Identification and Evaluation of Inhibitors Targeting the HIV-1 Integrase-LEDGF/p75 Interaction

By
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of
Master of Science in Medicine

Declaration

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

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Day of   , 2014

Dedication

I dedicate this dissertation to my parents for giving me the opportunity to follow my dream and always being by my side to help me through the difficulties. I love you and thank you.

My dearest Riaan, thank you for supporting me though this journey and even though you may not have understood you were always by my side. I love you unconditionally.

Publication and conference proceedings

Publications in DE accredited journals and book chapters

- Angela T. Harrison, Frederik H. Kriel, Maria A. Papathanasopoulos, Salerwe Mosebi, Shaakira Abrahams, Raymond Hewer. The evaluation of statins as potential inhibitors of the LEDGF/p75 – HIV-1 integrase interaction. *Submitted.*

Conference proceedings

- **Oral**

  - R Hewer, **A Harrison**, S Abrahams, S Mosebi, FH Kriel, MA Papathanasapoulos, MQ Fish. Identification of novel inhibitors that target HIV-1 integration. Oral presented at 6th South African AIDS conference, Durban, RSA, 18-21 June 2013.
  - R Hewer, **A Harrison**, S Abrahams, S Mosebi, MQ Fish, FH Kriel, MA Papathanasopoulos. Novel technologies and useful inhibitors for HIV drug discovery. Oral presented at 3rd National Conference on Science and Technology, Luanda, Angola, 11 -13 September 2013.

- **Poster**

  - **A Harrison**, MA Papathanasopoulos, FH Kriel, M Killick, S Mosebi, R Hewer. Identification of Novel Inhibitors of the Lens Epithelium Derived Growth Factor (LEDGF/p75)-HIV-1 Integrase (IN) Interaction. 6th South African AIDS conference, Durban, RSA, 19-21 June 2013.
  - R Hewer, **A Harrison**, MA Papathanasopoulos, FH Kriel, S Mosebi. Identification of a novel inhibitor of the HIV-1 integrase-LEDGF/p75 interaction. Poster presented at the 26th International Conference on Antiviral Research (ICAR) 2013, San Francisco, USA, 11-15 May 2013.

Abstract

The human immunodeficiency virus type 1 (HIV-1) integrase (IN) facilitates the irreversible integration of the viral chromosome into the host DNA in a two-step reaction process consisting of 3' end processing and strand transfer. The pre-integration complex (PIC) is a nucleoprotein complex consisting of HIV-1 IN, reverse transcriptase, matrix, nucleocapsid, viral protein R (Vpr) and various cellular host proteins. The dominant host protein that forms part of the PIC is known as lens epithelium derived growth factor (LEDGF/p75) that is ubiquitously expressed in the nucleus. The integration of HIV-1 into chromatinised template is stimulated by LEDGF/p75, while LEDGF/p75 stabilizes HIV-1 IN subunit-subunit interactions and promotes HIV-1 IN tetramerisation. This study aimed to identify and evaluate potential inhibitors of the HIV-1 IN-LEDGF/p75 interaction. A compound library (NCC-202) comprised of 281 compounds obtained from the NIH clinical collection was screened through an HIV-1 IN-LEDGF/p75 molecular model prepared on Accelrys Discovery Studio^{TM} 3.1. Recombinant HIV-1 IN and LEDGF/p75 were expressed in bacterial cells and purified by nickel affinity and cation exchange chromatography, and used to establish an AlphaScreen assay for compound screening. A total of twelve compounds were identified as possible HIV-1 IN-LEDGF/p75 inhibitors. Biochemical screening using an AlphaScreen assay was used and a total of six of the twelve inhibitors displaying inhibition above 50 % were identified. The best compound with an IC_{50} of 1.97 μM was identified as lovastatin. Lovastatin was found to have an EC_{50} of 6.54 μM in the antiviral assay but activity was likely attributed to cytotoxicity with a CC_{50} of 5.31 μM. Based on information from structurally similar statins, we hypothesised that a closed lactone ring in combination with a methyl group on the naphthalene structure elicited the likely inhibitory profile and that the lactone ring induced a large part of the cytotoxic effect of the compounds screened. Overall, we identified statins as potential inhibitors of the HIV-1 IN-LEDGF/p75 interaction, however the overlap of toxicity with antiviral effects renders statins in their current form unsuitable for antiretroviral treatment.

<u>Acknowledgements</u>

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List of abbreviations

°C	Degrees Celsius
ABTS	2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ACN	Acetonitrile
AIDS	Acquired Immune Deficiency Syndrome
ALLINI	Allosteric HIV-1 integrase inhibitors
Alpha	Amplified Luminescent Proximity Homogenous Assay
ARV	Antiretroviral
AZT	Zidovudine
BAF	Barrier to auto integration factor
BSA	Bovine serum albumin
CC_{50}	Cytotoxicity
CCD	Catalytic core domain
CCR5	Chemokine receptor 5
cpz	Chimpanzee
CTD	C terminal domain
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC_{50}	Half maximal effective concentration
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carcodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
FDA	US Food and Drug Administration
HAART	Highly active antiretroviral therapy

HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HMGA1	High mobility group protein A1
HTLV	Human T-lymphotropic virus
IBD	Integrase binding domain
IC_{50}	Half maximal inhibitor concentration
ICAM1	Intracellular adhesion molecule 1
IN	Integrase
INSTI	Integrase strand transfer inhibitor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilo bases
kDa	Kilo Daltons
LAV	Lymphadenopathy Associated Virus
LB	Luria Bertani
LC	Liquid chromatography
LCMS	Liquid chromatography mass spectrometer
LEDGF	Lens epithelium derived growth factor
LFA1	Lymphocyte function antigen -1
LTR	Long terminal repeat
M	Molar
mg	milligram
ml	millilitre
Mw	Molecular weight
MWCO	Molecular weight cut off
NaOH	Sodium hydroxide
NCC	NIH Clinical collection
NCINI	Non-catalytic site integrase inhibitors

nef	Negative factor gene
ng	nanogram
NHS	N-Hydroxysuccinimide
NIH	National Institutes of Health
nM	nanomolar
NMR	Nuclear magnetic resonance
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NtRTI	Nucleotide reverse transcriptase inhibitor
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAMPA	Parallel artificial membrane permeability assay
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC4	Positive cofactor 4
PI	Protease inhibitor
PIC	Pre integration complex
PMSF	Phenylmethanesulfonylfluoride
PogZ	Pogo transposable element with ZNF domain
pol	Polymerase gene
PR	Protease
PVDF	Polyvinylidenedifluoride
RE	Restriction enzyme
rev	Regulator of expression gene
RNA	Ribonucleic acid
RO5	Rule of 5
RT	Reverse transcriptase

SDS	Sodium dodecyl sulphate
SI	Selectivity index
SIV	Simian Immunodeficiency Virus
smm	Sooty mangabey monkeys
Sol	Soluble
SPR	Surface Plasmon resonance
ST	Strand transfer
tat	transactivator of transcription gene
TMB	3,3',5,5'-tetramethylbenzidine
TTBS	Tris buffered saline with tween 20
μg	microgram
μM	micromolar
UNAIDS	The Joint United Nations Programme on HIV and AIDS
UV	Ultra violet
vif	Viral infectivity factor gene
vpr	Viral protein R gene
vpu	Viral protein U gene
wt	wild type

Chapter 1: Literature review

Literature review

1.1 Introduction
1.1.1 History of HIV

Acquired immune deficiency syndrome (AIDS) was first identified in 1981 in young, previously healthy homosexual patients, and initially described as an immune deficiency state that was associated with multiple microbial infections (Gottlieb et al., 1981). The causative agent, Human Immunodeficiency Virus (HIV) was initially termed Lymphadenopathy Associated Virus (LAV) due to the isolation of the viral agent from a lymph node biopsy of a Kinshasa patient in 1983 (Barré-Sinoussi et al., 1983). LAV was later termed as Human T-lymphotropic virus (HTLV)-III as the genomic structure was similar to that of HTLV-I (Gallo et al., 1984). In 1984 HTLV-III was determined to be the causative agent of AIDS and was later renamed as HIV type 1 (HIV-1) in 1986 due to the immune depletion it caused (Coffin, 1986; Gallo et al., 1984; Popovic et al., 1984). In 1986 HIV-2 was isolated from commercial sex workers in west Africa (Marx, 1986).

Historical evidence for the zoonotic origins of HIV were found in a serum sample stored from 1959 obtained from a male in Kinshasa, that was retrospectively found to be closely related to a simian immunodeficiency virus (SIV) strain that originated from chimpanzees of the sub species *Pan Troglodytes* and was classified as HIV-1 in 1998 (Keele et al., 2006; Zhu et al., 1998). In 1960 a lymph node biopsy was stored from a female in Leopoldville and was recovered recently (Worobey et al., 2008). The comparison of HIV-1 sequences in the 1959 sample and 1960 samples showed a genetic variation of 12%. This suggests that HIV-1 was present in humans for a long time before 1960 (Worobey et al., 2008), and originated from multi-cross species transmissions of SIV from non-human primates into humans in Central and West Africa (Sharp et al., 1994).

1.1.2 Epidemiology of HIV
HIV prevalence

HIV/AIDS is a global pandemic with Sub-Saharan Africa more severely affected than any other region in the world. By the end of 2012, it was estimated that 35.3 million people world-wide were HIV positive (Joint United Nations Program and United Nations, 2013). Sub Saharan Africa accounted for 66% of infections, followed by South and South East Asia at 12 % (Joint United Nations Programme on HIV/AIDS and United Nations, 2012). In 2012, 1.6 million people were newly

infected with HIV worldwide, a 33% improvement on 2001 statistics of 3.4 million. Similarly, worldwide AIDS related deaths dropped from 2.3 million in 2005 to 1.6 million in 2012. In 2011, a total of 25 countries saw a reduction of more than 50% in newly infected individuals, with the Caribbean, part of Sub Saharan Africa, displaying a 42% reduction. North America and the Middle East saw 32 000 new infections in 2012. A total of 860 000 people were infected with HIV in Western and Central Europe whereas Australia had a lower HIV rate with a total of 35 000 people estimated to have HIV in 2012 and only 1133 new infections in 2011. Progress in HIV prevention is however uneven with a rise in new infections in the Middle East, North Africa, Eastern Europe and Central Asia (Joint United Nations Programme on HIV/AIDS and United Nations, 2012).

Nomenclature and phylogeny of HIV

HIV strains that have been sampled from various countries indicate high genetic variation (Sharp et al., 2001). HIV is subdivided into two broad types HIV type 1 (HIV-1) and HIV type 2 (HIV-2), with HIV-1 being the most prevalent (Sharp and Hahn, 2010). HIV-1 is most closely related to SIV found in chimpanzees (cpz) and gorillas while HIV-2 is related to SIV found in sooty mangabeys (smm). Thus, HIV types are classified based on their genetic organisation and evolutionary relations to their primate lentiviruses (Hirsch et al., 1989; Huet et al., 1990; Sharp and Hahn, 2010). Figure 1.1 shows that groups M, N and O arose from three independent transmissions of SIVcpz from central African chimpanzees and SIVsmm gave rise to HIV-2 groups A to H (Chen et al., 1997; Gao et al., 1999; Santiago et al., 2002). HIV-1 is divided into four lineages; groups M, N O and P. Group M is the main group with further subtypes of A to K, and 61 circulating recombinant forms (for an update see http://www.hiv.lanl.gov) ("HIV databases," access date 9 January 2014.) with some evidence to suggest that the genotypic subtype classification may have different disease phenotypes (Shehu-Xhilaga and Oelrichs, 2009). Subtype B predominates in Europe, America, Japan, Thailand and Australia (Bobkov et al., 2004) whereas subtype C predominates in Southern Africa, India and Nepal (McCormack et al., 2002; Neilson et al., 1999; Renjifo et al., 1998; Salminen et al., 1996). Group N represents non M and non O as identified in 1993 in Cameroon. Group O is known as the outlier group and is mostly confined to West Africa. Group P was discovered most recently in 2009 and is genetically more similar to SIV found in gorillas than the SIV in chimpanzees (Plantier et al., 2009). The virus was isolated from a Cameroonian

woman and one other human case has been identified in a Cameroonian male thus confirming the extremely rare circulation of group P (Vallari et al., 2011).

