour incubation.

The washing step was significantly more difficult and time consuming for the suspended M7-Luc cells compared to the adherent GHOST cells and there was an added risk that cells were washed out or damaged in the process.

#### 4.4.4.2 Results and Discussion

Figure 4.5 shows that there was a significant difference between the negative control group (M7-Luc cells) and the group that was incubated with DEAE-dextran and Pheroids in combination ($p < 0.01$). There was no difference in the viability of the cells between the groups incubated with DEAE-dextran or Pheroid alone, compared to the negative control group and to each other. This indicates that the Pheroid in combination with DEAE-Dextran probably induced cytotoxicity by disrupting the cell membranes. Therefore DEAE-Dextran can not be used to enhance the virulence in the presence of the Pheroid.

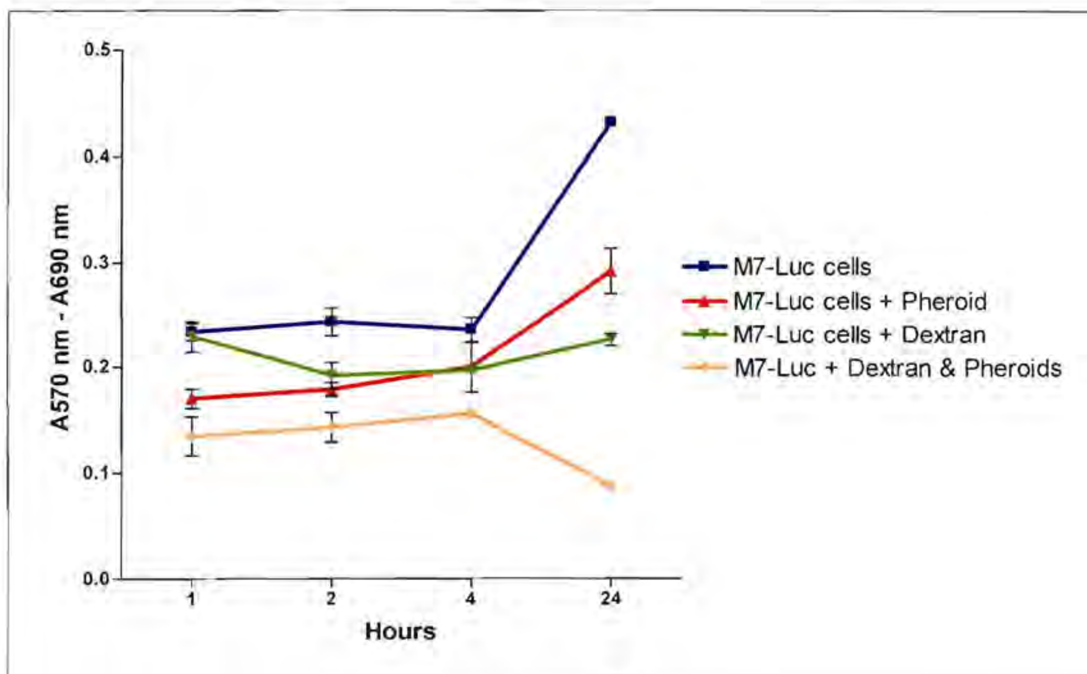



**Figure 4.5. Graph showing the MTT absorbance of uninfected M7-Luc cells after incubation for just one day. The Pheroid and/or DEAE-dextran were washed out after the indicated time to asses the effect of the constituents on the cells (n = 3).**

## 4.5 Pheroid concentrations

### 4.5.1 Filtration

Sterilisation is crucial for experimenting *in vitro*. Pathogens can be introduced into the wells by either poor technique or un-sterile additions to the cells. The growth medium contains antibiotics to fight against bacteria, fungi, yeasts and moulds but resistance may develop. Sterilisation by gamma-rays rendered stavudine useless as seen by Botha (2007), therefore this method of sterilisation was discontinued.

#### 4.5.1.1 Experimental design

Pheroids was filtered using a 0.8 μm filter. The size of the unfiltered and filtered Pheroid was determined with a Malvern particle size analyser. Prof A.F. Kotze operated the Malvern.

#### 4.5.1.2 Results and Discussion

The Pheroids are elastic as seen in figure 4.6 A & B. The problem with filter sterilisation was that a decrease in Pheroid concentration after filtration was visible with the naked eye; the suspension seemed less milky than before. This visible decline could also be explained by the decrease in vesicle sizes that were declining and were less obvious to see with the naked eye. The vesicle sizes and concentration would be influenced by the size of the filter, the strength with which the Pheroid is forced through the filter and also the age of the Pheroid since the vesicle sizes decline as the Pheroid matures (Uys, 2006). The Pheroids are elastic and can self-assemble (Uys, 2006) after breakage caused by filtration to render new vesicles, but since the Pheroids were diluted with media prior to filtration there were no certainty that the necessary amount of $N_2O$-gas was still within the system to ensure proper assembly and stability of the Pheroid vesicles (see Chapter two).

The Pheroid used during the term of this study was manufactured by different persons within a research environment. For this reason it was difficult to maintain uniformity of the Pheroid batches. See Addendum D for more results obtained by using the Malvern particle sizer with different Pheroid batches. The median particle

sizes varied from 1.059 µm to 1.747 µm. The process and equipment used for Pheroid formulation is critically important (Grobler *et al.*, 2007).

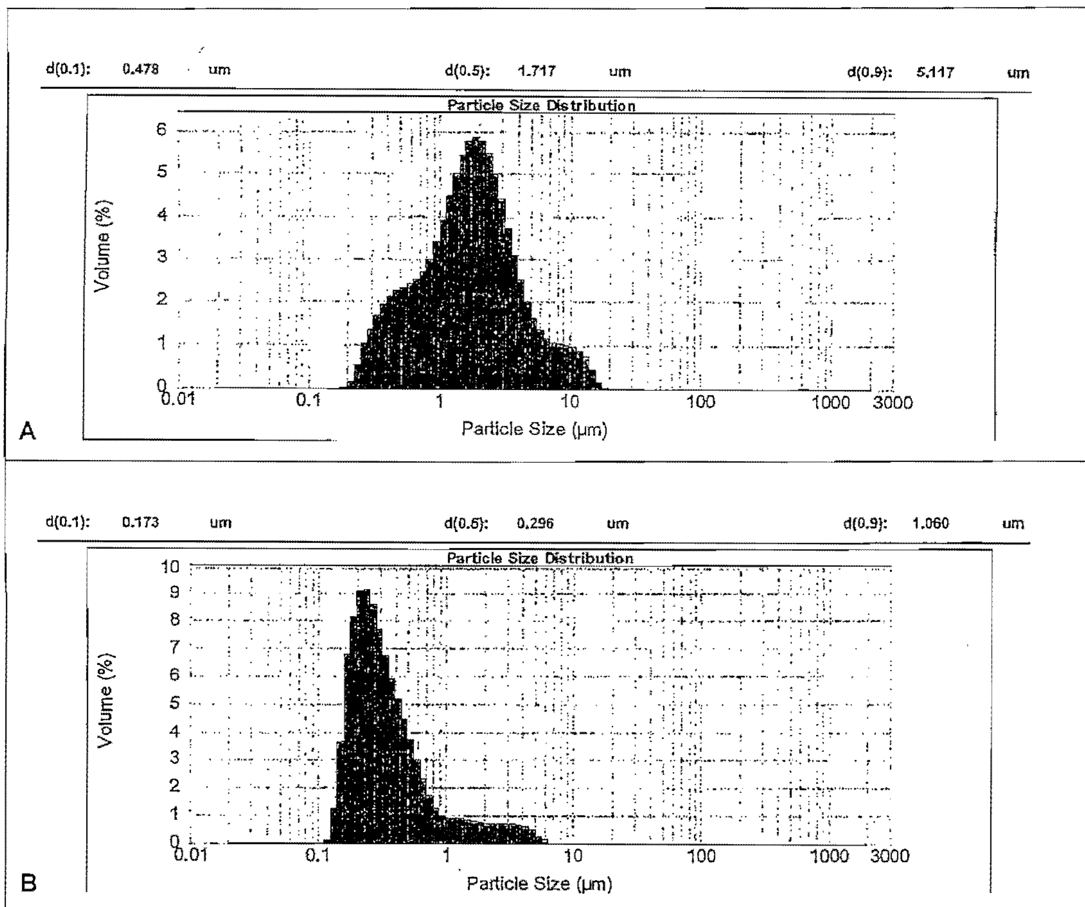



**Figure 4.6. Pheroid vesicle size determined using a Malvern particle sizer. This was done using the same Pheroid batch. A. The vesicle sizes (median = 1.717 µm) of Pheroids before filtration. B. Vesicle size (median = 0.296 µm) of Pheroids after filtration.**

## 4.5.2 Enhanced viability of the cells

An interesting observation was made when M7-Luc cells were incubated with relatively new Pheroids at low concentrations for three hours.

### 4.5.2.1 Experimental design

M7-Luc (7.5 x $10^4$ cells in a total volume of 250 µl per well) were incubated with 100 µl Pheroid for three hours before the cells were washed and centrifuged (1200 rpm for ten minutes) three times with cold PBS (± 5°C; retrieved from the refrigerator just before addition). The cells were incubated till day four. The MTT assay was done to asses the viability of the cells.

#### 4.5.2.2 Results and Discussion

The viability of the cells was significantly enhanced by the Pheroid formulation in comparison to the negative control group. This phenomenon was observed several other times although they were washed with media at room temperature. A possible explanation for the drastically enhanced viability of the cells in this specific study (Figure 4.7) was that the cells used the fatty acids within the Pheroid as additional energy to overcome the abuse of being washed by cold PBS in contrast to the control group which did not have the needed energy. See poster attached as Annexure A.2.

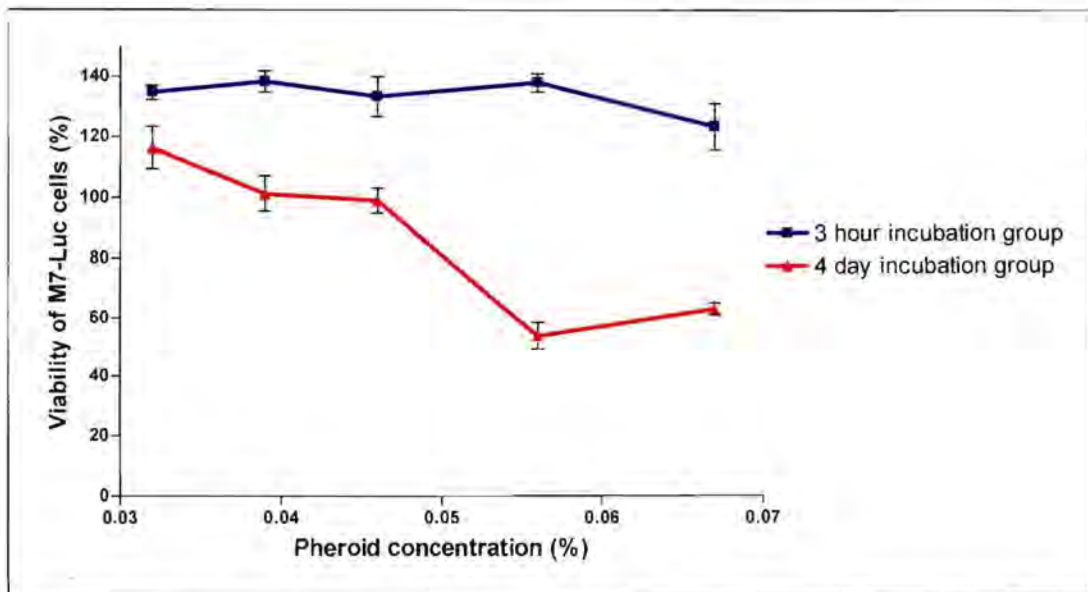



**Figure 4.7. Enhanced viability caused by incubation with newly made Pheroid at low concentrations and low incubation times (n = 3).**

### 4.5.3 Cytotoxicity of matured Pheroids on M7-Luc cells

In search of the optimal Pheroid concentration it was observed that different results were produced every time the experiment was done, even though the same batch was used. Therefore the results seen in Figure 4.7 were difficult to reproduce exactly.