The analysis of the occurrence of the genetic events in population history, known as a genetic clock, indicates that the most recent common ancestor of the HIV-1 group M dates back to the 1920s, and the characterization of the 1959 and 1960 samples highlights the subsequent substantial diversification of group M (Korber et al., 2000; Sharp and Hahn, 2010; Worobey et al., 2008; Zhu et al., 1998).
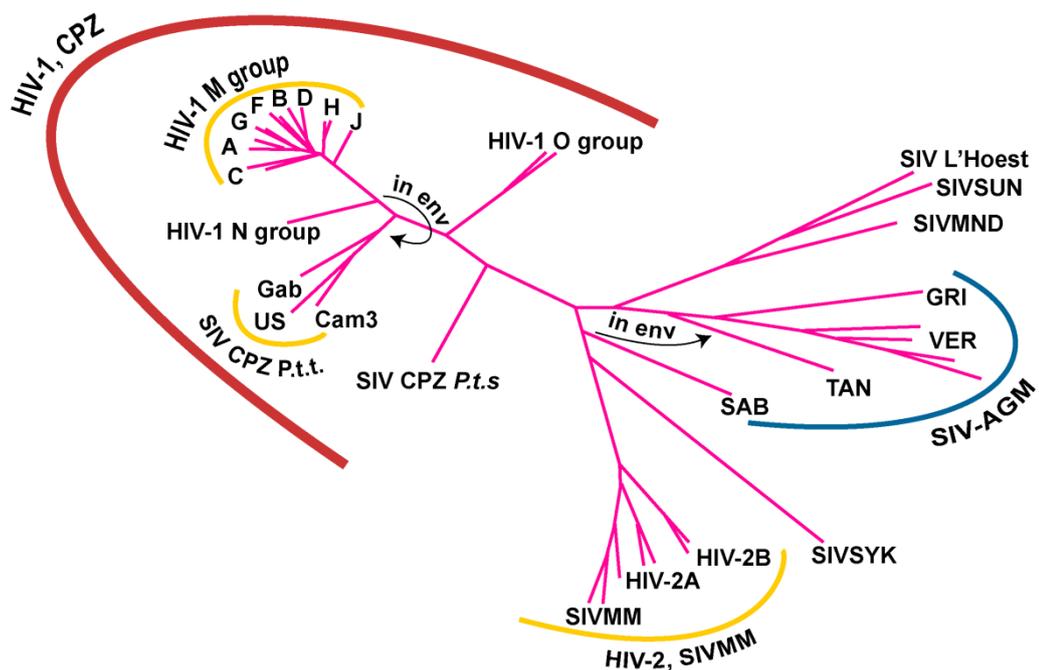
Figure 1.1: Maximum likelihood phylogenetic tree analysis showing the relationships between HIV-1, HIV-2 and SIV partial sequences. The tree was constructed utilising *pol* gene sequence. The figure was adapted from Human Retroviruses and AIDS research, 1999 (Korber et al., 1999).

1.1.3 Genetic and structural organization of HIV
Genetic organisation

HIV has been classified in the family *Retroviridae* with subfamily *Lentivirinae* and the genus of Lentivirus (Chiu et al., 1985; Vogt, 1997; Wain-Hobson et al., 1985).

The HIV lineage is as follows:

Viruses

    Vertebrate Viruses

        Retrovirus

            Family: Retroviridae

                Genus: Lentivirus

                    Serogroup: Primate lentivirus

                        Species: Human Immunodeficiency Virus

The HIV genome consists of nine genes, overlapping in a number of regions, encoding for proteins essential to the functioning of the virus (Fauquet and Fargette, 2005). The genomic organisation of HIV-1 is depicted in Figure 1.2, and shows the nine genes encoding for structural (Gag, Pol and Env), regulatory (Tat and Rev) and accessory (Vif, Vpu, Vpr and Nef) proteins that make up the HIV virion (Gelderblom, 1991). All HIV-1 genes, gene products and associated functions are listed in Table 1.1.
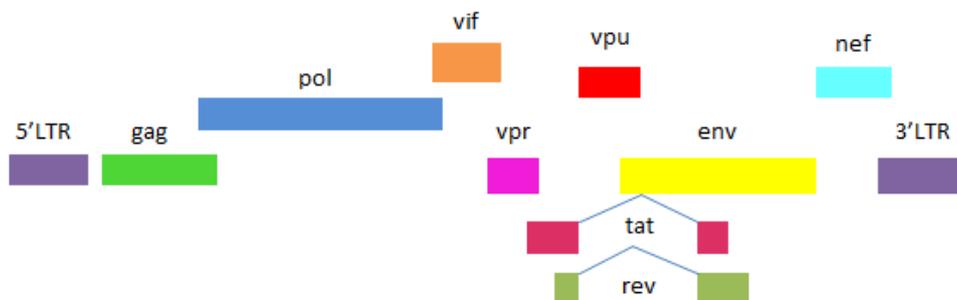
Figure 1.2: Genetic organisation of the HIV-1 genome (approximately 9.7kb), showing the three main structural genes *gag, pol* and *env* and regulatory (*tat, rev*) and accessory (*vif, vpr, vpu, nef)* genes needed for effective HIV replication.

Table 1.1: The gene product, size, function and location of HIV-1 structural, regulatory and accessory proteins.

Gene product	Size kDa	Function	Location
Structural gene Products			
Gag			
MA	p17	Membrane anchor, env interaction and nuclear transport of viral core (Foley et al., 2013)	Virion
CA	p24	Core capsid (Foley et al., 2013)	Virion
NC	p7	Nucleocaspid, binds RNA (Foley et al., 2013)	Virion
	p6	Binds VPR (Foley et al., 2013)	Virion
Pol			
Protease (PR)	p15	Cleavage of Gag-pol polyprotein precursor and maturation (Foley et al., 2013)	Virion
Reverse transcriptase(RT) RNaseH	p66,p51 p15	RNA-dependent and DNA dependent polymerase activities. RNaseH activity catalyses the cleavage of DNA bound RNA via a hydrolytic mechanism (Foley et al., 2013)	Virion
Integrase (IN)	p31	Mediates the insertion of the HIV-1 proviral cDNA into the genomic DNA (Foley et al., 2013)	Virion
Env			
Gp160	p160	Gp120 and gp41 precursor (Bernstein et al., 1995; Capon and Ward, 1991)	
Gp120	p120	CD4 and co-receptor binding (Bernstein et al., 1995; Capon and Ward, 1991)	Virion
Gp41	p41	Membrane fusion (Bernstein et al., 1995; Capon and Ward, 1991)	Plasma membrane
Accessory Gene Products			
Nef	p25~27	Down regulates cell surface CD4 and MHC-I molecules. Augments virus infectivity. Modulates signalling pathways of cells (Sherman and Greene, 2002; Tungaturthi et al., 2003; Yao et al., 2002; Zhang et al., 2001)	Plasma membrane
Vpr	p10~15	Nuclear localization of the pre-integration complex (PIC) , inhibits cell division, arrest cells in G2 phase of mitosis (Montal, 2003)	Virion, Nucleus

Vif	p23	Promotes virus infectivity, inhibits APOBEC3G (Sheehy et al., 2003)	Cytoplasm, Virion
Vpu	p17	Induces CD4 receptor degradation in the endoplasmic reticulum, thus enhancing budding (Fisher et al., 1987; Strebel et al., 1988)	Integral Membrane Protein
Regulatory Gene Products			
Tat	p14	Regulates viral gene expression (Seelamgari et al., 2004)	Primarily in nucleolus
Rev	p18	Nuclear transport of viral RNA, stability and utilization factor (Glushakova et al., 2001; Shaheduzzaman et al., 2002; Stoddart et al., 2003)	Primarily in nucleolus

Structural organisation

The structure of HIV-1 can be described as a classical retrovirus, consisting of two plus strands of HIV-1 RNA (ribonucleic acid) with each containing a copy of the nine genes of the virus. Figure 1.3 shows the structural makeup of HIV-1 where the RNA is enclosed in a capsid that consists of approximately two thousand copies of p24 viral protein (Goto et al., 1998). The capsid is then surrounded by the matrix (p17) which is further surrounded by a lipid bilayer formed from the cellular membrane of the host cell during budding of the newly formed virus particle, within which the viral envelope glycoproteins are embedded. The envelope glycoprotein subunit consists of two non-covalently linked membrane proteins, gp120, the external protein, and gp41, the trans membrane protein, which anchors the glycoprotein complex to the virion (Eckert and Kim, 2001). The envelope glycoprotein is the most variable part of the virus with gp120 divided into five highly variable regions (V) interspersed by five constant regions (C).
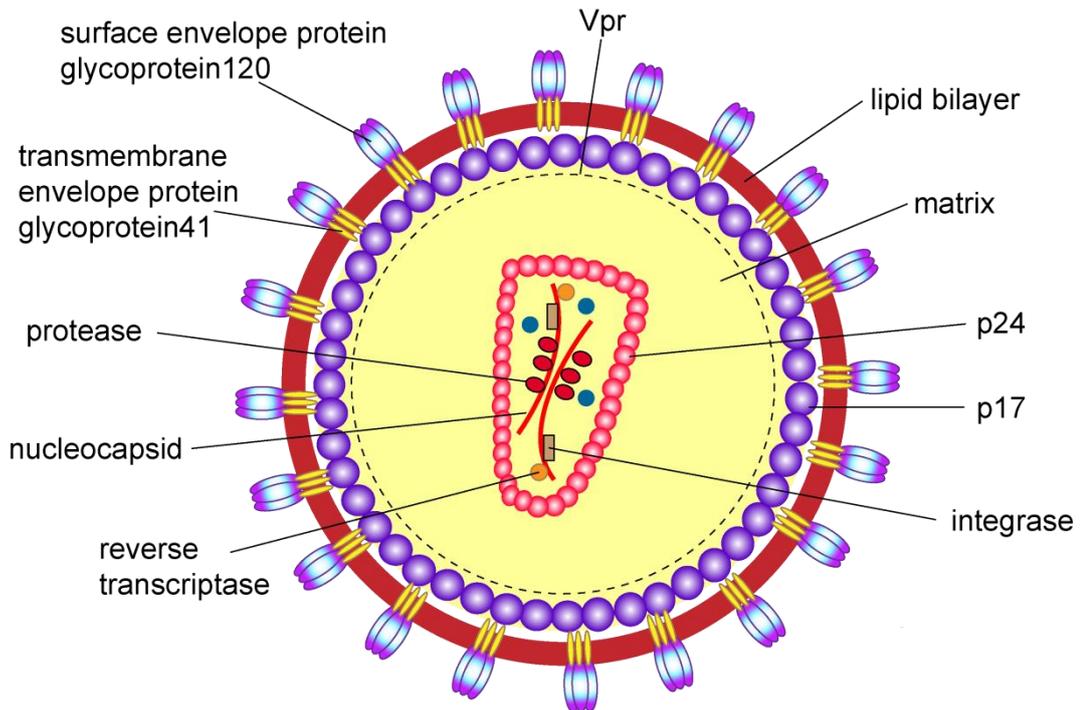
Figure 1.3: Representative structure of the mature HIV-1 virion showing structural and other proteins. Glycoproteins gp120 and gp41 together form the envelope that penetrates the lipid bilayer. P17 forms the matrix surrounding the capsid composed of p24 molecules encapsulating viral RNA, IN, PR and RT.

1.1.4 Lifecycle of HIV

The HIV lifecycle consists of numerous stages to successfully infect a host cell and replicate the HIV virion to maintain infection. The first stage in the lifecycle is viral entry followed by reverse transcription of viral RNA into cDNA followed by integration, transcription and translation of viral proteins, and finally virion assembly and budding (Figure 1.4).

The steps in the viral lifecycle include:

Viral entry

HIV-1 entry follows a sequential sequence whose purpose is to deliver virus and requires three main steps; namely, attachment to the host cell and CD4 binding, co-receptor binding and membrane fusion (Mondor et al., 1998; Moulard et al., 2000). Once the HIV-1 gp120 has bound to the CD4 receptor, it undergoes
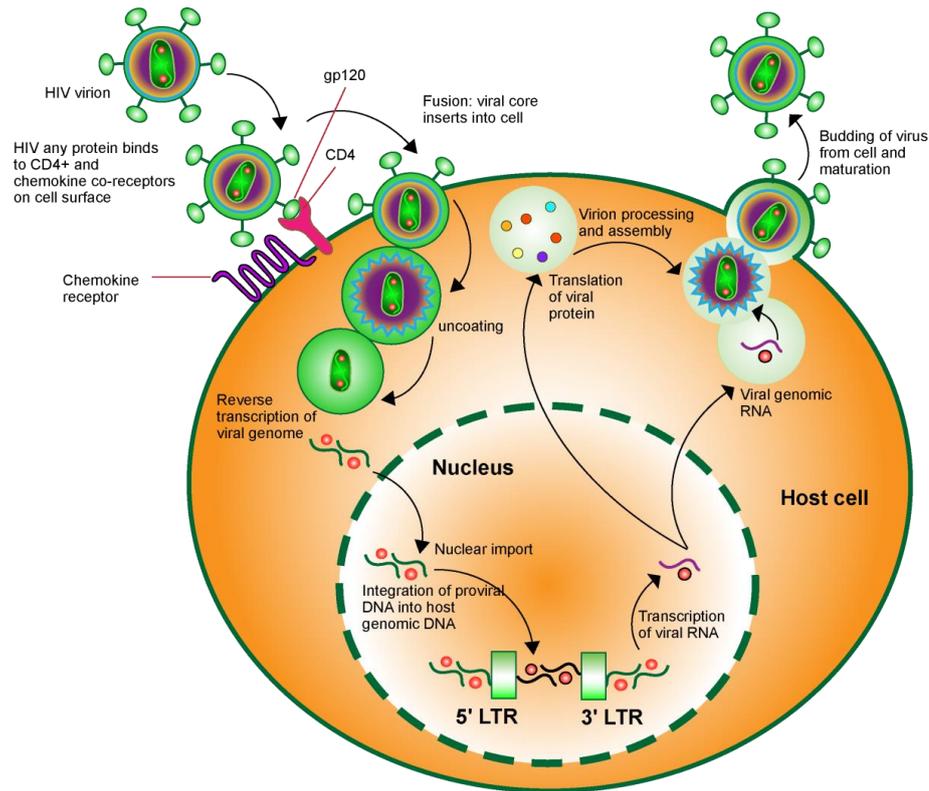
Figure 1.4: Typical lifecycle of HIV-1 showing entry, reverse transcription, integration, transcription, translation, assembly and budding. In the first step of the HIV-1 lifecycle the virion attaches to the host cell via the CD4 receptor and the chemokine receptor followed by viral entry. Once the virion has entered the host cell the nucleocapsid is uncoated allowing reverse transcription to occur where the viral DNA is then transported to the nucleus in the form of the PIC where integration will occur. The viral DNA is irreversibly integrated into host DNA, viral mRNA is transcribed and transported out of the nucleus where proteins are translated. The proteins assemble to form new virion particles and bud from the surface of the host cell, releasing immature virion particles.

conformational changes allowing it to bind to a co-receptor (Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996). The most common co-receptors are chemokine receptors CCR5 and CXCR4 (Doranz et al., 1996; Hoffman et al., 1998; Zhang et al., 1998). After the binding of gp120 to CD4 and the co-receptor, there is a conformational change in gp41 where the N-terminal is inserted into the target cell membrane (Eckert and Kim, 2001). This results in a fusion pore which allows the viral capsid to enter the cell (Chan et al., 1997; Weissenhorn et al., 1997). Once the virus has entered the cell, the capsid is uncoated and the viral genome is released in preparation for reverse transcription.

Literature review

Reverse transcription

Two enzymes are needed for transcription, namely DNA polymerase and RNaseH. Both enzymes are part of reverse transcriptase. DNA polymerase can copy the RNA or DNA and RNaseH degrades the RNA template. The viral RNA genome serves as a template for provirus formation by RT. HIV-1 contains a primer binding site approximately 180 nucleotides from the 5' end of the RNA that allows for the process of DNA synthesis (Fouchier and Malim, 1999; Gallay et al., 1997). DNA synthesis creates an RNA-DNA complex where the RNA is first reverse transcribed into single stranded DNA. RNaseH degrades the RNA as DNA is synthesized but cannot cleave a purine rich sequence that serves as the primer for the second strand of DNA synthesis (Gallay et al., 1997). The second cDNA strand is synthesised, 3' end processed by IN, and then forms a complex with proteins from both the host and virus known as the PIC (PIC; reviewed in more detail in section 1.3) and is actively transported to the nucleus for integration (Fouchier and Malim, 1999; Gallay et al., 1997).

Integration, transcription and translation

HIV-1 is either spliced and randomly integrated into the host DNA or it forms stable DNA circles (Bushman et al., 1990). Once the DNA is integrated into the host genome by strand transfer it is known as a provirus. The provirus has identical long terminal repeat (LTR) copies on either side of the coding region (Nekhai and Jeang, 2006). The 5' LTR now functions as the promoter regulating the production of RNA transcripts. The RNA transcripts may now be spliced in preparation for translation to form viral proteins or transported out of the nucleus in unspliced form to form new virion particles (Nekhai and Jeang, 2006).

Viral assembly and budding

The viral protease initiates cleavage of the Gag/Pol polyprotein. Immature viral polypeptides and full length HIV-1 RNA transcripts are assembled on the cell membrane to form immature particles. The lipid membrane of the new immature particle is formed from the host cells plasma membrane. Vpu facilitates the virion release from the cell membrane (Strebel et al., 1988). During the budding process, viral proteins within the immature particle are processed into their functional forms by protease and are rearranged into the mature particles (Shehu-Xhilaga and Oelrichs, 2009).

1.1.5 Drug targets of HIV-1 and drug intervention

Within the HIV-1 lifecycle, each of the steps described above is a potential target for intervention with antiretroviral therapy. Currently, antiretroviral drugs targeting entry, reverse transcription, integration and cleavage of the viral polyproteins by protease are in clinical use.

The first targeted stage is viral entry, with US Food and Drug Administration (FDA) approved inhibitors falling into two classes, including chemokine receptor antagonists and fusion inhibitors. Enfuvirtide (Fuzeon, Roche Diagnostics) is a gp41 binder (Kilby et al., 2002; Lalezari et al., 2003), preventing the fusion of the viral membrane with the host membrane. Maraviroc (Selzentry, Pfizer) binds to CCR5 preventing an interaction with gp120 (Lieberman-Blum et al., 2008). Several viral entry inhibitors are currently in clinical trials such as TNX-355/ibalizumab, a humanised anti CD4 monoclonal antibody that binds to CD4 and inhibits docking of the virus. PRO140 a humanised monoclonal antibody targeted against CCR5 has been fast tracked for approval and is currently in phase II clinical trials (Henrich and Kuritzkes, 2013).

The second targeted step for antiretroviral treatment is reverse transcription with RT being the first HIV-1 enzyme to be targeted for antiretroviral drug discovery (Mitsuya and Broder, 1987). There are three distinct groups of RT inhibitors, first are nucleoside reverse transcriptase inhibitors (NRTIs), secondly non-nucleoside reverse transcriptase inhibitors (NNRTIs) and lastly nucleotide reverse transcriptase inhibitors (NtRTIs). NNRTIs bind to the non-catalytic allosteric pocket on the enzyme whereas NRTIs block reverse transcription by binding to an alternative site, thereby referred to as non-competitive inhibitors, and inhibit the movement of the protein domains of RT which are needed for DNA synthesis. NRTIs consist of lamivudine, emtricitabine, abacavir, zalcitabine, zidovudine, azidothymidine, stavudine. Zidovudine was the first FDA approved antiretroviral treatment. NNRTIs efavirenz, nevirapine, delavirdine, etravirine and rilpivirine are all FDA approved (Arts and Hazuda, 2012).

Another target for intervention is assembly and maturation. Inhibitors targeting this step are known as protease inhibitors (PIs) and block the proteolysis of viral protein which is vital for the production of infectious viral particles. Protease inhibitors require a booster agent that does not directly reduce viral activity but

work as a pharmacokinetic enhancer (Wensing et al., 2010). Currently FDA approved PIs are saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, fosaprenavir, atazanavir, tipranavir and darunavir. Ritonavir was found to have negative side effects at full doses such as nausea and diarrhoea, but was found not to have these effects at lower doses. As a result ritonavir has been used as a boosting agent for other PI's (Zeldin and Petruschke, 2004).

The newest form of ARVs falls under the class of IN inhibitors also known as IN strand transfer inhibitors (INSTIs). The current FDA approved IN inhibitors are raltegravir ("FDA notifications Accelerated approval for raltegravir tablets," 2007), elvitegravir and dolutegravir a second generation IN inhibitor was recently approved by the FDA (Arts and Hazuda, 2012). New molecules are being designed which dually inhibit both RT and IN and are considered a type of 'portmanteau' inhibitors (Abbink and Berkhout, 2008; Das and Arnold, 2013).

Highly active antiretroviral therapy (HAART) is a highly aggressive form of treatment to suppress HIV-1 replication and HIV-1 disease progression. HAART consists of a combination of three drugs, two NRTIs and a NNRTI or PI. HAART was initially introduced in 1996 and has since significantly reduced the number of deaths worldwide. HAART prolongs lifespan of HIV-1 infected individuals by preventing the progression of HIV to AIDS (Bally et al., 2000). A major downfall to HAART is failure due to toxicity, poor response to therapy or patient non adherence to regimes that usually end in resistance to the drugs within the regime (Perelson et al., 1996). To date, these are the only FDA approved antiviral treatment areas. As HIV-1 is mutating and developing resistance genes to each of the current FDA approved drugs it is necessary to research novel areas of intervention.

1.2 The HIV-1 integrase enzyme and integration

The HIV IN enzyme and its cellular partners are of great interest in current drug discovery.

1.2.1 Structure and function of HIV-1 integrase

HIV-1 IN is responsible for the integration of viral DNA into host DNA (Coffin et al., 1997; Rice et al., 1996) which enables HIV to establish a permanent genetic reservoir. HIV-1 IN is a 32 kDa, 288 amino acid protein that is encoded by the *pol*

gene, and comprises the N-terminal domain, the catalytic core domain and the C-terminal domain. The N-terminal domain, consisting of amino acids 1-51, contains an HHCC motif that binds zinc, which has been implicated in promoting protein multimerization (Zheng et al., 1996). The catalytic domain consisting of amino acids 52-219, is important in enzymatic activity of HIV-1 IN and includes the nuclear localization signal which is critical in mediating the import of HIV-1 IN in the form of the PIC (Esposito and Craigie, 1999). The third domain known as the C-terminal domain, consisting of amino acids 220- 288, has a DNA binding site and is needed for the integration reaction as it is involved in DNA-IN complex stabilization (Esposito and Craigie, 1998; Lodi et al., 1995). HIV-1 IN contains several functional domains such as the nuclear localization signal (NLS) in the N terminal portion of MA p17 (Bukrinsky et al., 1993; Heinzinger et al., 1994), which is a critical sequence mediating the import of HIV-1 IN in the form of the PIC (Bouyac-Bertoia et al., 2001).

HIV-1 IN is functional in the multimeric form and catalyses two reactions shown in Figure 1.5. In the first reaction HIV-1 IN binds to the LTR at the ends of the viral DNA followed by the cleavage of two conserved nucleotides (CA) from the 3' ends of both LTRs. This is known as 3' processing and occurs in the cytoplasm as part of the PIC. The PIC is then actively transported to the nucleus where the resulting DNA, from 3' processing, serves as a substrate for the integration of the viral DNA into the host DNA. This is a process known as strand transfer which occurs simultaneously at both ends of the viral DNA (Brown, 1997; Brown et al., 1987; Craigie, 2001; Craigie et al., 1990; Engelman et al., 1991). Metal co-factors such as magnesium and manganese are needed for both 3' processing and strand transfer. HIV-1 IN shows strong non-specific nuclease activity in the presence of manganese. Furthermore, sequence variations as a result of reverse transcription errors are better tolerated by HIV-1 IN in the presence of magnesium (Marchand et al., 2003).
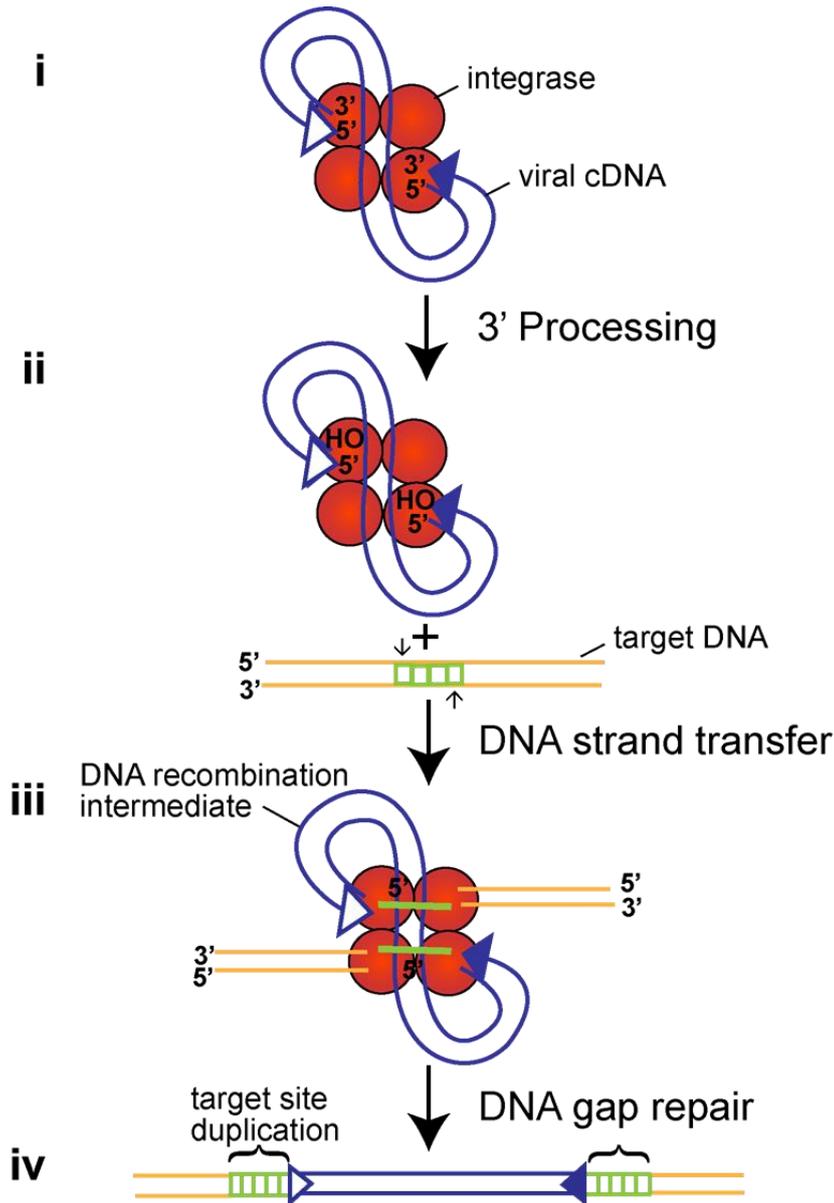
Figure 1.5: Schematic representation of the HIV-1 integration reaction. i) IN in tetrameric form is bound to viral cDNA and generates a CA overhang during 3' processing. ii) The pre- integration complex is transported to the nucleus where the strand transfer reaction is initiated by target DNA binding. iii) Binding is followed by nucleophilic attack and the formation of a phosphodiester bond leaving a 5' DNA flap. iv) DNA is repaired by host machinery which results in an integrated provirus.