### 4.5.3.1 Experimental design

Different dilutions of Pheroids (100 µl) were added to the 150 µl M7-Luc cells ($7.5 \times 10^4$ cells per well). The Pheroid was incubated with the cells for a total of four days or was washed out after three hours. The cells were washed and centrifuged (for five minutes at 2200 rpm) three times with growth media. MTT analysis was done on day four according to the methods described in section 3.5.1. The cells were also observed and photographed (with a Nikon Coolpix 990) under a Nikon TS100 inverted microscope (at a magnification of 40 x) to assess the structural differences between the cells.

### 4.5.3.2 Results and Discussion

When the results of the three experiments done at the three hour incubation period, were placed onto one graph; an interesting trend was seen (Figure 4.8). The viability of the cells decreased as the Pheroids matured. A significant difference was seen between the different matured-states of the Pheroid. The cytotoxicity caused by the Pheroid was extremely significant at day 29 compared to the nine day group ($p < 0.01$,) and nineteen day group ($p < 0.05$). There was not a significant difference between the nine and the nineteen days old Pheroids.

Increased viability of the M7-Luc cells were seen (at day nine and twenty nine) after four day incubation with the Pheroid instead of the expected cytotoxicity in Figure 4.9.

The cells used in Figure 4.9 (9 days old Pheroid) were observed under a microscope to asses the cell count and morphology. The photographs are portrayed in Figure 4.10. Figure 4.10 B shows decreased cell count caused by incubation for four days with 0.027 % (w/v) Pheroid. Unfortunately this phenomenon was not pondered on right away.

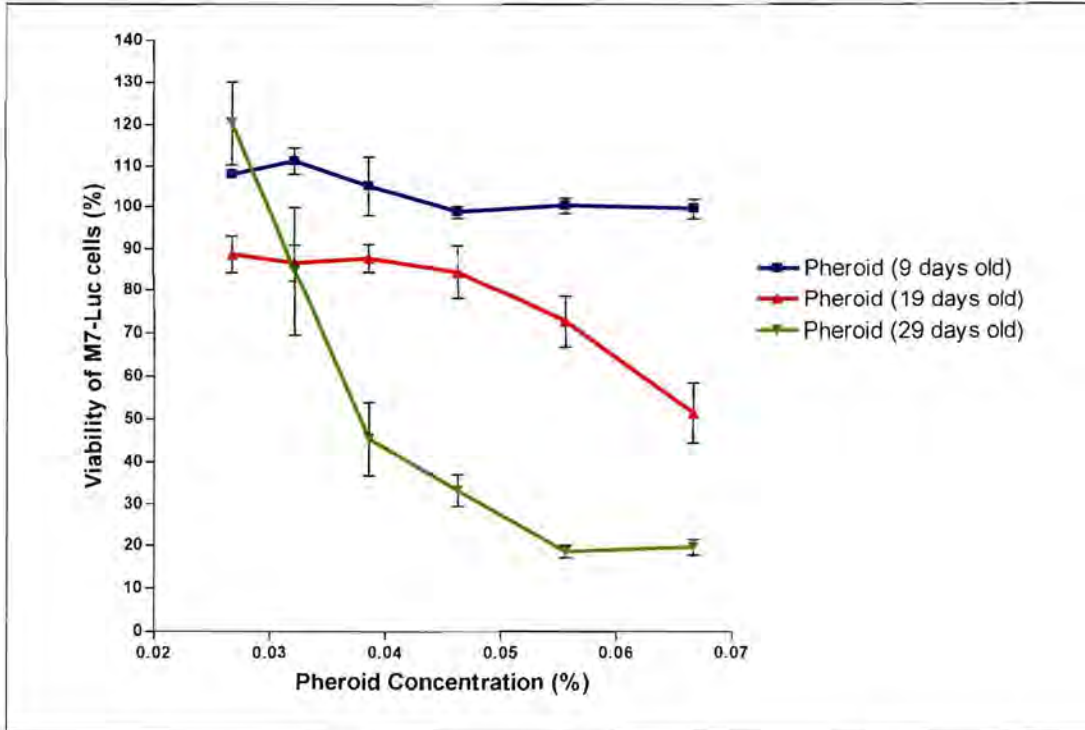



**Figure 4.8. Viability of uninfected M7-Luc cells incubated for three hours with the same Pheroid (batch V08016) containing no antioxidants at different dates (n = 3).**

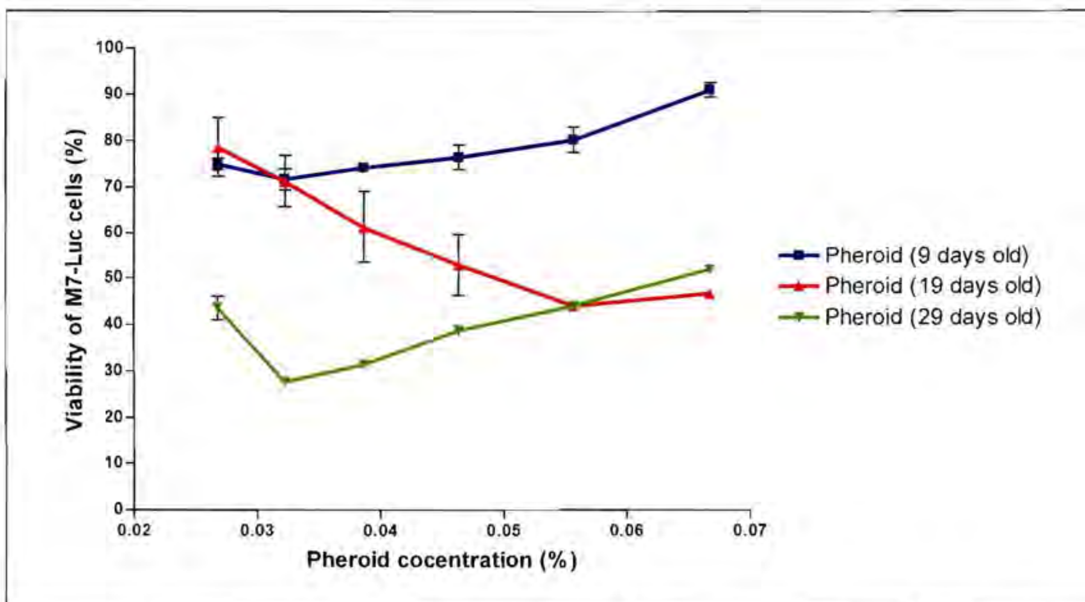



**Figure 4.9. Viability of uninfected M7-Luc cells incubated with Pheroid for four days. The same Pheroid batch containing no antioxidants, were used at different dates (n = 3).**

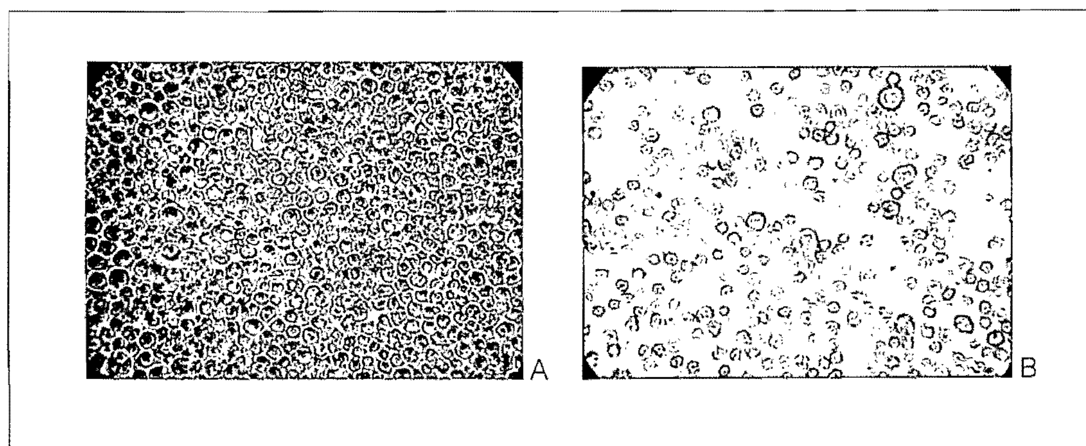



**Figure 4.10. Images of the suspended M7-Luc cells. A. Is the image of the negative control group after four days. B is the image of the cells incubated with Pheroid (9 days old) at a concentration of 0.027 % (w/v) for four days.**

## 4.5.4 Cytotoxicity of matured Pheroids on GHOST cells

The use of the GHOST cell line was not discontinued. This cell line functioned as an additional experimental group to asses the effect of the Pheroid on an anchorage dependent cell line and to evaluate the results obtained with the M7-Luc cells.

### 4.5.4.1 Experimental design

The same Pheroid batch was used on the GHOST cells as the M7-Luc cells in section 4.5.3. The diluted Pheroids (200 μl) were incubated with the cells (300 μl) for four days or three hours. The cells were washed and centrifuged three times with media to remove any Pheroids left after incubation for three hours. MTT analysis was performed on day four according to the methods described in section 3.5.1. The cells were also observed and photographed (with a Nikon Coolpix 990) under a Nikon TS100 inverted microscope (at a magnification of 40 x) to assess the structural differences between the living and the dead cells incubated with a high Pheroid concentration for the total four days.

### 4.5.4.2 Results and Discussion

The GHOST cells showed no significant difference between the three hour and four day incubation groups except at 29 days with the four day incubated group, as seen

in Figure 4.11. These cells did not show significantly increased viability as was the case with the M7-Luc cells at low Pheroid concentrations. The cell viability did not increase as dramatically after four day incubation at the high Pheroid concentrations like the M7-Luc cells. Figure 4.12 portrays the photos taken of the GHOST cells on day 19. Figure 4.12.A. is the cell control group. The cells are difficult to see with the naked eye, since they are a flat layer upon the surface. Figure 4.12.B. is the image of the cells incubated with a toxic Pheroid concentration (0.067 % (w/v), 19 days old) for four days. Take special notice that the individual cells can be seen and the cell membranes seem shrivelled-up.

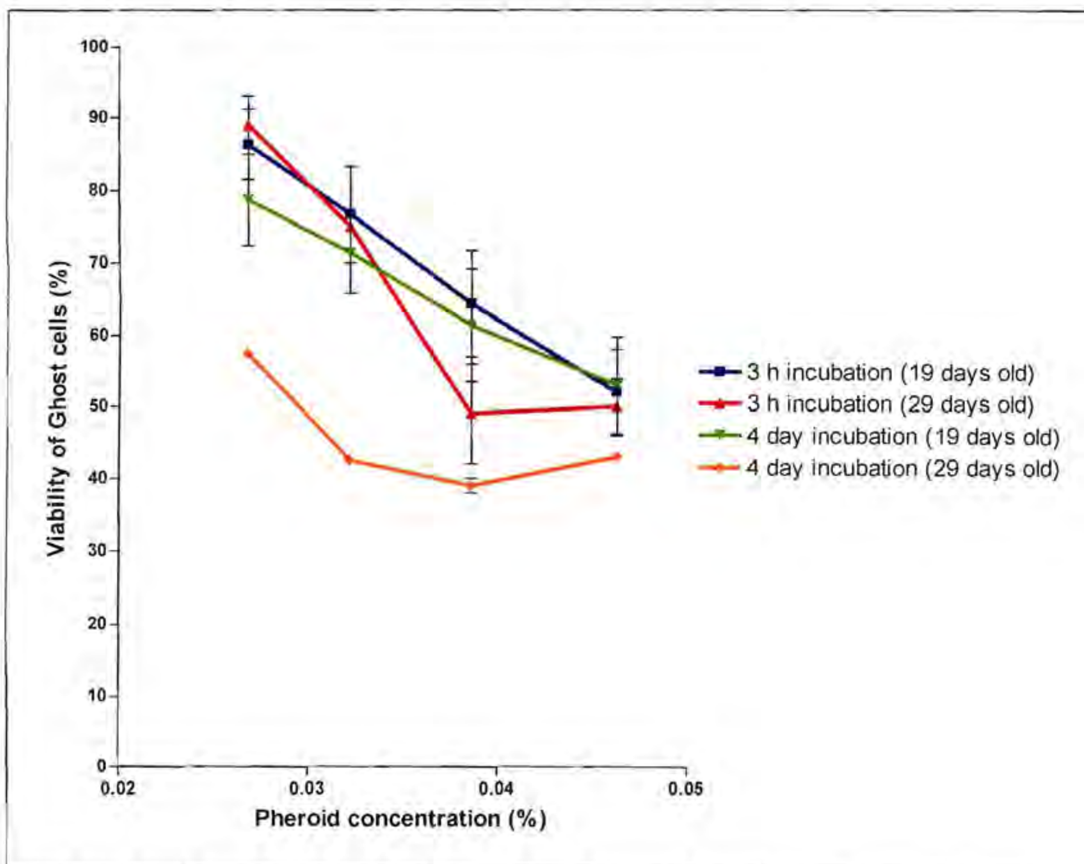



**Figure 4.11. The same Pheroid (batch V08016) containing no antioxidants were incubated with the uninfected GHOST cells at different dates (n = 2).**

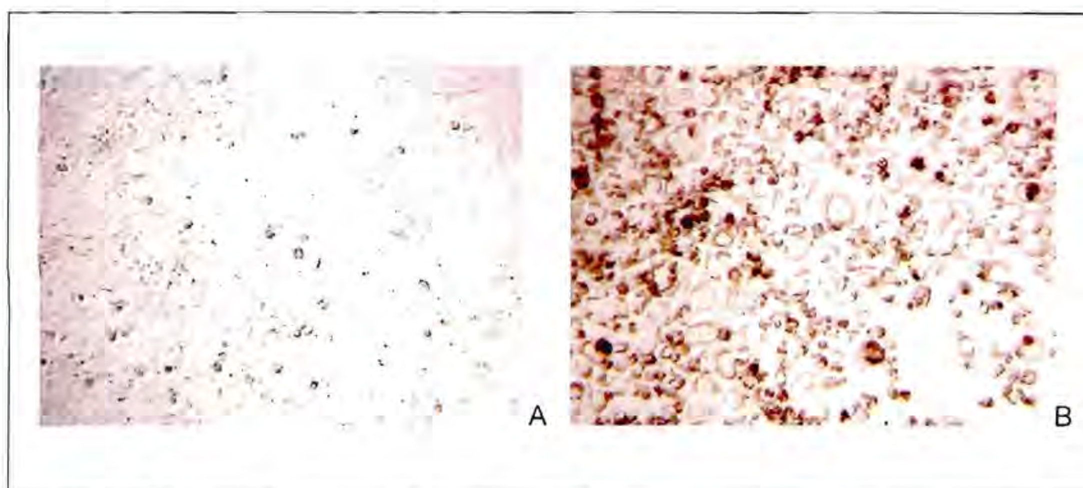



**Figure 4.12. Images of the adherent GHOST cells taken for the 19 day group. A. Is the image of the negative control group after four days. B is the image of the cells incubated with a toxic Pheroid concentration (0.046 % (w/v)) for four days.**

## 4.5.5 Antioxidation agents

The final Pheroid formulation for oral administration will have to contain antioxidants. The final formulation for entrapment of antiretroviral drugs was still not decided on, but development of this formula was taking place while this study was in progress. Kühn (2008) observed that the ABC entrapped in pro-Pheroids was stable, but further studies will be necessary to conclude the results. Since it was observed in section 4.5.3 and 3.5.4 that the Pheroid vesicles became more toxic over time antioxidants were added to all future Pheroid batches for this study. For this reason the effect of antioxidation agents (BHA and BHT) had to be assessed. Two Pheroid batches were produced on the same day. BHA (0.025 % w/w) and BHT (0.25 % w/w) were added to one batch (V08018). Another Pheroid batch (V08017) was made to evaluate the effect of the added anti-oxidation agents compared to the absence thereof on the cells.

### 4.5.5.1 Experimental design

Both M7-Luc (7.5 x $10^4$ cells in 250 µl per well) and GHOST (5 x $10^4$ cells in 500 µl per well) were prepared for this study. The Pheroids were added to the cells in the usual 40 % ratio to the total media volume. The three hour group were centrifuged

(2200 rpm for five minutes) and washed three times with media to remove any Pheroids from the wells.

#### 4.5.5.2 Results and Discussion

**Table 4.4. Viability (%) of M7-Luc and GHOST cells after incubation with Pheroid with added anti-oxidation agents and the absence thereof for three hours or four days (n = 3).**

| | Pheroid concentration (% (w/v)) | Three hour incubation | | Four day incubation | |
|---|---|---|---|---|---|
| | | Anti-oxidation agents absent | Anti-oxidation agents added | Anti-oxidation agents absent | Anti-oxidation agents added |
| M7-Luc cells | 0.023 | 95 ± 2 | 100 ± 4 | 95 ± 2 | 93 ± 2 |
| | 0.030 | 90 ± 1 | 95 ± 4 | 96 ± 2 | 91 ± 7 |
| | 0.039 | 96 ± 2 | 96 ± 3 | 92 ± 1 | 84 ± 1 |
| | 0.051 | 98 ± 1 | 94 ± 5 | 67 ± 1 | 90 ± 2 |
| | 0.067 | 102 ± 0 | 106 ± 3 | 62 ± 2 | 116 ± 5 |
| GHOST cells | 0.023 | 97 ± 1 | 104 ± 9 | 82 ± 4 | 68 ± 1 |
| | 0.030 | 85 ± 0 | 92 ± 5 | 75 ± 7 | 55 ± 1 |
| | 0.039 | 85 ± 1 | 90 ± 5 | 65 ± 0 | 52 ± 1 |
| | 0.051 | 81 ± 0 | 89 ± 0 | 63 ± 3 | 55 ± 1 |
| | 0.067 | 86 ± 0 | 84 ± 2 | 54 ± 2 | 57 ± 0 |

According to GraphPad® Instat, there were no significant statistical differences between the two Pheroid formulations on the viability of the M7-Luc cells, but the GHOST cells were a bit more sensitive. The GHOST cells only showed a significant difference between the three hour and four day incubation group's viability.

According to these results the addition of anti-oxidation agents were deemed safe to use for these cell cultures.

The Pheroid batch (V08018) which contained the antioxidants turned bright yellow some days after the Pheroid was made (as seen in Figure 4.13). A possible reason for this was that the antioxidants themselves were oxidised and that this reaction produced the yellow colour. The Pheroid batch which did not contain antioxidants remained white. In future, cognizance will also have to be taken of the shelf life of each of the raw materials used.

**Figure 4.13. Photograph taken of two Pheroid batches produced on the same day. V08017 was produced without additional antioxidants. V08018 was produced with added antioxidants (BHA and BHT). This picture was taken one month after the Pheroids was made.**

## 4.6 ABC and 3TC concentrations

Daluge (1997) determined ABC's *in vitro* cytotoxicity and sensitivity to HIV. The mean $CC_{50}$ within CEM cell lines was 160 µM and the mean $IC_{50}$ of sensitivity was 0.26 µM, measured with HIV-infected PBLs. The IC50 of 3TC ranged between 0.370 to 1.31 µM in MT4-cells. The $IC_{50}$ of 3TC ranged between 0.370 to 1.31 µM in MT4-cells. The $CC_{50}$ of 3TC was also determined in MT4-cells as 405 µM (Soudeyns *et al.*, 1991).

### 4.6.1 Experimental design

ABC and 3TC's toxicity and HIV's sensitivity to them, was evaluated within M7-Luc cells infected with SW7 virus. M7-Luc is a clone of CEM cells, which was used by Daluge for toxicity experimentation. PBLs were not used for this study; therefore HIV's sensitivity was analysed in M7-Luc, a T-lymphocyte cell line.

ABC and 3TC was dissolved in growth medium and filtered with a 0.2 µm filter to remove any debris to render a stock solution. Dilutions were also done using growth medium. One hundred microlitres of the diluted ABC or 3TC was added to 150 µl of infected or uninfected cells. The cells were incubated (37°C, 10 % $CO_2$) for a total of four days with the antiretroviral drugs. The MTT viability assay and luciferase assay was done to asses the efficacy of the drugs.

### 4.6.2 Results and Discussion

ABC and 3TC caused minor cytotoxicity at the concentrations used for this study, as was seen in Table 4.6.

The concentration in Figure 4.14 is given as the log value of ABC's and 3TC's concentration; this value and the coordinating concentration is given in Table 4.5.

To determine the $IC_{50}$ value of ABC and 3TC, the results were normalised and transformed to give a sigmoid curve using GraphPad® Prism. The $IC_{50}$ value of ABC and 3TC was extrapolated and determined as 0.2 and 0.023 µM respectively by GraphPad® Prism (Figure 4.14). This value differs from that of Daluge's and Soudeyns's findings, but $IC_{50}$ can differ from one cell line and virus type to another.

**Table 4.5. Cell toxicity caused by incubation with ABC or 3TC for four days indicated as the percentage viable cells (n = 4).**

| ABC Concentrations (μM) | Log ABC (log μM) | Viability average and SEM (%) of ABC | Viability average and SEM (%) of 3TC |
|---|---|---|---|
| 0.0001 | -4.000 | 91 ± 3 | 100 ± 1 |
| 0.001 | -3.000 | 90 ± 3 | 87 ±2 |
| 0.005 | -2.301 | 89 ± 3 | 84 ±2 |
| 0.01 | -2.000 | 89 ± 3 | 84 ±1 |
| 0.05 | -1.301 | 89 ± 4 | 85 ±1 |
| 0.1 | -1.000 | 88 ± 4 | 85 ±1 |
| 0.5 | -0.301 | 86 ± 2 | 85 ±1 |
| 1 | 0.000 | 85 ± 2 | 84 ±1 |
| 5 | 0.699 | 90 ± 4 | 83 ±1 |
| 10 | 1.000 | 92 ± 4 | 84 ±3 |