1.3 The pre-integration complex and host factors
1.3.1 Pre-integration complex

The PIC is a high molecular weight nucleoprotein complex comprised of viral and host proteins formed within the cytoplasm (Iordanskiy et al., 2006). The exact

composition of the PIC is unknown but in addition to cDNA, HIV-1 IN, matrix, nucleocaspid, RT and VPR, the dominant host proteins included in the PIC are barrier to auto integration factor (BAF), High mobility group chromosomal protein A1 (HMGA1) and Lens Epithelial Derived Growth Factor (LEDGF) p75 with all components essential for efficient integration of proviral DNA (Bukrinsky et al., 1993; Devroe et al., 2003; Gulizia et al., 1994). The viral cDNA remains associated with the PIC until integration is complete (Bukrinsky et al., 1992). The environment created by the PIC is ideal for 3' processing as cDNA is protected from degradation by cellular nuclease allowing the proviral DNA to be transported to the nucleus in an energy dependent reaction (Sherman and Greene, 2002). The PIC assists in the DNA strand transfer activity by the recruitment of cellular cofactor LEDGF/p75. LEDGF/p75 effectively tethers HIV-1 IN to chromosomal DNA as can be seen in Figure 1.6.

There are several residues that are involved in chemical bonds and hydrophobic contacts with cellular cofactors such as the LEDGF/p75 (Busschots et al., 2007; Cherepanov et al., 2005b; Hombrouck et al., 2007; Maertens, 2003; Rahman et al., 2007), HMGA1 and BAF. HMGA1 is a non-histone DNA-binding protein involved in the regulation of inducible gene transcription and microRNA expression (De Martino et al., 2009). Experimental removal of HMGA1 by gel filtration resulted in the loss of HIV-1 IN activity thereby indicating the essential need for HMGA1 for integration, once HMGA1 was added, the activity of HIV-1 IN was restored (Farnet and Bushman, 1997). Knockdown studies of BAF of HIV-1 IN did not affect HIV-1 replication (Lee and Craigie, 1998), indicating that BAF is not essential to the replication cycle. The proof-of-concept for HMGA1 inhibitors is present, however; BAF research seems futile as the knockdown of BAF showed no effect in HIV replication. Since LEDGF/p75 was identified as a binding partner of HIV-1 IN, several research groups have tried to identity novel drugs preventing the IN-LEDGF/p75 interaction (Cherepanov, 2002).
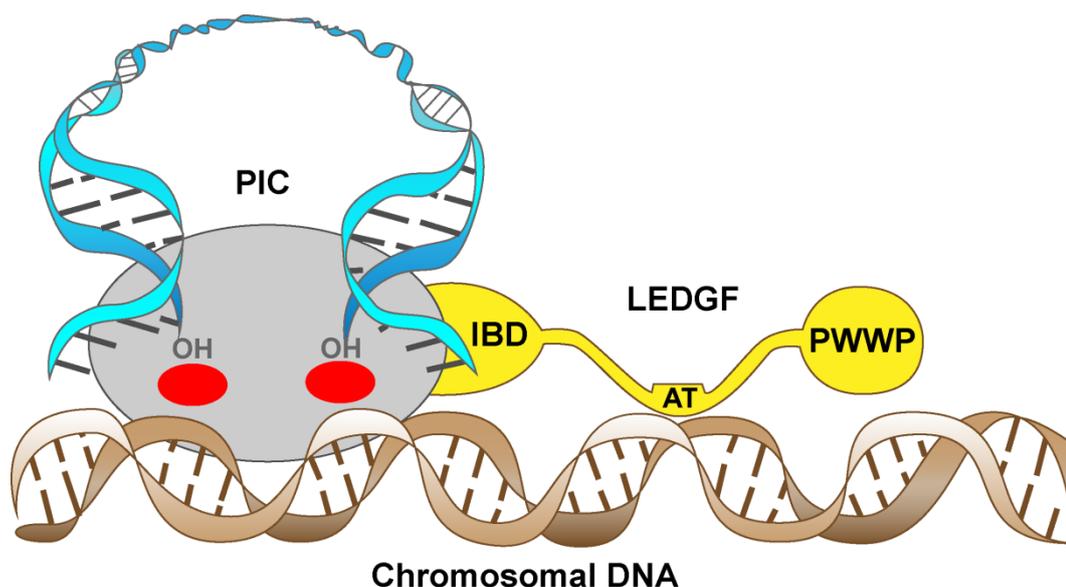
Figure 1.6: Representation of the pre-integration complex of HIV-1, indicating LEDGF/p75 bound to HIV-1 IN at the IN binding domain (IBD) and bound to chromosomal DNA at the AT hook. LEDGF/p75 contains a Pro-Trp-Trp-Pro (PWWP) motif at the N-terminal domain needed for tight association with chromosomal DNA (adapted from Hare and Cherepanov, 2009).

1.4 Structure and function of LEDGF/p75

LEDGF/p75 is a hepatoma derived growth factor protein that is ubiquitously expressed in the nucleus of cells (Fatma et al., 2004, 2001; Hui Ge et al., 1998; Kubo et al., 2002; Sharma et al., 2000; Shin et al., 2008; Shinohara et al., 2002; Yokoyama and Cleary, 2008). LEDGF/p75 was initially discovered by micro sequencing of a protein that was co-purified with the general transcriptional co-activator positive cofactor 4 (PC4) (Hui Ge et al., 1998). The following year LEDGF/p75 was isolated from a mouse lens epithelium library and reported to protect the cells from oxidative stress (Singh et al., 1999). It was thereby termed LEDGF (Dietz et al., 2002). LEDGF/p75 is the product of a splice variant of cDNA, the region codes for two proteins. The first is encoded by the 75 kDa species (p75) and the second variant is encoded by the smaller p52. Both proteins increase the activity of general transcription machinery *in vitro* and were thereby coined transcriptional co-activators p75 and p52. LEDGF/p75 and p52 share the same N-terminal 325 amino acids but differ in the C-termini. LEDGF/p75 is encoded by the gene PSIP1 (Singh et al., 2000) and contains a nuclear localization signal (NLS) which drives nuclear localization of HIV-1 IN

when both proteins are produced at high levels (Llano et al., 2004; Maertens, 2004; Vanegas et al., 2005).

The C-terminus of LEDGF/p75 consists of 205 amino acids with the IBD falling in this region. The IBD is crucial to the proteins virological and cellular significance (Cherepanov et al., 2004; Vanegas et al., 2005). LEDGF/p75 serves as a bi-functional tether, tethering chromatin at its N-terminal PWWP domain and tethering HIV-1 IN at the IBD on the C-terminus as seen in Figure 1.7 (Bartholomeeusen et al., 2008, 2007; H Ge et al., 1998; Hui Ge et al., 1998; Llano et al., 2006b; Maertens et al., 2006; Nishizawa et al., 2001; Turlure et al., 2006; Yokoyama and Cleary, 2008). The LEDGF/p75 interaction with HIV-1 IN through the IBD results in the interaction with a number of other proteins, mainly the cMyc interactor JPO2 (Bartholomeeusen et al., 2007; Maertens et al., 2006), the menin/MLL histone methyl transferase complex (Yokoyama and Cleary, 2008) and the pogo transposable element with ZNF domain (PogZ) (Bartholomeeusen et al., 2008). LEDGF/p75 protects JPO2 from proteolysis and tethers the protein to chromatin during all phases of the cell cycle (Bartholomeeusen et al., 2007; Bartholomeeusen et al., 2008; Maertens et al., 006; Yokoyama and Cleary, 2008), while the interaction with PogZ yields a tight chromatin bound complex during interphase but not during mitosis (Bartholomeeusen et al., 2008).
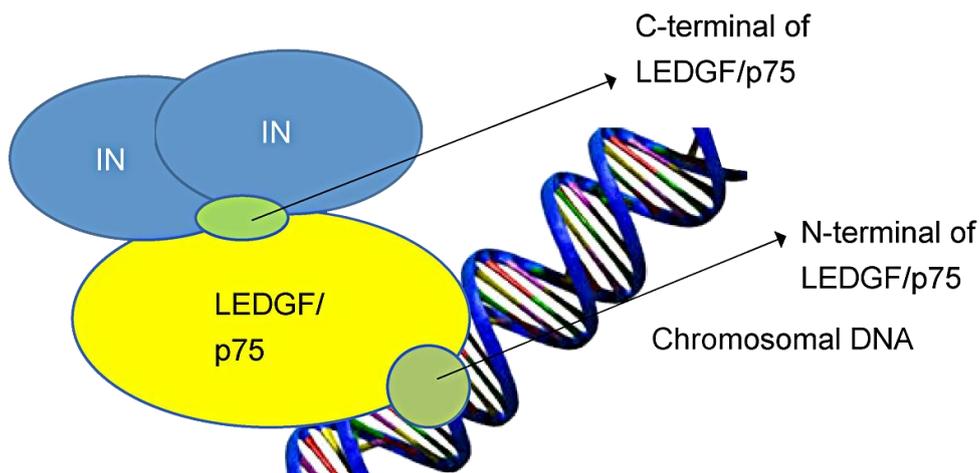
Figure 1.7: Schematic representation of the tethering function of LEDGF/p75. LEDGF/p75 attaches to chromosomal DNA via the N-terminal domain of LEDGF/p75 and attaches to the IBD at the C-terminal domain of LEDGF/p75 thereby tethering IN to DNA.

1.5 HIV-1 IN-LEDGF/p75 protein-protein interaction

Protein-protein interactions are intrinsic to every cellular process in cell based assays as well as in the human body. Proteins may interact with other protein or may interact with ligands. Interactions included in protein recognition are hydrogen bonding, hydrophobic interactions and electrostatic interactions. An understanding of these interactions is indispensable to rational drug design.

Hydrogen bonding is known as non-covalent interaction were a single hydrogen atom is shared between two atoms. The single hydrogen is covalently linked to the donor atom and electrostatically linked to acceptor atom. In protein interactions the donor and acceptor groups are the polar amino acid side chains. Various studies have shown that hydrogen bonding aids in the stabilization of protein molecules (Habermann and Murphy, 1996; Jiang and Lai, 2002; Makhatadze and Privalov, 1995).

Hydrophobicity is the repulsion of water from oil like substances. Hydrophobic interactions occur when non polar groups repel water and tend to aggregate in aqueous solutions due to unfavourable interactions with the water forming a barrier with the non-polar groups (Chandler, 2005; Matthews, 2001). In proteins, hydrophobic interactions drive protein folding as well as add a level of stability to the protein. Hydrophobic amino acids will cluster together in the core of the protein thereby folding and stabilizing as a result of hydrophobic interactions (Charton and Charton, 1982; Kauzmann, 1959).

Electrostatic interactions occur between charged substances; this can be like charged substances causing strong attraction or oppositely charged substances causing strong repulsion, providing they are within 4Å of one another. On a protein level, electrostatic interactions occur at the carboxyl termini of the amino acids or at ionisable side chains of specific amino acids (Dill, 1990). The integration of HIV-1 into chromatinised template is stimulated by LEDGF/p75 (Botbol et al., 2008) while LEDGF/p75 stabilizes HIV-1 IN subunit-subunit interactions and promotes HIV-1 IN tetramerisation (Gupta et al., 2010; McKee et al., 2008).

The HIV-1 IN-LEDGF/p75 protein-protein interaction consists of p75 residues 341-429 and the core domain of HIV-1 IN as seen in Figure 1.8 (Cherepanov et

al., 2005a, 2005b, 2004; Llano et al., 2004; Maertens, 2004; Vanegas et al., 2005). The elucidation of the crystal structure of the catalytic domain of HIV-1 IN in a complex with the IBD demonstrates a pair of LEDGF/p75-IBD molecules bound at a symmetry-related position at the interface of the HIV-1 IN dimer (Cherepanov et al., 2005a, 2005b). LEDGF/p75 effectively tethers the viral HIV-1 IN to the host cell chromatin through C-terminal interactions with the IBD and the N-terminal binding to chromatin thereby facilitating integration (Ciuffi and Bushman, 2006; De Rijck et al., 2010; Gijsbers et al., 2010; Marshall et al., 2007; Shun et al., 2007). Furthermore, LEDGF/p75 has been shown to stimulate HIV-1 IN activity using mini HIV DNA substrates (Cherepanov, 2002). The stimulatory effect of LEDGF/p75 on HIV-1 IN requires LEDGF/p75 to be bound to HIV-1 IN prior to DNA binding, which infers that the HIV-1 IN-LEDGF/p75 interaction occurs before reverse transcription (Yu et al., 2007).
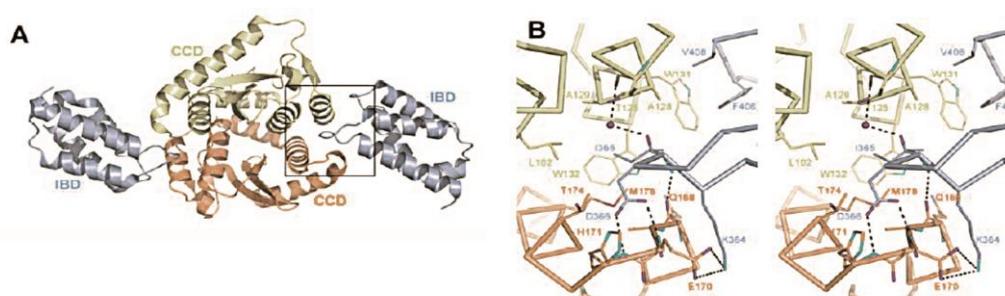
Figure 1.8: Crystallographic depiction of the IN binding domain, showing (A) the overall ribbon structure of the CCD-IBD complex where IN chains A and B are brown and beige, and the IBD subunits are in purple; and (B) the key inter molecular contacts of the CCD-IBD interface (adapted from Hare and Cherepanov, 2009)).

Mutations in HIV-1 IN can cause reduced activity of the enzyme if the mutation lies in the IBD. HIV-1 IN mutations of A128T and E170G in the IBD render HIV-1 IN resistant to an interaction with LEDGF/p75, further confirming the validity of the IBD/IN catalytic core domain function (Busschots et al., 2007; Cherepanov et al., 2005b). Additionally, IN mutations V165A, A179P, KR186,7AA , K159P, V176A and I203P cause impaired binding of host chromatin or LEDGF/p75 binding (Xu et al., 2008; Zheng et al., 2010). The reduced affinity on HIV-1 IN KR186,7AA for LEDGF/p75 is due to disabled oligomerisation of HIV-1 IN (Berthoux et al., 2007; McKee et al., 2008). V165 is involved in the HIV-1 IN-

LEDGF/p75 interaction interface and A179 was identified as a new amino acid in the LEDGF/p75 binding site critical for the IN-LEDGF/p75 interaction (Busschots, 2004; Cherepanov et al., 2005b; Rahman et al., 2007). HIV-1 IN with mutations of K159P, V176A and I203P lost the ability to bind both chromatin and LEDGF/p75, while mutations of H171A, L172A and EH170, 1AA impaired the ability to interact with LEDGF/p75 but retained the ability to bind chromatin. This suggests that HIV-1 IN can bind chromatin independently of LEDGF/p75. Viruses harbouring HIV-1 IN mutants incapable of binding chromatin on LEDGF/p75 completely lost infectivity whereas virus harbouring HIV-1 IN mutations capable of chromatin binding but not LEDGF/p75 sustained low levels of HIV-1. Even in the latter case the level of HIV-1 infectivity was highly reduced (Zheng et al., 2010) indicating the importance of LEDGF/p75 to the HIV-1 integration reaction.

1.6 LEDGF/p75-integrase inhibitors

Due to the increase in mutations in HIV-1 IN against current INSTIs, focus has shifted to novel mechanisms of action with the design of allosteric inhibitors or interaction inhibitors targeting essential cofactors of integration such as the HIV-1 IN-LEDGF/p75 interaction. Depletion of LEDGF/p75 from cells by knockout techniques drastically reduced HIV-1 infection in those cells (Llano et al., 2006a; Schrijvers et al., 2012; Shun et al., 2007; Vandekerckhove et al., 2006) providing proof-of-concept evidence that the HIV-1 IN-LEDGF/p75 interaction is a feasible target for anti HIV therapy. Various approaches have been used to design and identify small molecule inhibitors for the HIV-1 IN-LEDGF/p75 interaction. Most efforts so far have been successful with *in silico* screening and structurally based de novo design leading to the identification of HIV-1 IN-LEDGF/p75 inhibitors (Christ et al., 2012; De Luca et al., 2010; Du et al., 2008; Fan et al., 2011; Peat et al., 2012).

LEDGINS are small molecules that are inhibitors of the HIV-1 IN-LEDGF/p75 interaction and bind to the LEDGF/p75 binding site on the HIV-1 IN catalytic core domain, allosterically inhibiting the strand transfer activity of HIV-1 IN (Christ et al., 2012). This interference and inhibition suggests that LEDGINS could potentially block integration and therefore the replication of HIV-1 within the cell. LEDGINs binding may restrict IN oligomeric flexibility, affecting the formation of the nucleoprotein complex active in integration of viral DNA into host DNA, known

as the intasome. It was found that LEDGINs do stabilize HIV-1 IN and promote dimerization (Christ et al., 2012). This most likely restricts the multimerisation dynamics of HIV-1 IN needed to bind viral DNA productivity (Christ et al., 2012). To date there is only one HIV-1 IN-LEDGF/p75 inhibitor (BI2244336) (Gilead, USA) in phase I clinical studies which was recently licenced to Gilead for further investigations and developments (Tsiang et al., 2012).

Peptides are another source of HIV-1 IN-LEDGF/p75 inhibition, however, these pose problems with stability and bioavailability (Adessi and Soto, 2002). Initial work involved peptides derived from the LEDGF/p75 sequence that binds to HIV-1 IN (Al-Mawsawi et al., 2008; Hayouka et al., 2010; Rhodes et al., 2011). A peptide was reported to compete with LEDGF/p75 for HIV-1 IN binding with an IC_{50} of 25µM. With this peptide, the strand transfer activity and 3' processing inhibition was less pronounced and activity was completely lost when the DNA-IN complex was assembled prior to the presence of the peptide, suggesting that the peptide disrupts the initial DNA binding of HIV-1 IN (Hayouka et al., 2007). Although most work to date has focused on finding inhibitors that bind to HIV-1 IN to prevent LEDGF/p75 binding, Desimmie *et al.* recently described LEDGF/p75 binders (Desimmie et al., 2012).

It is now evident that molecules reaching high potency show a dual mechanism of action, actively blocking the HIV-1 IN-LEDGF/p75 interaction as well as displaying strand transfer inhibition (Christ et al., 2012, 2010; Kessl et al., 2012; Tsiang et al., 2012). Allosteric HIV-1 IN inhibitors (ALLINIs) are a new class of HIV-1 antiviral agents that show multimode mechanisms of action by allosterically modulating HIV-1 IN multimerization and interfering with HIV-1 IN-LEDGF/p75 binding (Christ et al., 2012). ALLINI pressure has revealed an A128T substitution in the HIV-1 IN as a primary mechanism of resistance to IN inhibitors (Feng et al., 2013). BI2244336 falls under a class of new agents known as non-catalytic site IN inhibitors (NCINIs). These compounds bind to a conserved pocket on HIV-1 IN that is the target for LEDGF/p75, however, this falls outside of the catalytic site for HIV-1 IN. Tests conducted with this compound displayed antiviral activity against recombinant viruses that show known HIV-1 IN inhibitor and NNRTI resistance. BI2244336 was found to have an EC_{50} of 14 nM in PhenoSense assays screened with 200 clinical isolates and retained its activity against 40

raltegravir resistant isolates with an EC_{50} of 26 nM (Fenwick et al., 2011; Simoneau et al., 2011; Yoakim et al., 2011). BI2244336 is in phase Ia clinical trials, being tested on healthy non HIV-1 infected individuals.

Recently Hu and co-workers identified atorvastatin (Lipitor, Pfizer, USA) as an inhibitor of the HIV-1 IN-LEDGF/p75 interaction via molecular docking and then biologically determined an IC_{50} of 8.90 µM (Hu et al., 2012). In the same study, they evaluated fluvastatin (Lescol) but found this statin to be lacking notable inhibitory activity (Hu et al., 2012). Statins have been reported to have antiviral activity through clinical studies however various mechanisms of action have been suggested (Amet et al., 2008; del Real, 2004; Ganesan et al., 2011; Giguère and Tremblay, 2004; Jain and Ridker, 2005; Mazière et al., 1994).

1.7 Statins
1.7.1 Structure and function of statins
Statins are a class of drugs that are used to lower cholesterol by the inhibition of the HMG-CoA reductase enzyme and are therefore known as HMG-CoA reductase inhibitors (Christians et al., 1998). The structural guidelines for a compound to be classed as a statin are a dihydroxyheptanoic acid unit and a ring system with different substituents as shown in Figure 1.9 (Moghadasian, 1999).
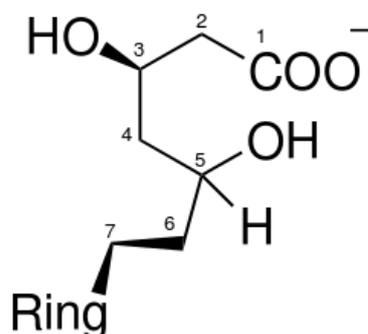
Figure 1.9: Representative structure of the basic statin pharmocophore.

Statins have been grouped into two groups based on their structure; type one statins have a substituted decalin-ring structure and are naturally occurring as seen in Figure 1.10. Statins in this class include lovastatin, simvastatin and

pravastatin (Istvan and Deisenhofer, 2001) and are derived from fungi *Pleurotus ostreatus* (Alarcón et al., 2003), *Aspergillus terreus,* (Alberts et al., 1980) and bacterium *Nocordia autotrophica* (Yoshino et al., 1986) respectively. Simvastatin and lovastatin are what is known as a prodrug, they are in a structurally inactive form when ingested but are then metabolized to their active form to inhibit HMG CoA reductase. Both simvastatin and lovastatin have a closed lactone ring in their pro-drug form and once metabolized have an open hydroxyl acid (Hamelin and Turgeon, 1998).
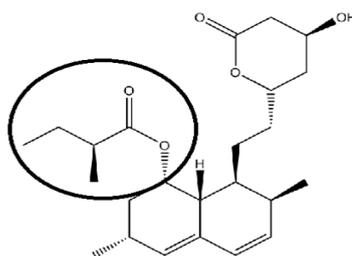
Figure 1.10: Structure of type one lovastatin displaying the representative butyryl group of all type one statins.

Type two statins are synthetic and have larger groups attached to the dihydroxyheptanoic acid unit as seen in Figure 1.11. The main difference between type one and type two is the replacement of the butyryl group of statins with the fluorophenyl group in type two. The fluorophenyl group has additional polar groups that cause a tighter bind to the HMG CoA reductase enzyme. Statins in this group are fluvastatin, cerivastatin, atorvastatin and rosuvastatin (Istvan and Deisenhofer, 2001).
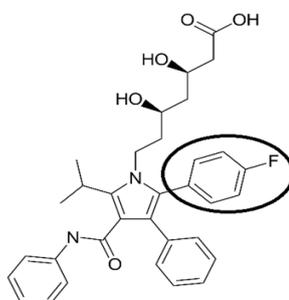
Figure 1.11: Atorvastatin of type two statins displaying the representative fluorophenyl group of all type two statins.

1.7.2 Antiviral activity of statins

Statin compounds have been found to exhibit antiviral effects against HIV-1 (del Real, 2004; Ganesan et al., 2011; Giguère and Tremblay, 2004; Jain and Ridker, 2005) replication of sub genomic hepatitis C virus (HCV)-1b replicons (Ikeda et al., 2006), suppress RNA replication of Japanese fulminant hepatitis-1 (JFH-1) HCV (Amemiya et al., 2008), poliovirus (Liu et al., 2006), cytomegalovirus (Potena et al., 2004) and respiratory syncytial virus (Gower and Graham, 2001).

The antiviral activity of statins in HIV-1 has been shown in some *in vitro* studies, however, the exact mechanism of action is debatable (Amet et al., 2008; Giguère and Tremblay, 2004; Jain and Ridker, 2005; Mazière et al., 1994). The antiviral effect on HIV-1 has been attributed to the disruption of lipid rafts (Ganesan et al., 2011), effects on protein prenylation and inhibition of lymphocyte function antigen -1 (LFA-1) and intracellular adhesion molecule-1 (ICAM-1) (del Real, 2004; Ganesan et al., 2011; Iyengar et al., 1998), disruption of CCR5 and RANTES expression levels in CD4+ T lymphocytes *in vitro* (Nabatov et al., 2007) and down regulation of RHO activity (del Real, 2004). Amet *et al* reported that statins increase intracellular Gag and decrease virus release by HIV-1 cells (Amet et al., 2008). The diminished geranylation, a form of prenylation, may be the principal mechanism of statin induced reduction of HIV-1 release (del Real, 2004). Conversely, groups have reported preliminary data suggesting the failure of statins to control *in vitro* and *in vivo* levels of HIV-1 (Moncunill et al., 2005; Probasco et al., 2008; Sklar et al., 2005). Moncunill and co-workers studied the anti HIV activity of various statin compounds but the statins showed no anti HIV-1 activity at sub-toxic concentration. Further studies on limited exposure to statins showed antiviral activity of Lovastatin to levels of 3.9 µg/ml with cytotoxicity at 19 µg/ml (Moncunill et al., 2005). Probasco and co-workers studied the effects of atorvastatin on anti HIV-1 activity and HIV-1 in cerebrospinal fluid in a human clinical pilot study, however no appreciable decrease in HIV-1 replication was observed (Probasco et al., 2008). Sklar and co-workers studied the effects of pravastatin on HIV-1 infected patients but the results showed no consistent effect of pravastatin on viral load (Sklar et al., 2005).