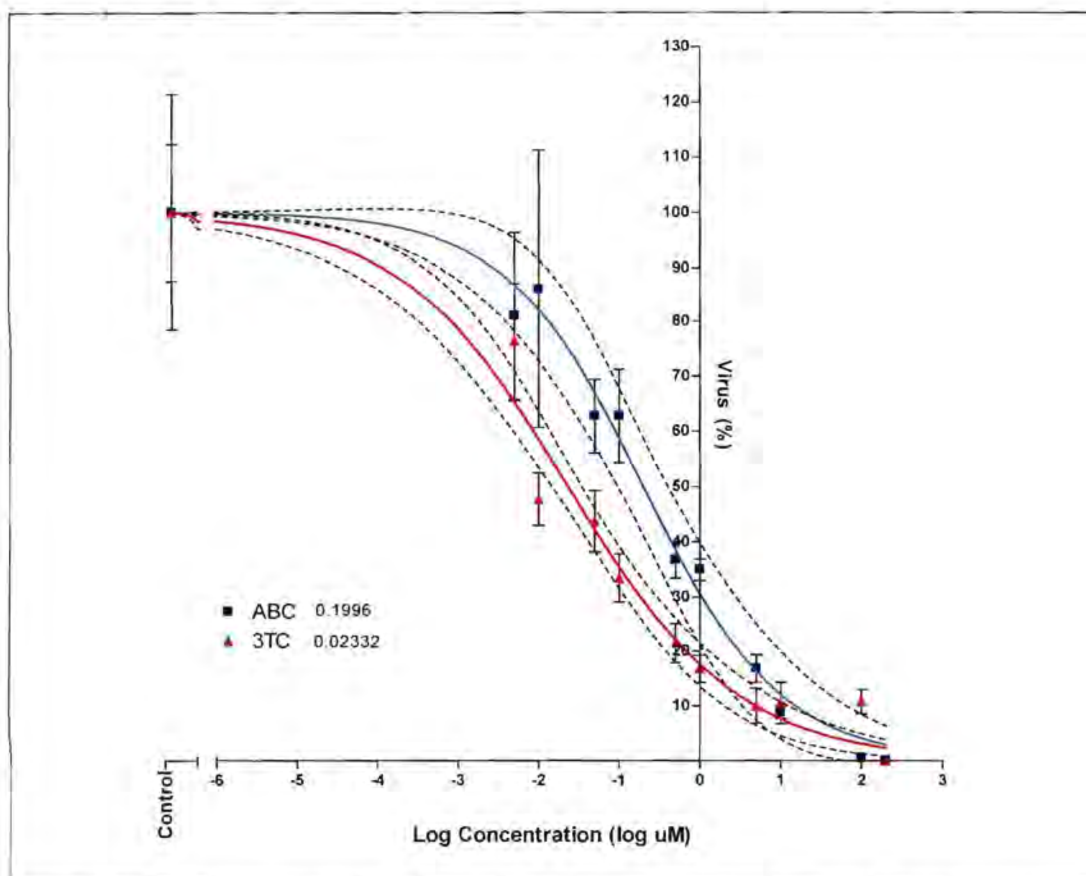



**Figure 4.14. $IC_{50}$ of ABC and 3TC incubated for four days in M7-Luc cells infected with SW7 for four days (n = 4).**

## 4.7 Combination of ABC with Pheroid

### 4.7.1 Experimental design

Evaluating the efficacy of the ABC entrapped within the Pheroid was problematic. The ABC was entrapped for one hour before it was added to the cells during this experiment. Previous experiments (results not shown here) were done with Pheroid not containing anti-oxidants and the ABC was entrapped for periods shorter than one hour. These entrapment times were insufficient, but it was not possible to entrap the ABC for any longer than an hour because of time constraints in the lab. M7-Luc cells infected with SW7-virus were used for this experiment. A stock solution of ABC and a 0.033 % (w/v) solution of Pheroid were prepared and diluted with growth medium. The total volume of each well was 250 µl; 100 µl of this volume consisted of

the ABC/Pheroid combination. The cells were incubated with the ABC/Pheroid combination for the total of four days.

### 4.7.2 Results and Discussion

Figure 4.15. shows a steep decline in viral replication levels in the cells incubated with the Pheroid. The two assays did not show good correlation, but it was acceptable.

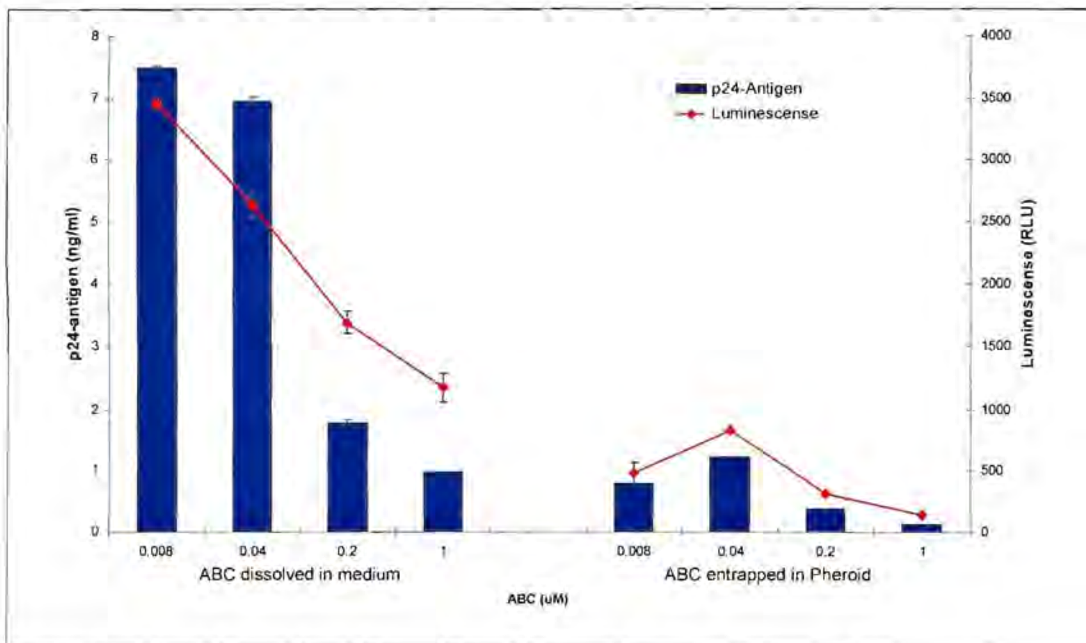



**Figure 4.15. Viral replication measured with the p24-antigen assay ($R^2$ = 0.997) and luciferase assay, within the M7-Luc cells after incubation with ABC dissolved in media or entrapped (for one hour) in the Pheroid (n = 3). The incubation period was 4 days.**

In order to asses the immense viral inhibition in Figure 4.15 caused by the Pheroid the viability of the cells were measured with the MTT assay.

Enhanced cell viability was noted (Figure 4.16) with the ABC entrapped within the Pheroid compared to the ABC-media solution ($p < 0.01$).

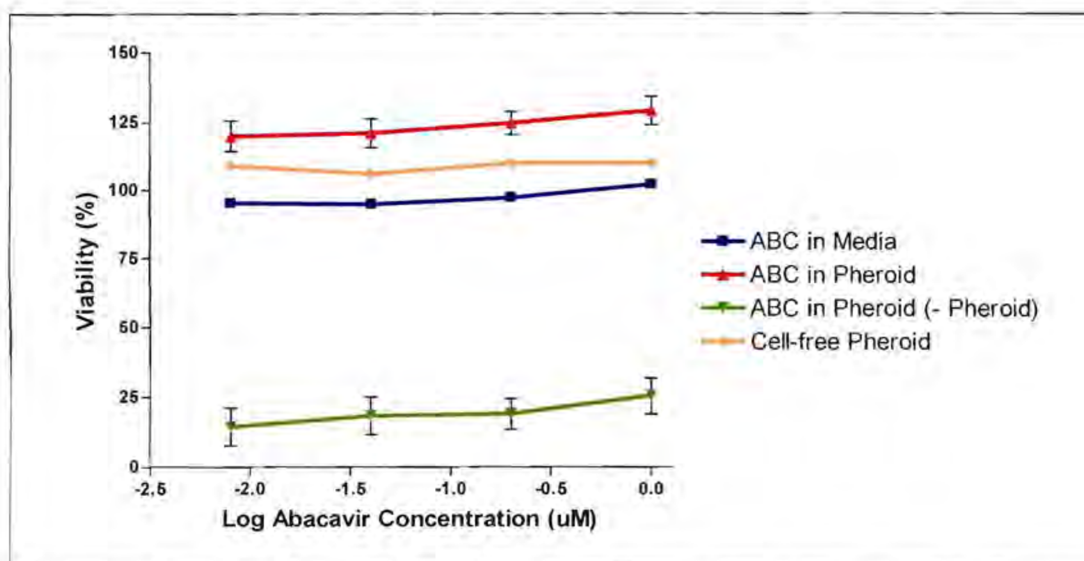



**Figure 4.16. Cell viability of cells incubated for four days with ABC in medium (blue line) or Pheroid (0.033% w/v) (red line) measured with the MTT viability assay. The Pheroid containing the ABC was also analysed in a cell-free system (orange line). The green line represents the viability of the cells after the influence of the Pheroid was subtracted.**

To ensure that the cell viability was correct, the same ABC entrapped Pheroid formulation was analysed in a cell-free environment to establish any interference with the MTT viability assay. The cell-free Pheroid reduced the MTT to the dark blue formazan and produced results ± 10 % higher than the cell control's values ($p < 0.01$). It is important to note that this Pheroid was made with added anti-oxidants.

If the cell-free Pheroid values were subtracted from the values obtained from the ABC entrapped Pheroid within the M7-Luc cells, it resulted in very poor cell viability ($p < 0.01$). This reduced cell viability portrayed the true cell count seen under the microscope. This firstly shows that the Pheroid interferes with the MTT assay and secondly that the Pheroid concentration used was still too high.

Vitamin E and antioxidants such as BHA and BHT found within the Pheroid possess intrinsic reductive potential; it can reduce the MTT tetrazolium ring to the insoluble dark blue formazan (Bruggisser *et al.*, 2002). This is probably the reason for the enhanced viability observed when the Pheroid was present during the MTT assay. To minimise the interference of the Pheroid with analytical methods the Pheroid will have to be removed prior to analysis by a washing step. For this reason the

enhanced viability observed in the three hour incubation group can be trusted since the Pheroid was removed from the cells prior to analysis.

## 4.8 Conclusion

Two different transfected continuous cell lines were chosen for this study; namely the anchorage dependent GHOST cells and the suspended M7-Luc cells. Both these cell lines were best infected with the SW7 virus in the absence of infection enhancers. Infection enhancers were not added to the cells to improve viral infection since it was observed that the Pheroid in combination with DEAE-dextran or Polybrene showed signs of cytotoxicity. A possible explanation was that this combination severely damaged the cells by disrupting the cell's membrane.

Insufficient GFP expression was observed on day three. This indicated that inadequate viral replication have taken place. The total incubation time was extended from three days (as proposed by Botha, 2007) to four days.

Sterile conditions were absolutely critical for *in vitro* analysis. But sterilisation of the Pheroid by filtration was discontinued since the repeatability of the Pheroid concentration was questionable.

ABC was chosen as the test substance for this study since it showed low cytotoxicity in cell cultures (Daluge *et al.*, 1997) and is water soluble which would not present solubility problems in growth media. The cytotoxicity and efficacy of 3TC was also determined. The drugs were dissolved and both the drug and/or Pheroid were diluted in growth medium to simplify experimentation. ABC was entrapped in the Pheroid and its *in vitro* efficacy and toxicity was tested on HIV-infected and uninfected cell cultures.

Incubation of the M7-Luc cells with low Pheroid concentrations for short incubation times revealed enhanced viability of the cells in comparison to the control group's cells. Cell viability of the M7-Luc cells were also enhanced in a concentration dependent manner after four day incubation with the Pheroid, although decreased cell numbers was observed with a microscope as the Pheroid concentrations increased. Unfortunately this phenomenon was not pondered on right away. It was also observed, while establishing the optimal Pheroid concentration that the cell

viability of the cells which was incubated for three hours with the Pheroid, decreased as the Pheroid batch matured. For this reason, all future Pheroid batches were made with added anti-oxidants.
The $IC_{50}$ of ABC (dissolved in medium) was determined as 0.2 μM in M7-Luc cells infected with the SW7 virus. This correlated with Daluge's $IC_{50}$ of 0.26 μM determined in HIV-infected PBL's. The $IC_{50}$ of 3TC was also determined in SW7 infected M7-Luc cells; it was measured as 0.023 μM. This value differed greatly with Soudeyns's findings, but $IC_{50}$ can differ from one cell line and virus type to another. Neither ABC nor 3TC were toxic to the cells at the concentrations used for this study.

Evaluating the efficacy of the ABC entrapped in the Pheroid was problematic. Development of a method for the determination of drug loading and drug release from the Pheroid is necessary for future studies (also mentioned by Lubbe, 2007). The ABC was entrapped within the Pheroid, an hour before this emulsion was added to the cells. Entrapment of one hour or less may not be sufficient.

The efficacy of ABC entrapped within the Pheroid is still inconclusive and further studies will have to be done.

# Chapter 5:

## Summary and Conclusion.

This section will conclude the contents of this study and provide suggestions for future investigation with Pheroid™ technology.

## 5.1 Introduction

South Africa is the country with the largest number of HIV infections in the world. There is still no cure for HIV-1 infection, but anti-HIV medicine can prolong life and enhance the quality of life of an HIV infected person. Patient adherence to antiretroviral therapy is extremely low due to the severe side-effects caused by these drugs. This is one of the causes of resistance of HIV to these drugs. Pheroid™ technology is a patented delivery system. The entrapment of an active within the Pheroid™ would generally provide a safer, more effective formulation than the active alone (Grobler, 2004). This could mean that the amount of drug needed for treatment of HIV can be decreased while producing lower adverse effects and reducing the price of treatment. Pheroid vesicles were used during this study.

## 5.2 Summary

The main purpose of this study was to optimise and validate *in vitro* cell viability and efficacy assays. The MTT assay was used to asses the viability of the cells and to determine the toxicity of the antiretroviral drugs and Pheroid™ on the cells. The p24-antigen and luciferase assays were used to determine *in vitro* efficacy. Several problems presented itself while evaluating the efficacy of ABC and/or the Pheroid.

Practical and logistical problems were experienced in the cause of the study. One of the major problems was that the Pheroid was manufactured by the Phertech group in Potchefstroom at the NWU, but the cell culturing, viral infections and MTT assays were done at the NICD in Johannesburg. Transporting and storing the Pheroid correctly was problematic. The Pheroid froze several times when stored in the refrigerator at the NICD. This meant that a new batch had to be manufactured before further investigation could proceed. Due to Eskom's unannounced electricity interruptions several experiments were repeated more than once.

The other logistical problem was that because of the time spent in commuting to and from the NICD, there was not adequate time to entrap the ABC into the Pheroid and to perform the experimental work on the same day. The ABC and/or Pheroid were diluted with complete growth medium which was freshly prepared at the NICD prior to the experiment.

The HIV-specific assays proved valuable for assessment of viral replication inhibition. One direct/HIV-specific and one indirect assays were used to asses the inhibition of HIV replication. The p24 antigen ELISA is an HIV-1 specific "sandwich" based assay which required a lot of washing steps and were rather expensive to use. The Luciferase assay was used on the M7-Luc cells. This assay was sensitive, inexpensive and easy to use. The measurements obtained with these HIV-specific assays were repeatable and correlated in an acceptable manner.

The MTT viability assay was used to measure the toxicity caused by the Pheroid and/or the drug in the cells. The MTT assay is a quantitative colorimetric assay widely used to measure the viability of cells. This assay is used routinely to determine cell viability at the NICD and was used in this study although Botha (2007) found the assay unreliable in conjunction with the Pheroid. Botha suggested that another viability assay should be used to asses the efficacy of Pheroid entrapped antiretrovirals.

The optimal Pheroid concentration proved to be both illusive and elusive using the MTT viability assay and is still undecided. The Pheroid was shown to interfere with the MTT assay. Unfortunately this possible solution to the lack of repeatability found was considered rather late in the study. Vitamin E and antioxidants such as BHA and BHT, incorporated in the Pheroid, are natural anti-oxidants that possess intrinsic reductive potential (Bruggisser *et al.*, 2002). It reduces the MTT tetrazolium ring to the dark blue formazan product which was misinterpreted as enhanced viability when the Pheroid was present during analysis. To prevent this problem an additional washing step must be introduced prior to analysis. The additional wash may diminish the interference of the Pheroid with analytical methods as indicated by preliminary results.

## Conclusion

It would be advisable to package the ABC into the Pheroid during production for future studies. Furthermore, the Pheroid formulation for entrapment of antiretroviral drugs will have to be optimised and the addition of antioxidants to the Pheroid formulation will have to be assessed

It would seem that the scientific world is still in a comfort zone regarding the MTT viability assay. The use of this assay has already been criticised by several people. The MTT viability assay should be used with caution especially in combination with substances with intrinsic reductive potential such as vitamin E and other antioxidants. Alternative cell viability assays will have to be found to evaluate antiretroviral products, particularly since the inclusion of anti-oxidants may alleviate some of the side effects of these drugs. In future all Pheroid products should be pre-screened in a cell-free system to evaluate any interference with the analytical methods.

The *in vitro* efficacy of ABC entrapped within the Pheroid is still inconclusive and further studies are required.

A drug generally has to be analysed *in vitro* for effectiveness and toxicity in order to evaluate the *in vivo* effects of the drug. Most of the promising results obtained by using the Pheroid were observed in an *in vivo* system. Unfortunately there is still no valid animal infection model for HIV in South Africa to assess the efficacy of the antiretrovirals entrapped in the Pheroid.

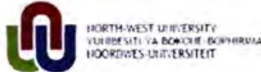
# Annexure A

Annexure A.1 is a copy of the poster presented at the 28th Annual Conference of the Academy of Pharmaceutical Sciences held at Club Myconos from 4 to 7 September 2007.

Annexure A.2 is a copy of the poster presented at the 29th Annual Conference of the Academy of Pharmaceutical Sciences held at Hunters Rest from 22 to 26 September 2008

## Annexure A.1.

# A 30 day stability study on stavudine in a commercially available ARV product

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Anne Grobler, Awie Kotze, Wilna Liebenberg

Innovation Fund Project T60042, Unit for Drug Research and Development,
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## Introduction

Despite progress in preventing HIV transmission from pregnant mothers to their babies, approximately 1500 children worldwide were daily infected with the virus in 2006. According to the 2006 IEA World Energy Outlook, about 75 % of the people in sub-Saharan Africa have no access to electricity, thus no access to the refrigerators necessary to store paediatric HIV medicines. Decomposition of stavudine in Africa's harsh climatic conditions, leads to the degradation of stavudine to thymine. The SA Department of Health still prescribes stavudine as part of the first line drug regime, because of its low cost in comparison to other antiretrovirals (ARVs). Zerit® oral solution (Bristol-Myers Squibb) is the only preparation on the market with stavudine for children.

## Aims

There is a global need for a combination product, consisting of the drugs required to suppress AIDS in children. The combination product will have to incorporate effective ARVs, which are stable once in solution. The aim of the study was therefore to evaluate the stability of stavudine in solution under different temperature conditions.

## Experimental design

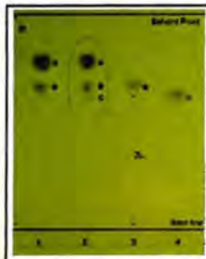
Two analytical methods have been used to evaluate the stability of stavudine, i.e. HPLC and TLC.

Thin-layer Chromatography (TLC) analysis:
- TLC was performed according to the method for stavudine capsules in USP30:NF25.
- Zerit® oral solution (batch no. 32/20.2.8/268) was reconstituted and stored in the refrigerator (2-3°C) and in a room at 16 - 24°C.
- Free stavudine and thymine were diluted in water and were used as references on the Alugram® Sil G/UV254 TLC plate, until the mobile front reached 10 cm from the starting line.

High Pressure Liquid Chromatography (HPLC) analysis:
- The HPLC analysis was performed on a Shimadzu LC-20AD instrument. The mobile phase used was ammonium acetate and methanol (94:6) with a Onyx Monolithic C18 (50x4.6 mm) column.

## Results

The TLC Results:



1. Zerit® Oral Solution (A & B) stored in the fridge
2. Zerit® Oral Solution (A, B & C) stored at 16 - 25°C
3. Stavudine (B) standard dissolved in water.
4. Thymine (C) standard dissolved in water.

**Figure 1:** TLC demonstration of the decomposition of stavudine after 30 days.

The TLC plate was observed under a UV light (254 nm). The Rf-value of each spot was determined, using:

$$R_f = \frac{Z_s}{Z_f}$$

The Rf of the different spots of Zerit® Oral Solution were compared to the values of the standards.

| Spot | A | B | C |
|---|---|---|---|
| Compound | Zerit® Oral solution constituents | Stavudine | Thymine |
| Rf value | 8 | 7 | 6.5 |

The HPLC Results:

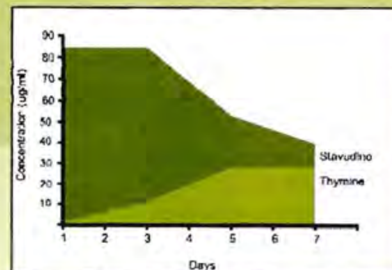



**Figure2:** Concentration of stavudine and thymine after decomposition at 37 °C for one week.

| Day: | 1 | 3 | 5 | 7 |
|---|---|---|---|---|
| Stavudine | 83.39778 | 83.35964 | 52.28937 | 38.71366 |
| Thymine | 0 | 9.744304 | 26.80351 | 27.35073 |

## Conclusions

- The results of the HPLC study showed that the stavudine concentration in Zerit® oral solution declined to less than 40% within one week under high temperature conditions (37°C).
- At room temperature, degradation of stavudine to thymine was also observed with the TLC method.
- Stavudine in Zerit® at lower temperatures (3°C) remained relative stable, which indicates that it is necessary for stavudine in solution to be kept in a refrigerator, as specified by the manufacturer.
- Stavudine can therefore not be considered for future ARV combination products in solution, due to its poor stability.

## References

DUNGE, A., CHAKRABOTI, A.K. & SINGH, S. 2004. Mechanistic explanation to the variable degradation of stavudine and zidovudine under hydrolytic, oxidative and photolytic conditions. Journal of pharmaceutical and biomedical analysis, 35 (2004):965-970. Available: ScienceDirect

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## Annexure A.2.

# Enhancement of cell viability by a Pheroid™ formulation

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## Introduction

Human immunodeficiency virus (HIV) is the causative agent for acquired immunodeficiency syndrome (AIDS). The excessive increase in AIDS incidences around the world and the severity of the toxicity caused by the drugs used in treatment, have fueled the need for enhanced anti-viral therapy. There is an even greater need for oral formulations for babies and children. Pheroid™ technology is a drug delivery system, which may besides enhancing efficacy of the added anti-HIV drugs, also improve patient compliancy and overcome toxicities.

## Purpose

The aim of this study was to determine the optimal concentration and incubation time for the Pheroid™ to be used with Anti-HIV drugs.

## Methods

The cells used in this assay (M7-Luc cells) is a CEMx174 cell clone that was transfected to contain Tat-responsive reporter genes that express luciferase (Luc) and green fluorescence protein (GFP). After entry of HIV into the cell, the cell shows green under a fluorescence microscope. Relative luciferase activity is used to measure viral infection.

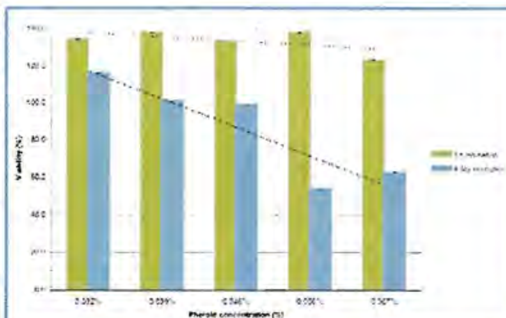
The cells were grown in a 96-well plate in the presence and absence of the Pheroid™ formulation, for a total of three hours and also four days. All groups were done in triplicate. The three hour experimental group was washed with PBS and supplemented with RPMI 1640 media and then left till day 4. A incubation time of three hours was chosen to mimic the *in vivo* situation where a circulation system is

Image (A) is M7-Luc cells without any addition of Pheroid™. Image (B) shows M7-Luc cells incubated with a toxic concentration of Pheroid™.