1.8 Techniques to analyse small molecule inhibitors of the LEDGF/p75-IN interaction

1.8.1 AlphaScreen technology

Alpha (Amplified Luminescent Proximity Homogenous Assay) technology is a highly flexible, no wash assay used to study protein interactions and complexes up to 200 nm. This bead-based assay allows the study of biological targets such as enzymes, receptor ligand interactions, low affinity interactions, second messenger levels, RNA, DNA, proteins, peptides, sugars and small molecules. Two bead types are needed for the assay. The first bead is a donor bead containing phthalcyanine, a photosensitizer that converts ambient oxygen to a reactive excited form of O_2 upon illumination at 680 nm. The second bead, known as the acceptor bead, contains a thioxene derivative which allows for light emission. A singlet oxygen can diffuse 200 nm in solution. Once an acceptor bead is in a close enough proximity to the donor bead by means of protein association, then energy transferred from the singlet oxygen to the thioxene derivative results in the production of light ("AlphaScreen® for Protein-Protein Interaction Assays | PerkinElmer."). Two forms of the Alpha technology are currently in use, namely the AlphaScreen and the AlphaLISA with the difference between the two assays being the acceptor beads. The thioxene derivative present on the acceptor bead of the AlphaScreen assay is Rubrene, which emits light between 520- 620 nm. In the AlphaLISA assay the acceptor beads thioxene derivative is Europium and emits light at 615 nm (Beaudet et al., 2008).

This study focused on setting up an AlphaScreen assay to investigate the interaction of LEDGF/p75 and HIV-1 IN, with the aim of identifying and further characterization of possible inhibitors of the interaction. The proposed reagents for the assay include recombinant LEDGF/p75 containing a histidine tag which binds to the nickel chelating acceptor bead, recombinant HIV-1 IN able to bind biotinylated DNA capable of binding to streptavidin donor beads, as seen in Figure 1.12. Once the assay is optimized, it is expected that when the proteins interact, light is emitted between 520 nm and 620 nm, and when an inhibitor of interest competes or breaks the bond between LEDGF/p75 and HIV-1 IN, the fluorescence will drop, allowing for the determination of percentage inhibition and dose responses.
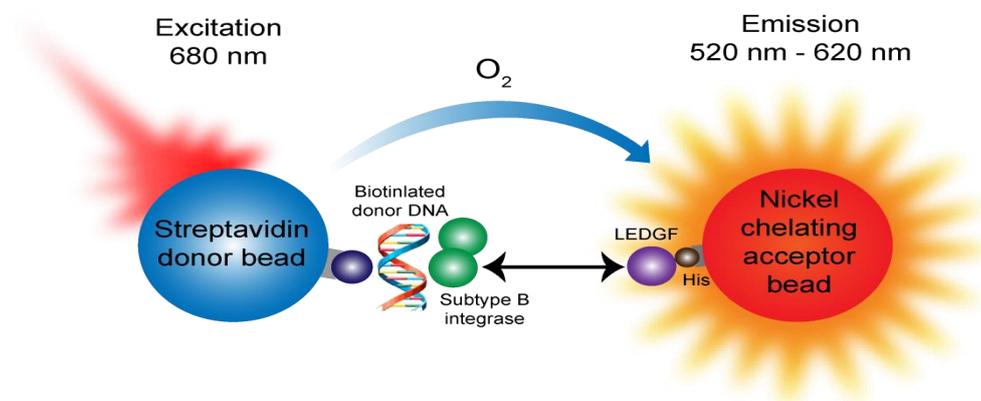
Figure 1.12: Schematic representation of the HIV-1 IN-LEDGF/p75 interaction in an AlphaScreen assay. The streptavidin donor bead links to the biotin labelled donor DNA-IN complex which then interacts with the HIS tagged LEDGF/p75 bound to the nickel chelating acceptor bead.

1.9 Objectives

The overall objective of this study was to identify and evaluate potential inhibitors of the HIV-1 IN-LEDGF/p75 interaction. This was accomplished by fulfilment of the following aims:

1. Isolation and purification of recombinant LEDGF/p75 and HIV-1 IN with high purity and sufficient yields.
2. Formulation of a novel biological assay to identify potential inhibitors of the HIV-1 IN-LEDGF/p75 interaction.
3. Screening of compounds identified by molecular modelling using the newly developed AlphaScreen assay to identify potential inhibitors.
4. *In vitro* screening of potential inhibitors for antiviral efficacy.

Chapter 2: Materials and Methods

Material and methods

2.1 Reagents used in this study

E. cloni EXPRESS® BL21 (DE3) (pLysS) and *E. cloni* 10G SOLOs competent cells (Lucigen Corporation, USA) were used in transformation with recombinant plasmids of pLEDGF/p75 (pFT-1-LEDGF, obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: catalogue number 11396), HIV-1 IN subtype B (IN) (pINSD.His.Sol obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: catalogue number 2958), and pogo transposable element with zinc finger (ZNF) domain (pPogZ) (pPB-His-hPOGZ, Applied Biological Materials, catalogue number PV032018). The HIV-1 subtype C IN expressing plasmid (from isolate 05ZAFV6) was designed and available in our laboratory.

The NCC-202 clinical collection library comprised of 281 compounds for repurposing was purchased from Evotec A.G (Germany). Compound structures were used in molecular modelling studies, and potential hit compounds were used in subsequent analyses. Additional statins tested for antiviral activity included 10mg tablets of lovastatin (Lovachol, Aspen, SA), simvastatin (Zocor, Merck, USA), atorvastatin (Lipitor, Pfizer, USA) and pravastatin (Pravachol, Aspen, SA), mevastatin (Santa Cruz).

A set of control compounds were sourced for use as positive controls in various assays, and included: Raltegravir (NIH AIDS Research and Reference Reagent Program, catalogue number 11680) for the HIV-1 IN strand transfer assay and antiviral assay, (2-(6-chloro-2-methyl-4-phenylquinolin-3-yl) pentanoic acid (CX05168) (ChemScene, USA, catalogue number CS-0685) for the AlphaScreen, Auronophin (Sigma Aldrich, USA) for the cytotoxicity assay, Zidovudine (NIH AIDS Research and Reference Reagent Program, catalogue number 3485) for the reverse transcriptase assay and chloramphenicol (Dulfecha Biochemie, Spain) for the solubility and permeability assay. All compounds were above 95% purity as specified by suppliers.

Mammalian cell lines, MT-4 cells (antiviral and cytotoxicity testing) and HeLa (cytotoxicity testing), were also obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: catalogue numbers 120 and 153, respectively. Expanded and titrated HIV-1$_{NL4-3}$ was available in our laboratory.

2.2 Recombinant protein expression, purification and modification

2.2.1 Protein expression of HIV-1 IN, LEDGF/p75 and PogZ

2.2.1.1 Bacterial transformation

E. cloni EXPRESS® BL21 (DE3) (pLysS) and *E. cloni* 10G SOLOs competent cells (Lucigen Corporation, USA) were each transformed with recombinant plasmids of pLEDGF/p75 , HIV-1 IN subtype B (IN), and pogo transposable element with zinc finger (ZNF) domain (pPogZ). BL21 cells were used to express recombinant protein, whereas 10G SOLOs were used for recombinant plasmid purification. The transformation protocol was followed according to manufacturer's instructions. Briefly, BL21 (DE3) (pLysS) cells and 10G SOLOs were thawed on ice and 49 µl of cells were added to 0.1 µg of the respective plasmid. The cells were incubated on ice for 30 minutes and then heat shocked at 42°C for 45 seconds before returning to ice for two minutes. Room temperature recovery media (Lucigen Corporation, USA) was then added to a final volume of 1 ml and the mixture was vigorously shaken for one hour at 37°C. Luria Bertani (LB) Agar (Laboratorios Conda, Spain) plates were made containing the respective antibiotic; 100 µg/ml ampicillin (Melford, USA) for pLEDGF/p75 and pINSD.His.Sol and 30 µg/ml kanamycin (Roche Diagnostics, Switzerland) for pPogZ. Expression bacterial plates contained 35 µg/ml chloramphenicol (Dulfecha Biochemie, Spain) in addition to the primary antibiotic. Room temperature LB agar plates were spread with 100 µl of transfected BL21 cell culture and the plates were then incubated overnight at 37°C (Bartholomeeusen et al., 2008; Jenkins et al., 1996; Vandekerckhove et al., 2006).

2.2.1.2 Production of glycerol stocks

A single colony was isolated from the LB agar plate and inoculated into 10 ml LB Broth (Laboratorios Conda, Spain) containing antibiotics relevant for each plasmid, and 35 µg/ml chloramphenicol was added for BL21 (DE3) (pLysS) cultures. The cultures were incubated at 37°C with vigorous shaking until the optical density (OD) reached 0.8 at 600 nm (xMark spectrophotometer, Bio-Rad, USA) for pLEDGF/p75 and 0.6 for pINSD.His.Sol and pPogZ. Aliquots at a ratio of 1:1 transformed bacteria and autoclaved 60% glycerol (Sigma Aldrich, USA)

were combined ensuring the mixture was homogeneous. Glycerol stocks were then stored at -80 °C for future use.

2.2.1.3 Plasmid preparation

The glycerol stock of the transformed 10G SOLOs was thawed on ice for five minutes and 20 µl was added to 10 ml LB broth containing ampicillin or kanamycin, dependant on the plasmid. The culture was then incubated overnight at 37°C with vigorous shaking. Recombinant plasmid extraction was performed utilising a Stratagene plasmid mini prep kit (Agilent technologies, USA) according to manufacturer's protocol. Briefly, a 1.5 ml aliquot of cell culture was centrifuged at 3000 xg for one minute. The supernatant was discarded and 100 µl of solution 1 (50 mM Tris HCl pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA), 50 ug/ml RNase A) was added to the spin cup and vortexed until the cell pellet was completely resuspended. Once homogeneous, 100 µl of solution 2 (0.2 M NaOH, 1 % sodium dodecyl sulphate (SDS)) was then added and the micro tube and inverted several times. Lastly, 125 µl of solution 3 (chaotropic salt solution, buffer recipe not provided due to propriety mix belonging to Stratagene) was added and gently mixed. The micro tube was then centrifuged at 14000 xg for five minutes. The supernatant was transferred to a micro spin cup and pellet discarded. The micro spin cup was centrifuged at 14000 xg for 30 seconds. A volume of 750 µl of endonuclease wash was added to the micro spin cup and centrifuged at 14000 xg for 30 seconds. The filter throughput was discarded and 750 µl of wash buffer (10 mM Tris HCl pH 7.5,100 mM NaCl, 2.5 mM EDTA) was added and the spin cup was centrifuged at 14000 xg for 30 seconds. The spin cup was removed and placed in a fresh 1.5 ml micro tube. A volume of 50 µl of elution buffer (10 mM Tris base pH 8, 1 mM EDTA) was added and incubated in the spin cup for 5 minutes at room temperature. Finally the micro tube was centrifuged at 14000 xg for 30 seconds and the flow through was read on a NanoDrop 2000 (Thermo Scientific, USA) to determine purity and concentration of the extracted plasmid DNA. Aliquots were then stored at -80°C.

2.2.1.4 Agarose gel electrophoresis and plasmid digest

Purity and size of each recombinant plasmid was determined by agarose gel electrophoresis. A 1 % agarose gel (Sigma Aldrich, USA) was made with TBE (90 mM Tris-HCl (Sigma Aldrich, USA), 90 mM boric acid (Sigma Aldrich, USA), 2 mM EDTA (Sigma Aldrich, USA)) buffer, and 0.5 µl of SYBR gold (Life Technologies, USA) was added to the gel mix for staining purposes. The gel mix

was heated until homogeneous and poured into the casting set with comb inserted and allowed to cool for 30 minutes. Samples for each plasmid were prepared according to manufacturer's instructions. Briefly 150 ng of the whole undigested plasmid was added to 1 µl of 6x loading buffer (Fermentas, USA) as sample one. The second and third sample was of a single digest with one of the restriction enzymes (RE) (Fermentas, USA) for each plasmid. LEDGF/p75 plasmid was digested using RE *Bgl*II and *EcoR*1; HIV-1 IN utilized *BamH*1 and *Nde*I. A total of 150 ng of plasmid was incubated with 16 µl of Nuclease free water, 2 µl of reaction buffer R (Fermentas, USA) and 1 µl of RE and mixed gently. The mixture was then incubated for two hours at 37°C. The final sample contained a double digest which consisted of 15 µl of nuclease free water, 2 µl of Tango^TM buffer (Fermentas, USA), and 1 µl of DNA and 1 µl of each RE for the plasmid. The solution was mixed gently and incubated at 37°C for two hours. Subsequently, 2 µl of 6x loading dye (Fermentas, USA) was added to 12 µl of RE digested and undigested samples and loaded onto the agarose gel. A DNA Ladder (Fermentas, USA) was loaded as a reference. The gel was run at 80 V for approximately two hours and the gel was analysed using a Bio-Imaging Minibus Pro (DNR Bio-Imaging systems, Israel) using gel capture (DNR acquisitioning and imaging software) with an ultra violet (UV) plate and UV lamp to confirm the presence and size of the desired plasmid.