Cell viability was analyzed on day four, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT (5 mg/ml) was added to each well and left to incubate. The mitochondria of viable cells metabolises MTT to insoluble formazan crystals which precipitates within the cells. After two hours, a mixture of 20% SDS (sodium dodecyl sulphate) and 50% DMF (Dimethylformamide) in distilled water, was added to each well to kill the cells and solubilize the formazan crystals. The dissolved formazan crystals gives a purple colour, which are measured using a spectrophotometer at a wavelength of 570 nm and 690 nm. Viability data are presented as the percentage of viable cells in the experimental groups, compared to the negative control group.

Insoluble formazan crystals in cells before the addition of SDS & DMF.

## Results



Viability (%) of M7-Luc cells after three hour and a four day incubation with different concentrations of Pheroid™.

## Discussion

At low concentrations and short incubation times, the Pheroid™ formulation enhances cell viability (P<0.01). The Pheroid™ formulation is toxic when incubated with cells at high concentrations and extended incubation times (P=0.011), since there is no circulation system to prevent the fatty Pheroid™ formulation from smothering the cells and to extract toxic by-products. Hence the concentration and incubation time of the Pheroid™ with the cells had to be optimised.

The essential fatty acids in the Pheroid™ formulation enhance the viability of the cells by either binding to the cell membrane, improving the integrity of the membrane and/or by diffusing into the cell, entering the mitochondria and providing the cell with additional energy created by the cleaving of fatty acids through β-oxidation and the Krebs cycle. This gives the cells incubated with the Pheroid™ formulation at low concentrations and short incubation times, additional energy to overcome any physical abuse done to them by additional washing steps. Further studies are being done to evaluate the possible enhanced efficacy of anti-HIV drugs entrapped in the Pheroid™ formulation.

## References

MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 16:55-63p.

GARRET, R.H. & GRISHAM, C.M. 1997. Principles of Biochemistry with a human focus. Singapore: Brooks/Cole, Thomson Learning. 893 p.

# Annexure B

Annexure B. 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 procedure to be followed when working in the unit.

The Centre for Disease Control (1999a) requires that manipulation of concentrated HIV stocks should be conducted in a Biosafety Level 3 (BSL 3) laboratory. For this reason, the study could not be performed at the NWU, Potchefstroom campus. This study was conducted at the National Institute of Communicable Diseases (NICD), Sandringham. They kindly provided the use of their expertise, assays, systems, laboratory facilities, equipment and reagents. The provided AIDS unit safety manual was followed strictly.

NATIONAL HEALTH LABORATORY SERVICE

## 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* |
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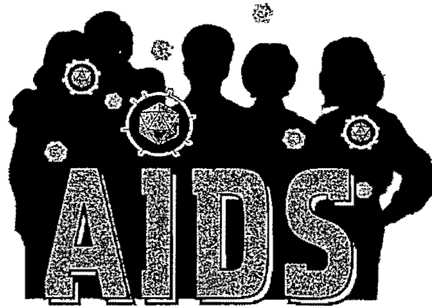
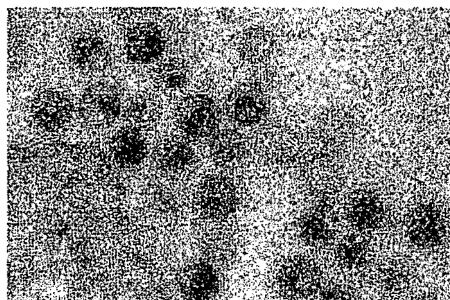



# AIDS UNIT SAFETY MANUAL

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



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**Appendix**


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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 before 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 $\mu l$ volume can contain $10^{12}$-$10^{13}$ viral molecules. Thus, if 1 $\mu l$ is aerosolized, $10^{10}$-$10^{11}$ molecules are released. To dilute $10^{10}$ molecules to less than 1 molecule per 100$\mu l$ (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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**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

**Indemnity 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 they 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 is herby granted.

I Helanie van der Merwe ____________ hereby certify that I understand fully, the contents of this indemnity form, and accept the conditions thereof.

Signed at Potchefstroom on this 18 day of October 2007.

19/10/07



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 C

Annexure C.1 is a copy of the Certificate of Analysis for Abacavir

Annexure C.2 is a copy of the Certificate of Analysis for Lamivudine

## Annexure C.1.

25 AUG 07 16:52 abc 04023707143 P. 2

# SRI SAI NIKITHA PHARMA PVT. LTD.

Office : 425/3RT, S.R.Nagar, Hyderabad - 500 038. A.P. India.
Tel : 0091 40 23801672 / 73, Fax : 0091 40 23707143
E-mail : brcreddy_08@yahoo.co.in

## CERTIFICATE OF ANALYSIS

| | | | |
|---|---|---|---|
| Product Name | ABACAVIR SULPHATE | Page No. | 1 of 2 |
| Batch Number | CAB3070929 | A.R.NO. | 07CB00436 |
| Mfg.Date | July 2007 | Date of Release | 20 August '07 |
| Quantity | 1.00KG | Retest Date | June 2009 |

| S.NO | TEST | SPECIFICATIONS | RESULT |
|---|---|---|---|
| 1. | Description | A white to off – White crystalline powder | An off –White crystalline powder |
| 2. | Solubility | Soluble in water, slightly soluble in Methanol | Soluble in water, slightly soluble in Methanol |
| 3. | Identification:<br>A) By IR. | The IR absorption spectrum of sample recorded as KBr pellet should exhibit maxima only at the same wave numbers as that of similarly recorded spectrum of the Abacavir sulfate working standard. | The IR absorption spectrum of sample recorded as KBr pellet exhibits maxima only at the same wave numbers as that of similarly recorded spectrum of the Abacavir sulfate working standard. |
| | B) By HPLC | The retention time of the Abacavir peak in the chromatogram of the sample solution should correspond to that of the standard solution, as obtained in the Assay. | The retention time of the Abacavir peak in the chromatogram of the sample solution corresponds to that of the standard solution as obtained in the assay. |
| | C) Test for Sulfate | A copious dense White precipitate should form; indicating the presence of sulfate ions. | A copious dense white precipitate formed; indicating the presence of Sulfate ions. |
| 4. | Water (% w/w, by KF) | Not more than 1.0 | 0.38 |
| 5. | Specific Optical Rotation $[\alpha]^{25}_{D}$ (c=0.5 in Methanol, on anhydrous basis) | Between -32.0° and -38.0° | -36.96° |
| 6. | Residue on ignition (determined on 1.0 g, % w/w) | Not more than 0.3 | 0.08 |
| 7. | Heavy Metals (ppm) | Not more than 20 | Less than 20 |
| | PREPARED BY | CHECKED BY | APPROVED BY |
| Signature: | [signature] | [signature] | [signature] |
| Date: | 20/08/07 | 20/08/2007 | 20.08.07. |

REPRESENTED BY:
D B Fine Chemicals (Pty) Limited
P.O. Box 788
RIVONIA 2128
Johannesburg
South Africa

## Annexure C.2.

Feb 08 13:20 srisai nikitha 04023707143 p.4

# SRI SAI NIKITHA PHARMA PVT. LTD.

Office : 425/3RT, S.R.Nagar, Hyderabad - 500 038. A.P. India.
Tel : 0091 40 23801672 / 73, Fax : 0091 40 23707143
E-mail : brcreddy_08@yahoo.co.in

## CERTIFICATE OF ANALYSIS

| Product Name | LAMIVUDINE | Quantity | 3 KGS |
|---|---|---|---|
| Batch Number | 002022007 | Date of Analysis | 06.02.08 |
| Mfg.Date | Feb' 2008 | Date of Expiry | Jan' 2011 |

| S.No | TEST | SPECIFICATIONS | RESULT |
|---|---|---|---|
| 1 | Description | A White to Off-white powder | Off-white powder |
| 2 | Solubility | Soluble in water | Complies |
| 3 | IR Spectrum | The IR spectrum exhibit maxima at the same wave numbers as the Lamivudine working standard spectrum | complies |
| 4 | Melting Range | Melts between 172°C and 178°C | 174°C to 176°C |
| 5 | Water by KF | Not more than 0.50% w/w | 0.42% w/w |
| 6 | Related substances by HPLC | Single impurity not more than 0.50% w/w<br>Total impurities not more than 1.00% w/w | 0.25%<br>0.30% |
| | PREPARED BY | CHECKED BY | APPROVED BY |
| Sign. | [signature] | [signature] | [signature] |
| Date | 08/02/08 | 08-02-08 | 08-02-08 |

REPRESENTED BY:
D B Fine Chemicals (Pty) Limite
P.O. Box 786
RIVONIA 2128
Johannesburg
South Africa

# Annexure D

Annexure D is composed of copies of the results obtained with the Malvern Mastersizer of the manufactured Pheroid batches to demonstrate the differences between the batches made.

| | |
|---|---|
| Annexure D1: | Batch V08011 |
| Annexure D2: | Batch V08012 |
| Annexure D3: | Batch V08013 |
| Annexure D4: | Batch V08022 |

## Annexure D.1. Batch: V08011

# MASTERSIZER

## Result Analysis Report

Sample Name:
LINDEL VESICLES FOR TC

Sample Source & type:
Isotec

Sample bulk lot ref:

SOP Name:
Isotec Nutrition Samples using Hydro 2000

Measured by:
Micron Scientific

Result Source:
Measurement

Measured:
27 May 2008 11:20:07 AM

Analysed:
27 May 2008 11:20:08 AM

---

Particle Name:
Oleic Acid

Particle RI:
1.458

Dispersant Name:
Water

Accessory Name:
Hydro 2000MU (A)

Absorption:
0.1

Dispersant RI:
1.330

Analysis model:
General purpose

Size range:
0.020 to 2000.000 um

Weighted Residual:
4.233 %

Sensitivity:
Enhanced

Obscuration:
11.94 %

Result Emulation:
Off

---

Concentration:
0.0034 %Vol

Specific Surface Area:
4.21 m²/g

Span :
2.288

Surface Weighted Mean D[3,2]:
1.425 um

Uniformity:
0.778

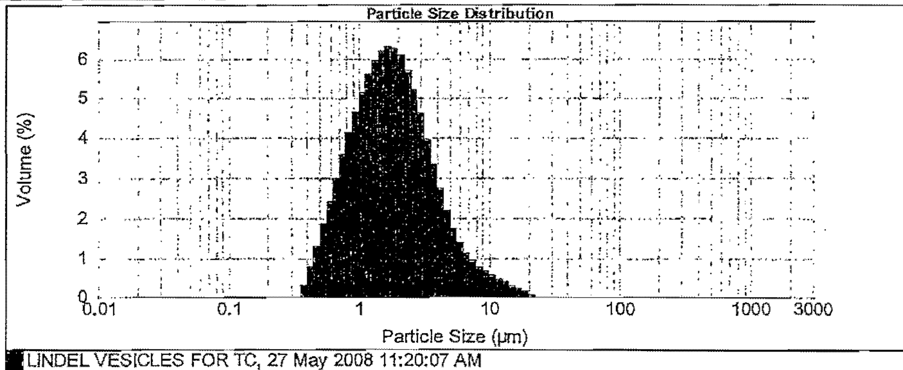
Vol. Weighted Mean D[4,3]:
2.443 um

Result units:
Volume

d(0.1): 0.719 um d(0.5): 1.747 um d(0.9): 4.716 um

---

Particle Size Distribution




LINDEL VESICLES FOR TC, 27 May 2008 11:20:07 AM

| Size (μm) | Volume In % |
|---|---|
| 0.020 | 0.00 |
| 0.022 | 0.00 |
| 0.025 | 0.00 |
| 0.028 | 0.00 |
| 0.032 | 0.00 |
| 0.036 | 0.00 |
| 0.040 | 0.00 |
| 0.045 | 0.00 |
| 0.050 | 0.00 |
| 0.056 | 0.00 |
| 0.063 | 0.00 |
| 0.071 | 0.00 |
| 0.080 | 0.00 |
| 0.089 | 0.00 |
| 0.100 | 0.00 |
| 0.112 | 0.00 |
| 0.126 | 0.00 |
| 0.142 | |

| Size (μm) | Volume In % |
|---|---|
| 0.142 | 0.00 |
| 0.159 | 0.00 |
| 0.178 | 0.00 |
| 0.200 | 0.00 |
| 0.224 | 0.00 |
| 0.252 | 0.00 |
| 0.283 | 0.00 |
| 0.317 | 0.00 |
| 0.356 | 0.30 |
| 0.399 | 0.79 |
| 0.448 | 1.29 |
| 0.502 | 1.84 |
| 0.564 | 2.41 |
| 0.632 | 2.99 |
| 0.710 | 3.57 |
| 0.796 | 4.13 |
| 0.893 | 4.66 |
| 1.002 | |

| Size (μm) | Volume In % |
|---|---|
| 1.002 | 5.15 |
| 1.125 | 5.58 |
| 1.262 | 5.92 |
| 1.416 | 6.17 |
| 1.589 | 6.29 |
| 1.783 | 6.26 |
| 2.000 | 6.06 |
| 2.244 | 5.70 |
| 2.518 | 5.20 |
| 2.825 | 4.61 |
| 3.170 | 3.97 |
| 3.557 | 3.33 |
| 3.991 | 2.73 |
| 4.477 | 2.20 |
| 5.024 | 1.76 |
| 5.637 | 1.41 |
| 6.325 | 1.14 |
| 7.096 | |

| Size (μm) | Volume In % |
|---|---|
| 7.096 | 0.93 |
| 7.962 | 0.78 |
| 8.934 | 0.66 |
| 10.024 | 0.56 |
| 11.247 | 0.47 |
| 12.619 | 0.39 |
| 14.159 | 0.30 |
| 15.887 | 0.23 |
| 17.825 | 0.15 |
| 20.000 | 0.06 |
| 22.440 | 0.00 |
| 25.179 | 0.00 |
| 28.251 | 0.00 |
| 31.698 | 0.00 |
| 35.566 | 0.00 |
| 39.905 | 0.00 |
| 44.774 | 0.00 |
| 50.238 | |

| Size (μm) | Volume In % |
|---|---|
| 50.238 | 0.00 |
| 56.368 | 0.00 |
| 63.246 | 0.00 |
| 70.963 | 0.00 |
| 79.621 | 0.00 |
| 89.337 | 0.00 |
| 100.237 | 0.00 |
| 112.468 | 0.00 |
| 126.191 | 0.00 |
| 141.589 | 0.00 |
| 158.866 | 0.00 |
| 178.250 | 0.00 |
| 200.000 | 0.00 |
| 224.404 | 0.00 |
| 251.785 | 0.00 |
| 282.508 | 0.00 |
| 316.979 | 0.00 |
| 355.656 | |

| Size (μm) | Volume In % |
|---|---|
| 355.656 | 0.00 |
| 399.052 | 0.00 |
| 447.744 | 0.00 |
| 502.377 | 0.00 |
| 563.677 | 0.00 |
| 632.456 | 0.00 |
| 709.627 | 0.00 |
| 796.214 | 0.00 |
| 893.367 | 0.00 |
| 1002.374 | 0.00 |
| 1124.683 | 0.00 |
| 1261.915 | 0.00 |
| 1415.892 | 0.00 |
| 1588.656 | 0.00 |
| 1782.502 | 0.00 |
| 2000.000 | |

Operator notes: *HELANIE VAN DER MERWE*
*BN: V08011*
*MD: 29/04/2008*

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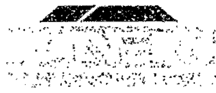
Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

File name: Examples
Record Number: 1010
27 May 2008 11:20:57 AM

**Annexure D.2.** Batch: V08012



# MASTERSIZER 2000

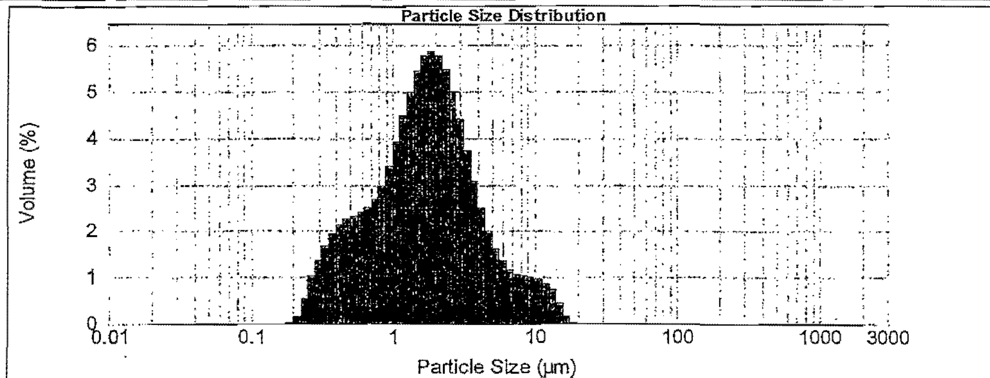
## Result Analysis Report

| | | |
|---|---|---|
| **Sample Name:** LINDEL VESICLES FOR TC | **SOP Name:** Isotec Nutrition Samples using Hydro 2000 | **Measured:** 27 May 2008 11:07:25 AM |
| **Sample Source & type:** Isotec | **Measured by:** Micron Scientific | **Analysed:** 27 May 2008 11:07:26 AM |
| **Sample bulk lot ref:** | **Result Source:** Measurement | |

| | | | |
|---|---|---|---|
| **Particle Name:** Oleic Acid | **Accessory Name:** Hydro 2000MU (A) | **Analysis model:** General purpose | **Sensitivity:** Enhanced |
| **Particle RI:** 1.458 | **Absorption:** 0.1 | **Size range:** 0.020 to 2000.000 um | **Obscuration:** 0.54 % |
| **Dispersant Name:** Water | **Dispersant RI:** 1.330 | **Weighted Residual:** 4.778 % | **Result Emulatior** Off |

| | | | |
|---|---|---|---|
| **Concentration:** 0.0001 %Vol | **Span :** 2.703 | **Uniformity:** 0.89 | **Result units:** Volume |
| **Specific Surface Area:** 5.28 m²/g | **Surface Weighted Mean D[3,2]:** 1.137 um | **Vol. Weighted Mean D[4,3]:** 2.466 um | |

**d(0.1):** 0.478 um **d(0.5):** 1.717 um **d(0.9):** 5.117 un




| Size (μm) | Volume In % | Size (μm) | Volume In % | Size (μm) | Volume In % | Size (μm) | Volume In % | Size (μm) | Volume In % | Size (μm) | Volume In % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.020 | 0.00 | 0.142 | 0.00 | 1.002 | 3.91 | 7.096 | 1.06 | 50.238 | 0.00 | 355.656 | 0.00 |
| 0.022 | 0.00 | 0.159 | 0.00 | 1.125 | 4.46 | 7.962 | 1.01 | 56.368 | 0.00 | 399.052 | 0.00 |
| 0.025 | 0.00 | 0.178 | 0.01 | 1.262 | 5.00 | 8.934 | 0.98 | 63.246 | 0.00 | 447.744 | 0.00 |
| 0.028 | 0.00 | 0.200 | 0.13 | 1.416 | 5.45 | 10.024 | 0.95 | 70.963 | 0.00 | 502.377 | 0.00 |
| 0.032 | 0.00 | 0.224 | 0.53 | 1.589 | 5.75 | 11.247 | 0.85 | 79.621 | 0.00 | 563.677 | 0.00 |
| 0.036 | 0.00 | 0.252 | 1.02 | 1.783 | 5.87 | 12.619 | 0.74 | 89.337 | 0.00 | 632.456 | 0.00 |
| 0.040 | 0.00 | 0.283 | 1.34 | 2.000 | 5.77 | 14.159 | 0.45 | 100.237 | 0.00 | 709.627 | 0.00 |
| 0.045 | 0.00 | 0.317 | 1.67 | 2.244 | 5.47 | 15.887 | 0.14 | 112.468 | 0.00 | 796.214 | 0.00 |
| 0.050 | 0.00 | 0.356 | 1.93 | 2.518 | 5.00 | 17.825 | 0.02 | 126.191 | 0.00 | 893.367 | 0.00 |
| 0.056 | 0.00 | 0.399 | 2.12 | 2.825 | 4.40 | 20.000 | 0.00 | 141.589 | 0.00 | 1002.374 | 0.00 |
| 0.063 | 0.00 | 0.448 | 2.24 | 3.170 | 3.74 | 22.440 | 0.00 | 158.866 | 0.00 | 1124.683 | 0.00 |
| 0.071 | 0.00 | 0.502 | 2.32 | 3.557 | 3.08 | 25.179 | 0.00 | 178.250 | 0.00 | 1261.915 | 0.00 |
| 0.080 | 0.00 | 0.564 | 2.40 | 3.991 | 2.49 | 28.251 | 0.00 | 200.000 | 0.00 | 1415.892 | 0.00 |
| 0.089 | 0.00 | 0.632 | 2.51 | 4.477 | 1.99 | 31.698 | 0.00 | 224.404 | 0.00 | 1588.656 | 0.00 |
| 0.100 | 0.00 | 0.710 | 2.71 | 5.024 | 1.61 | 35.566 | 0.00 | 251.785 | 0.00 | 1782.502 | 0.00 |
| 0.112 | 0.00 | 0.796 | 3.00 | 5.637 | 1.33 | 39.905 | 0.00 | 282.508 | 0.00 | 2000.000 | |
| 0.126 | 0.00 | 0.893 | 3.41 | 6.325 | 1.15 | 44.774 | 0.00 | 316.979 | 0.00 | | |
| 0.142 | | 1.002 | | 7.096 | | 50.238 | | 355.656 | | | |

**Operator notes:** *HELANIE*
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*PD 19/05/2008*

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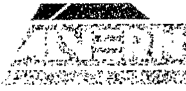
Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

File name: Exampl
Record Number: 1(
27 May 2008 11:0

## Annexure D.3. Batch: V08013



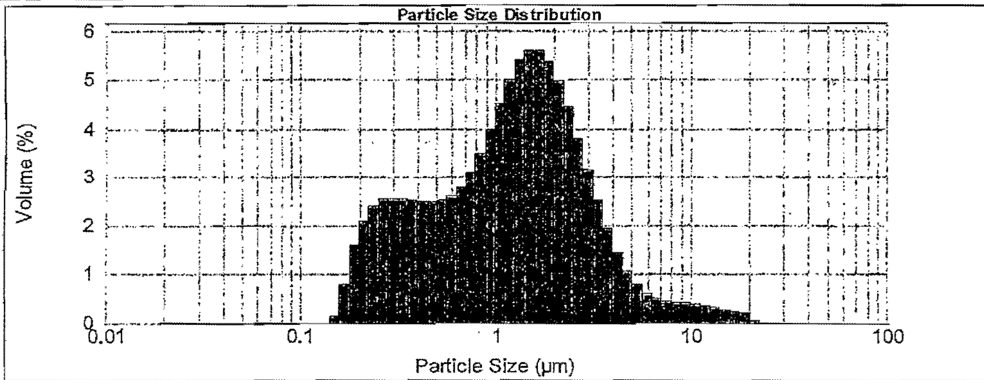
# Result Analysis Report

| | | |
|---|---|---|
| **Sample Name:**<br>LINDIL VESICLES | **SOP Name:**<br>Isotec Nutrition Samples using Hydro 2000 | **Measured:**<br>04 June 2008 10:54:03 AM |
| **Sample Source & type:**<br>Isotec | **Measured by:**<br>Micron Scientific | **Analysed:**<br>04 June 2008 10:54:05 AM |
| **Sample bulk lot ref:** | **Result Source:**<br>Measurement | |

| | | | |
|---|---|---|---|
| **Particle Name:**<br>Oleic Acid | **Accessory Name:**<br>Hydro 2000MU (A) | **Analysis model:**<br>General purpose | **Sensitivity:**<br>Enhanced |
| **Particle RI:**<br>1.458 | **Absorption:**<br>0.1 | **Size range:**<br>0.020 to 2000.000 um | **Obscuration:**<br>12.45 % |
| **Dispersant Name:**<br>Water | **Dispersant RI:**<br>1.330 | **Weighted Residual:**<br>6.216 % | **Result Emulatio**<br>Off |

| | | | |
|---|---|---|---|
| **Concentration:**<br>0.0032 %Vol | **Span :**<br>2.493 | **Uniformity:**<br>0.931 | **Result units:**<br>Volume |
| **Specific Surface Area:**<br>J4 m²/g<br>804 | **Surface Weighted Mean D[3,2]:**<br>0.746 um | **Vol. Weighted Mean D[4,3]:**<br>1.809 um | |