2.2.1.5 Protein expression
BL-21 (DE3) (pLysS) expression cells containing the plasmid were grown overnight at 37°C in LB broth containing appropriate antibiotics and chloramphenicol. Cells were then inoculated at 1:100 into LB broth and incubated with shaking at 37°C for pINSD.His.Sol and pPogZ to an optical density of 0.6 at 600 nm, and 32°C for pLEDGF/p75 until reaching an optical density of 0.8-0.9 at 600 nm. Cells were induced to express recombinant protein by the addition of 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Scientific, USA) final concentration and grown for a further three hours at appropriate temperatures. The pLEDGF/p75 culture temperature was decreased to 28°C for this step and samples were taken every hour to monitor expression. In addition, glucose (Sigma Aldrich, USA) positive and glucose negative samples were run simultaneously to determine if the presence of glucose prevented early expression before IPTG was added. Cell pellets were then harvested and frozen

at -80°C (Bartholomeeusen et al., 2008; Jenkins et al., 1996; Vandekerckhove et al., 2006).

2.2.1.6 Sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis

An electrophoresis tank was set up and filled with sodium dodecyl sulphate (SDS) tank buffer (25 mM Tris-HCl, 192 mM Glycine (Sigma Aldrich, USA), 0.1 % SDS (Sigma Aldrich, USA)). Crude samples taken at baseline and 60 minute intervals after induction were prepared by adding 10 µl of sample to 5 µl of 2x Sample buffer (Life Technologies, USA). The samples were boiled for two minutes and placed on ice. Samples were loaded onto a 4 - 20 % precast TGX gel (Bio-Rad, USA) and run at 80 V for approximately two hours. A quick stain solution (0.08 % coomassie brilliant blue R-250 (Thermo Scientific, USA), in deionised water, pH 3) and deionised water were heated on a hot plate until reaching 70°C. The gel was removed from the casting set and was immersed in the hot water with gentle shaking for five minutes. The hot water was replaced and this step was repeated three times. Finally the water was removed, the quick stain was added and the gel incubated with gentle shaking for 15 minutes. The gel was then rinsed with room temperature water and viewed on a Bio-Imager to determine if recombinant proteins at the desired resolution were present and over-expressed.

2.2.1.7 Protein extraction

Cell pellets from bacterial expression, (section 2.2.1.5), were thawed on ice for 30 minutes. Thawed cells were lysed in lysis buffer A (1 M NaCl (Saarchem, SA), 25 mM Tris-HCl pH7.4, 0.5 mM phenylmethanesulfonylfluoride (PMSF) (Thermo Scientific, USA), 5 mM imidazole (Sigma Aldrich, USA)) for LEDGF/p75, lysis buffer B (1 M NaCl, 20 mM Hepes (Life Technologies, USA) pH 7.2, 5 mM imidazole, 10 mM $MgCl_2$ (Sigma Aldrich, USA), 0.25% CHAPS (Sigma Aldrich, USA), 1 mM PMSF) for HIV-1 IN and lysis buffer C (20 mM Tris-HCl pH7.4, 200 mM NaCl, 0.5 mM PMSF, 5 mM imidazole) for PogZ. Cell lysates were then homogenised for one minute and aliquoted for sonication. Sonication was performed at 75 % at 0.6 cycles (Labsonic M ultrasonic processor, Sartorius, Germany). Each tube was sonicated for one minute for three cycles. Sonicated cell lysates were then centrifuged at 3200 xg for 30 minutes at 4°C. The supernatant was pooled and pellets were discarded.

2.2.1.8 Column chromatography
Nickel affinity chromatography

A HiTrap^TM IMAC HP (GE Healthcare, UK) affinity column was connected to an ÄKTAprime plus (GE HealthCare, UK) and equilibrated with lysis buffer A, B or C as used in the extraction of the recombinant proteins from bacterial lysis. The supernatant was loaded onto the column and a concentration gradient was set from 0 % buffer A, B or C respectively to 100 % elution buffer A, B or C containing 200 mM imidazole over a 50 ml gradient.

Collected fractions were run on an SDS PAGE gel as described in section 2.2.1.6, to determine which fractions contained the desired protein. The SDS PAGE gel was viewed and fractions containing an over expressed protein at the size were pooled and concentrated using an Amicon ultra-filtration device with a 10 kDa cut off (Millipore Merck, USA). The sample was then loaded onto a PD-10 desalting column (GE Healthcare, UK) equilibrated with either HIV-1 IN storage buffer (1 M NaCl, 20 mM Hepes (pH 7.5), 0.1 mM EDTA, 1 mM DTT (dithiothreitol) (Thermo Scientific, USA), 10 % glycerol (Sigma Aldrich, USA) pH 7.4) or PogZ storage buffer (150 mM NaCl, 25 mM Tris-HCl, 2 mM DTT, 10% glycerol, pH 7.6) or buffers to be used in subsequent columns (see sections 2.2.1.8 II below). Fractions were pooled and read on the NanoDrop 2000 to determine concentration. Glycerol was added to a final percentage of 10 % to pooled fractions and stored at -80°C. LEDGF/p75 samples needed additional purification steps were pooled.

Ion exchange chromatography

LEDGF/p75 samples were buffer exchanged into a sodium phosphate buffer A (50 mM NaH$_2$PO$_4$ (Saarchem, SA), pH 7.2) and pooled. An SP-Sepharose Fast Flow column (GE Healthcare, UK) was utilised and equilibrated with sodium phosphate buffer D and the pooled fractions were loaded onto the column. Fractions were then eluted in sodium phosphate elution buffer (50 mM NaH$_2$PO$_4$, 1 M NaCl pH 7.2) over a 50 ml gradient from 0 M NaCl to 1 M NaCl. Fractions were collected and run on an SDS PAGE gel as previously described.

Fractions containing LEDGF/p75 were pooled and concentrated using an Amicon ultra-filtration device with a 10 kDa cut off. The sample was then loaded onto a PD-10 column and buffer exchanged to a size exclusion buffer (250 mM NaCl, 25

mM Tris-HCl, pH 7.4, 2 mM DTT). Fractions were then read on a NanoDrop 2000 to determine concentration.

Size exclusion chromatography

The Superdex 200 (GE HealthCare, UK) size exclusion column was equilibrated at 0.5 ml/min overnight with a LEDGF/p75 size exclusion buffer (150 mM NaCl, 25 mM Tris-HCl, 2 mM DTT, 0.01% Tween 20 (Sigma Aldrich, USA), pH 7.6). The sample was loaded and allowed to elute on the ÄKTAprime plus (GE HealthCare, UK) at 0.2 ml per minute and 2.5 ml fractions were collected. Samples were run on an SDS-PAGE gel as previously described and the samples were concentrated using an Amicon ultra-filtration device with a 10 kDa cut off (Millipore Merck, USA). Glycerol was added to a final percentage of 10% to fractions to be stored at -80°C for future use.

2.2.2 Validation of recombinant protein purification

2.2.2.1 SDS PAGE gel to monitor protein purification

Samples were collected at each stage of purification to monitor protein purity. Samples were boiled with 2X sample buffer (Bio-Rad, USA) for two minutes and loaded onto a 4 - 20% TGX gel (Bio-Rad, USA). The gel was run at 80 V in SDS tank buffer for two hours. After completion, the gel was quick stained and destained (section 2.2.1.6) to view the protein bands on the gel. Purity of the recombinant proteins was determined using Gel Quant Express Analysis software (DNR Bio-Imaging Technologies, Israel).

2.2.2.2 Western blot

An SDS PAGE gel for each expressed protein was transferred to a polyvinylidenedifluoride (PVDF) membrane utilizing an iBlot gel transfer system (Life Technologies, USA). Once transferred, the gel was placed in a standard coomasie stain (50 % methanol, 10 % acetic acid (ACE Chemical Company, Australia), 0.05 % Brilliant Blue R-250) to confirm transfer. The membrane was placed in a blocking buffer (5 % bovine serum albumin (BSA) (Sigma Aldrich, USA) Tris buffered saline solution containing Tween 20 (TTBS) (50 mM Tris-HCl, 150 mM NaCl, 0.05 % Tween 20, pH 7.6) overnight. Once blocked, the membrane was washed 3 times for 5 minutes in TTBS with slight agitation. The membrane was placed into a 2000x dilution of primary antiserum to HIV-1 IN

(142-153) (NIH AIDS Reagent Program, catalogue number 3514); monoclonal mouse anti-PSIP (Abcam, UK) or monoclonal mouse anti-PogZ antibody (Abcam, UK) respective of the protein used made up in blocking buffer and incubated with slight shaking for two hours. At this point the membrane was washed three times for five minutes in TTBS buffer and then placed into a 20 000x dilution of secondary goat anti mouse antibody (Abcam, UK) made up in blocking buffer for 90 minutes. Once complete, the membrane was washed three times for five minutes in TTBS and placed into a chemiluminescent substrate solution (Pierce Biotechnology, USA) in the dark for 15 minutes. The membrane was then viewed on the ChemiDoc™ MP System (Bio-Rad, USA) by exposure for one minute.

2.2.3 Modifications of purified recombinant proteins

2.2.3.1 6XHIS tag cleavage

The 6XHIS tag on HIV-1 IN subtype B and C were cleaved by thrombin. Briefly, a benzamidine column (GE Healthcare, UK) was regenerated with three bed volumes of alternating high pH benzamindine buffer (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and a low pH benzamidine buffer (0.1 M sodium acetate (Sigma Aldrich, USA), 0.5 M NaCl, pH 4.5). The column was then re-equilibrated with five bed volumes of HIV-1 IN binding buffer (25 mM Hepes buffer, pH 7.5, 1 M NaCl, 1 mM DTT and 10 % glycerol). Thrombin (Sigma Aldrich, USA) was made up in HIV-1 IN binding buffer with the addition of 25 mM $CaCl_2$ (Sigma Aldrich, USA) to a final ratio of 123 units of thrombin per mg of protein. The protein was incubated in 1 ml final volume binding buffer containing 25 mM $CaCl_2$ and thrombin for 4 hours with slight shaking at 26°C. The sample was loaded onto the benzamidine column and eluted with the binding buffer. Fractions were immediately collected and run on a precast TGX gel (Bio-Rad, USA). The gel was stained as previously described to view which fractions contained the cleaved HIV-1 IN. The fractions containing HIV-1 IN were pooled and concentrated down with use of the Amicon ultra-filtration device with a 10 kDa cut off (Millipore Merck, USA) to approximately 1 ml. The HIV-1 IN was buffer exchanged with a PD-10 column into an HIV-1 IN storage buffer for future use. The thrombin on the column was removed with 0.05 M Glycine, pH 3.5.

A 4 - 20 % precast TGX prestained gel (Bio-Rad, USA) was run with 6XHIS tagged HIV-1 IN and cleaved HIV-1 IN and was transferred to a PVDF membrane

for Western blotting as explained in section 2.2.2.2. The membrane was then blocked overnight with blocking buffer and subsequently incubated in a 1000x dilution of primary monoclonal mouse anti-HIS antibody (Abcam, UK) then into a 5000x dilution of secondary goat anti mouse (Abcam, UK) to confirm the 6XHIS tag on the uncleaved protein and the absence of a tag on the cleaved protein. The membrane was viewed as previously described.

2.2.3.2 Biotinylation of HIV-1 IN and PogZ

HIV-1 IN and PogZ were biotinylated for use in the non DNA AlphaScreen assay. A biotin protein labelling kit (Roche Diagnostics, Switzerland) was utilized and biotinylation was performed according to the manufacturer's protocol. Briefly purified HIV-1 IN and PogZ were buffer exchanged by dialysis using a 10 kDa molecular weight cut off (MWCO) snake skin dialysis tubing (Pierce Biotechnology, USA) to a 0.01 M (1X) phosphate buffered saline (PBS) solution pH7.4. Biotin was made up to 20 mg/ml in DMSO both supplied with the kit. A ratio of 1:10 was used to calculate the quantity of biotin according to the table supplied. A total of 10 units of biotin was incubated with 1 unit of HIV-1 IN or PogZ. Biotin was then added to the protein and incubated for two hours at room temperature with gentle agitation. The supplied G25 column (Pierce Biotechnology, USA) was blocked with biotin blocking buffer, supplied in the kit, and equilibrated with 30 ml of 1x phosphate buffered saline (PBS) (Sigma Aldrich, USA) buffer. The biotin-protein solution was added to the column and allowed to elute. Fractions were collected and read on the Nanodrop 2000 to determine the protein concentration. NaCl, DTT and glycerol were added and samples were aliquoted and stored at -80°C for future use.