**d(0.1):** 0.289 um **d(0.5):** 1.265 um **d(0.9):** 3.443 un




LINDIL VESICLES, 04 June 2008 10:54:03 AM

| Size (μm) | Volume In % |
|---|---|
| 0.020 | 0.00 |
| 0.022 | 0.00 |
| 0.025 | 0.00 |
| 0.028 | 0.00 |
| 0.032 | 0.00 |
| 0.036 | 0.00 |
| 0.040 | 0.00 |
| 0.045 | 0.00 |
| 0.050 | 0.00 |
| 0.056 | 0.00 |
| 0.063 | 0.00 |
| 0.071 | 0.00 |
| 0.080 | 0.00 |
| 0.089 | 0.00 |
| 0.100 | 0.00 |
| 0.112 | 0.00 |
| 0.126 | 0.00 |
| 0.142 | 0.00 |

| Size (μm) | Volume In % |
|---|---|
| 0.142 | 0.13 |
| 0.159 | 0.80 |
| 0.178 | 1.57 |
| 0.200 | 2.07 |
| 0.224 | 2.38 |
| 0.252 | 2.52 |
| 0.283 | 2.54 |
| 0.317 | 2.52 |
| 0.356 | 2.50 |
| 0.399 | 2.48 |
| 0.448 | 2.48 |
| 0.502 | 2.50 |
| 0.564 | 2.60 |
| 0.632 | 2.78 |
| 0.710 | 3.06 |
| 0.796 | 3.48 |
| 0.893 | 3.96 |
| 1.002 | |

| Size (μm) | Volume In % |
|---|---|
| 1.002 | 4.49 |
| 1.125 | 4.99 |
| 1.262 | 5.38 |
| 1.416 | 5.58 |
| 1.589 | 5.58 |
| 1.783 | 5.36 |
| 2.000 | 4.96 |
| 2.244 | 4.43 |
| 2.518 | 3.81 |
| 2.825 | 3.15 |
| 3.170 | 2.51 |
| 3.557 | 1.93 |
| 3.991 | 1.44 |
| 4.477 | 1.05 |
| 5.024 | 0.78 |
| 5.637 | 0.60 |
| 6.325 | 0.49 |
| 7.096 | |

| Size (μm) | Volume In % |
|---|---|
| 7.096 | 0.44 |
| 7.962 | 0.42 |
| 8.934 | 0.40 |
| 10.024 | 0.38 |
| 11.247 | 0.35 |
| 12.619 | 0.32 |
| 14.159 | 0.27 |
| 15.887 | 0.22 |
| 17.825 | 0.21 |
| 20.000 | 0.05 |
| 22.440 | 0.00 |
| 25.179 | 0.00 |
| 28.251 | 0.00 |
| 31.698 | 0.00 |
| 35.566 | 0.00 |
| 39.905 | 0.00 |
| 44.774 | 0.00 |
| 50.238 | |

| Size (μm) | Volume In % |
|---|---|
| 50.238 | 0.00 |
| 56.368 | 0.00 |
| 63.246 | 0.00 |
| 70.963 | 0.00 |
| 79.621 | 0.00 |
| 89.337 | 0.00 |
| 100.237 | 0.00 |
| 112.468 | 0.00 |
| 126.191 | 0.00 |
| 141.589 | 0.00 |
| 158.866 | 0.00 |
| 178.250 | 0.00 |
| 200.000 | 0.00 |
| 224.404 | 0.00 |
| 251.785 | 0.00 |
| 282.508 | 0.00 |
| 316.979 | 0.00 |
| 355.656 | |

| Size (μm) | Volume In % |
|---|---|
| 355.656 | 0.00 |
| 399.052 | 0.00 |
| 447.744 | 0.00 |
| 502.377 | 0.00 |
| 563.677 | 0.00 |
| 632.456 | 0.00 |
| 709.627 | 0.00 |
| 796.214 | 0.00 |
| 893.367 | 0.00 |
| 1002.374 | 0.00 |
| 1124.683 | 0.00 |
| 1261.915 | 0.00 |
| 1415.892 | 0.00 |
| 1588.656 | 0.00 |
| 1782.502 | 0.00 |
| 2000.000 | |

**Operator notes:** *HELANIE VAN DER MERWE*
*BN: V08013*
*EXP: 03/07/08*

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Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

File name: Examp
Record Number: 1
04 Jun 2008 10:5

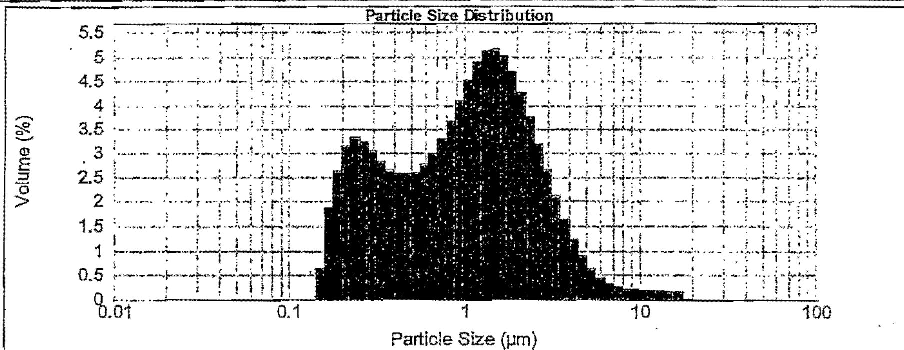
**Annexure D.4.** Batch: V08022

# MASTERSIZER 2000

## Result Analysis Report

| | | | |
|---|---|---|---|
| **..ple Name:** Lindil Vesicles Helanie | **SOP Name:** Pheroid vesicles using Hydro 2000 MU | **Measured:** 13 October 2008 11:26:40 AM | |
| **Sample Source & type:** EFF | **Measured by:** Silverani | **Analysed:** 13 October 2008 11:26:41 AM | |
| **Sample bulk lot ref:** V08022 | **Result Source:** Measurement | | |
| **Particle Name:** Oleic Acid | **Accessory Name:** Hydro 2000MU (A) | **Analysis model:** General purpose | **Sensitivity:** Enhanced |
| **Particle RI:** 1.458 | **Absorption:** 0.1 | **Size range:** 0.020 to 2000.000 um | **Obscuration:** 22.94 % |
| **Dispersant Name:** Water | **Dispersant RI:** 1.330 | **Weighted Residual:** 6.766 % | **Result Emulation:** Off |
| **Concentration:** 0.0060 %Vol | **Span :** 2.612 | **Uniformity:** 0.927 | **Result units:** Volume |
| **Specific Surface Area:** .79 m²/g | **Surface Weighted Mean D[3,2]:** 0.613 um | **Vol. Weighted Mean D[4,3]:** 1.482 um | |

**d(0.1):** 0.238 um **d(0.5):** 1.059 um **d(0.9):** 3.004 um




Lindil Vesicles Helanie, 13 October 2008 11:26:40 AM

| Size (μm) | Volume In % |
|---|---|
| 0.020 | 0.00 |
| 0.022 | 0.00 |
| 0.025 | 0.00 |
| 0.028 | 0.00 |
| 0.032 | 0.00 |
| 0.036 | 0.00 |
| 0.040 | 0.00 |
| 0.045 | 0.00 |
| 0.050 | 0.00 |
| 0.056 | 0.00 |
| 0.063 | 0.00 |
| 0.071 | 0.00 |
| 0.080 | 0.00 |
| 0.089 | 0.00 |
| 0.100 | 0.00 |
| 0.112 | 0.00 |
| 0.126 | 0.00 |
| 0.142 | |

| Size (μm) | Volume In % |
|---|---|
| 0.142 | 0.64 |
| 0.159 | 1.66 |
| 0.178 | 2.63 |
| 0.200 | 3.16 |
| 0.224 | 3.33 |
| 0.252 | 3.25 |
| 0.283 | 3.04 |
| 0.317 | 2.81 |
| 0.356 | 2.65 |
| 0.399 | 2.57 |
| 0.448 | 2.55 |
| 0.502 | 2.61 |
| 0.564 | 2.75 |
| 0.632 | 2.98 |
| 0.710 | 3.30 |
| 0.796 | 3.67 |
| 0.893 | 4.10 |
| 1.002 | |

| Size (μm) | Volume In % |
|---|---|
| 1.002 | 4.53 |
| 1.125 | 4.89 |
| 1.262 | 5.12 |
| 1.416 | 5.15 |
| 1.589 | 5.00 |
| 1.783 | 4.70 |
| 2.000 | 4.27 |
| 2.244 | 3.76 |
| 2.518 | 3.20 |
| 2.825 | 2.64 |
| 3.170 | 2.12 |
| 3.557 | 1.63 |
| 3.991 | 1.23 |
| 4.477 | 0.89 |
| 5.024 | 0.63 |
| 5.637 | 0.45 |
| 6.325 | 0.33 |
| 7.096 | |

| Size (μm) | Volume In % |
|---|---|
| 7.096 | 0.26 |
| 7.962 | 0.22 |
| 8.934 | 0.20 |
| 10.024 | 0.19 |
| 11.247 | 0.19 |
| 12.619 | 0.17 |
| 14.159 | 0.16 |
| 15.887 | 0.14 |
| 17.825 | 0.02 |
| 20.000 | 0.00 |
| 22.440 | 0.00 |
| 25.179 | 0.00 |
| 28.251 | 0.00 |
| 31.698 | 0.00 |
| 35.566 | 0.00 |
| 39.905 | 0.00 |
| 44.774 | 0.00 |
| 50.238 | |

| Size (μm) | Volume In % |
|---|---|
| 50.238 | 0.00 |
| 56.368 | 0.00 |
| 63.246 | 0.00 |
| 70.963 | 0.00 |
| 79.621 | 0.00 |
| 89.337 | 0.00 |
| 100.237 | 0.00 |
| 112.468 | 0.00 |
| 126.191 | 0.00 |
| 141.589 | 0.00 |
| 158.866 | 0.00 |
| 178.250 | 0.00 |
| 200.000 | 0.00 |
| 224.404 | 0.00 |
| 251.785 | 0.00 |
| 282.508 | 0.00 |
| 316.979 | 0.00 |
| 355.656 | |

| Size (μm) | Volume In % |
|---|---|
| 355.656 | 0.00 |
| 399.052 | 0.00 |
| 447.744 | 0.00 |
| 502.377 | 0.00 |
| 563.677 | 0.00 |
| 632.456 | 0.00 |
| 709.627 | 0.00 |
| 796.214 | 0.00 |
| 893.367 | 0.00 |
| 1002.374 | 0.00 |
| 1124.683 | 0.00 |
| 1261.915 | 0.00 |
| 1415.892 | 0.00 |
| 1588.656 | 0.00 |
| 1782.502 | 0.00 |
| 2000.000 | |

**Operator notes:** *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place.*

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Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007546

File name: Examples
Record Number: 1256
13 Oct 2008 11:27:41 AM

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**C**

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