2.3. Theoretical, biochemical and biological screening of test compounds

2.3.1. Theoretical screening of test compounds for HIV-1 IN/LEDGF/p75 inhibition

2.3.1.1. Molecular modelling of NCC collection

The NCC-202 clinical collection library comprised of 281 compounds for repurposing was screened virtually with Accelrys Discovery Studio™ software package (licensed from Accelrys Inc., San Diego, CA, 2010, USA) for potential

binding to HIV-1 IN. The PDB code 2B4J model (IN-LEDGF/p75) was downloaded (http://www.rcsb.org/pdb) and LEDGF/p75 was removed from the HIV-1 IN-LEDGF/p75 complex and prepared by building missing loops, addition of hydrogen atoms, bond order assignment, and optimization of bond lengths, bond angles, torsion angels and non-bonded interactions. Known HIV-1 IN-LEDGF/p75 inhibitors (27) identified by Bruno Simoneau *et al* consisting of known IC_{50}, EC_{50}, CC_{50} and Kd values were used to train the model (Simoneau et al., 2011). Subsequently, the 281 molecules were screened through the optimised and validated model and a total of twelve compounds were identified. These twelve compounds were sent forward for biological testing as possible HIV-1 IN-LEDGF/p75 inhibitors. Because lovastatin was identified as one of the 12 potential IN-LEDGF/p75 inhibitors, additional statins were included in subsequent experiments.

2.3.1.2. Osiris molecular property explorer

The HIV-1 IN-LEDGF/p75 inhibitors selected by molecular modelling and statins were theoretically screened using Osiris Molecular Property Explorer available online at http://www.organic-chemistry.org/prog/peo/. The compounds were assessed and the water-octanol partitioning co-efficiency (cLogP), aqueous solubility (LogS), molecular weight, fragment based drug-likeness, overall drug score, tumourigenicity, mutagenicity, irritancy and possible effects on reproduction were determined.

2.3.1.3. Lipinski's Rule of Five

All potential HIV-1 IN-LEDGF/p75 inhibitors selected by molecular modelling and statins were assessed theoretically in accordance with the four rules of Lipinski's rule of five. The compounds were inspected using the Accelrys' Discovery Studio 3.1 package utilizing the ADMET function to determine the number of hydrogen bond donors and acceptors, and the Osiris molecular property explorer was used to determine molecular weight and the Log P values. With a total of four parameters and each parameter weighing equally a score out of four was determined.

2.3.1.4 Statin property determination

The melting point, logP, logS, physiological charge, hydrogen atom acceptor and donor, polar surface area, rotatable bond count, refractivity and polorisability

were determined for all statin compounds using ChemSpider available online at
http://www.chemspider.com/ , Osiris Molecular Property Explorer available online
at http://www.organic-chemistry.org/prog/peo/ and Accelrys Discovery Studio™
software package (licensed from Accelrys Inc., San Diego, CA, 2010, USA).

2.3.2. Preparation of test compounds

The 12 NCC-202 compounds were supplied as 10 mM stock solutions, and were
further diluted in DMSO to the required concentrations. Lovastatin, simvastatin,
atorvastatin and pravastatin 10 mg tablets were crushed separately in a mortar
and pestle until finely ground. Distilled water (dH_2O) was added to a volume of 30
ml and thoroughly mixed until homogeneous. The mixture was added to an
extraction vessel and 10 ml of methanol (Sigma Aldrich, USA) was passed
through with slight agitation. This process was repeated three times to maximise
the amount of compound extracted. One gram of ammonium sulphate (Sigma
Aldrich, USA) was then added to the combined methanol to ensure no water
residue was present and was passed through 0.22 µM filter paper. The methanol
containing the respective compound was then placed on an evaporator to
remove all solvent and leave the compound powder behind.

A total of 10 mg of each purified compound powder was analysed by nuclear
magnetic resonance (NMR) (400 MHz Bruker Avance Spectrometer at 298K
equipped with a BBI 5 mm probe) and 1 mg of each purified compound was
analysed by mass spectroscopy (Dionex Ultimate 3000 Rapid Separation LC
system equipped with a C-18 pre-coated column and coupled to a microTOF QII
Bruker mass spectrometer fitted with an electrospray source) to confirm purity of
the extracted compound. Finally aliquots of 10 mg of compound were dissolved in
DMSO (Sigma Aldrich, USA) to a volume of 1 ml for experimental use (Chaudhari
et al., 2007; Deak et al., 2002; Önal and Sagirli, 2006), and stored at -20°C until
used. The process was repeated several times to obtain sufficient yields for
derivatisation.

Statin derivatives were also synthesized. Lovastatin lactone was hydrolysed to
the β-hydroxy acid form by using 52 mg of lovastatin, isolated from Lovachol
tablets (as described in 2.3.2), and dissolving in a total of 10 ml dH_2O. A total of
0.1 ml 10 M sodium hydroxide (NaOH) (Saarchem, SA) solution was added to

bring the final NaOH concentration to 0.1 M and the mixture was allowed to stir overnight, after which the pH was adjusted to 7.5 with hydrochloric acid (HCl) (ACE Chemical Company, Australia). A mixture of water, acetonitrile (ACN) (Sigma Aldrich, USA) and ethyl acetate (Sigma Aldrich, USA) in a 1:1:1 ratio was used to precipitate the lovastatin β-hydroxy acid product which was then filtered through a 22 µM filter and dried. The final product was run on the NMR (400MHz Bruker Avance Spectrometer equipped with a broadband inverse (BBI) 5mm probe) and through mass spectroscopy (Dionex Ultimate 3000 Rapid Separation LC system equipped with a C-18 pre-coated column and coupled to a micrOTOF QII Bruker mass spectrometer fitted with an electrospray source) to confirm the β-hydroxy acid form and purity (Deak et al., 2002; Huang et al., 2010a; Önal and Sagirli, 2006).

Pravastatin acid was condensed to the lactone form by taking 100 mg pravastatin acid, isolated from Aspen Pravachol tablets (as described in 2.3.2), and mixing with 5 ml ethyl acetate after which 2 µl of trifluoroacetic acid (ACE Chemical Company, Australia) was added to the suspension and the reaction was stirred overnight. The reaction was then dried and a mixture of water, ACN and ethyl acetate in 1:1:1 ratio was used to precipitate the product which was then filtered through a 22 µM filter paper, dried and run on the NMR (400MHz Bruker Avance Spectrometer equipped with a broadband inverse (BBI) 5mm probe) and through mass spectroscopy (Dionex Ultimate 3000 Rapid Separation LC system equipped with a C-18 pre-coated column and coupled to a micrOTOF QII Bruker mass spectrometer fitted with an electrospray source) to confirm pravastatin lactone product and purity (Deak et al., 2002; Önal and Sagirli, 2006; Tanaka and Terahara, 1982). Both lovastatin acid and pravastatin lactone were dissolved in DMSO at a concentration of 10 mg/ml and stored at -20°C for future use.

Only compounds that were found to be above 95 % pure by means of NMR and mass spectroscopy were used in subsequent experiments.

2.3.3. Biochemical screening of HIV-1 IN-LEDGF/p75 inhibitors

2.3.3.1. Development of the HIV-1 IN-LEDGF/p75 AlphaScreen assay

2.3.3.1.1 Evaluation of the HIV-1 IN-LEDGF/p75 interaction by size exclusion chromatography

The Superdex 200 (GE HealthCare, UK) size exclusion column was calibrated with size exclusion markers (GE Healthcare, UK). Both low molecular weight and high molecular weight markers were utilized. Proteins aprotonin (6 500 g/mol), ribonuclease A (13 700 g/mol), carbonic anhydrase (29 000 g/mol), ovalbumin (43 000 g/mol), conalbumin (75 000 g/mol), andolase (158 000 g/mol), ferritin (440 000 g/mol), thyroglobulin (669 000 g/mol) and blue dextran (2 000 000 g/mol) were used to calibrate the size exclusion column and applied at a rate of 0.5 ml per minute in a size exclusion sodium phosphate buffer (0.1 M $NaH_2PO_4.H_2O$, 0.1 M Na_2HPO_4, pH7.4). The void volume of the column was determined with the use of blue dextran. Once markers had been eluted a standard curve was constructed for future use in determining the size of eluted proteins.

The column was then equilibrated overnight in a size exclusion interaction buffer (150 mM NaCl, 25 mM Tris-HCl, 1 mM DTT, 1 mM $MgCl_2$ and 0.01% Tween 20, pH 7.6). A total of 20 µM HIV-1 IN and 20 µM LEDGF/p75 were allowed to associate at room temperature for four hours with slight shaking. Thereafter the complex was loaded onto the column and allowed to elute over a four hour period at 0.5 ml/min.

Western blot analyses were conducted as described in section 2.2.2.2, using a total of 20 µM HIV-1 IN and 20 µM LEDGF/p75 (both with the His tags attached) that were allowed to incubate together in a size exclusion interaction buffer for four hours at room temperature with slight shaking. The PVDF membrane was probed with a 2 000x dilution of primary monoclonal mouse anti-HIS antibody (Abcam, USA) and a 20 000x dilution of secondary goat anti mouse antibody (Abcam, USA).

Material and methods

2.3.3.1.2 HIV-1 IN-DNA-LEDGF/p75 AlphaScreen assay set-up

An AlphaScreen assay previously described by Christ and co-workers was optimized for use (Christ et al., 2010). Briefly, 1.5 µM HIV-1 IN was incubated with 0.3 µM biotinylated donor DNA for 45 minutes at room temperature with gentle shaking. A total of 300 nM biotinylated HIV-1 IN-DNA was incubated with 100 nM HIS tagged LEDGF/p75 for one hour in AlphaScreen assay buffer (150 mM NaCl, 25 mM Tris, 2 mM $MgCl_2$, 1 mM DTT, and 0.01% Tween 20 and 0.02% BSA, pH 7.4) at 26°C with gentle shaking. Nickel chelating acceptor beads (Perkin Elmer, USA) and streptavidin donor beads (Perkin Elmer, USA) were added to a final concentration of 10 µg/ml and incubated at 30°C in the dark with gentle shaking. A final assay volume of 100 µl was used originally and later scaled down to 50 µl in ½ area Opti-Plates (Perkin Elmer, USA). Once incubation was complete the plate was read between 520-620 nm on the EnSpire® plate reader (Perkin Elmer, USA). Controls included; beads alone, HIV-1 IN-DNA alone, LEDGF/p75 alone, HIV-1 IN-DNA and LEDGF/p75 alone, each protein in isolation with beads alone, In addition, CX05168 which is a known HIV-1 IN-LEDGF/p75 inhibitor was used as the control compound to validate the assay. Initially a single dose of 100 µM final concentration of CX05168 was incubated with 300 nM IN for 30 minutes at 26°C with slight shaking. Subsequently, 100 nM LEDGF/p75 was added and incubated for one hour at 26°C with slight shaking. Once incubations were completed 10 µg/ml final concentration of both acceptor and donor beads were added and incubated at 30°C with shaking in the dark for one hour, the plate was then read on the EnSpire® plate reader (Perkin Elmer, USA). Once the inhibition of the HIV-1 IN-LEDGF/p75 interaction was seen a dose response curve was determined to obtain a half maximal inhibitor concentration (IC_{50}) for CX05168. A total of nine serial dilutions ranging from 0.39 µM to 100 µM were run on the assay. The percentage inhibition for each dose was determined with use of the controls and plotted against the log of the concentration. The data obtained was plotted on a sigmoidal curve using Origin 8.1 (Origin Lab Corporation, UK) and an IC_{50} dose was determined.

2.3.3.1.3 HIV-1 IN-LEDGF/p75 AlphaScreen assay set-up

A final concentration of 300 nM HIV-1 IN was incubated with 100 nM HIS tagged LEDGF/p75 for one hour at 26°C with gentle shaking. Nickel chelating acceptor beads and streptavidin donor beads were added to a final concentration of 10 µg/ml for one hour in the dark with gentle shaking at 30°C. The plate was then

read on the EnSpire® plate reader (Perkin Elmer, USA) as described above. Both subtype B and C HIV-1 IN was run in the assay. Similarly, CX05168 was used as the control compound to validate the assay as described above in section 2.3.3.1.2.

2.3.3.1.4. PogZ- LEDGF/p75 binding AlphaScreen assay set-up

A PogZ- LEDGF/p75 binding AlphaScreen assay was set up to confirm that the identified potential inhibitors bound HIV-1 IN and not LEDGF/p75.

The AlphaScreen assay was set up in ½ area Opti-Plates with PogZ replacing HIV-1 IN. A PogZ and LEDGF/p75 stock solution was prepared in AlphaScreen assay buffer and 300 nM PogZ was added to 100 nM LEDGF/p75 and allowed to incubate at room temperature with shaking for one hour. AlphaScreen nickel chelating acceptor beads and streptavidin donor beads were added to a final concentration of 10 µg/ml with the final assay volume of 50 µl. The plate was then incubated at 30°C for one hour in the dark with shaking and the plate was then read as previously described on the EnSpire® plate reader (Perkin Elmer, USA) and data was stored.

2.3.3.1.5 AlphaScreen assay compound screening

The three newly developed AlphaScreen assays was set up in the scaled down version of a 50 µl final volume.

For the initial HIV-1 IN-DNA-LEDGF/p75 AlphaScreen assay, HIV-1 IN was incubated with DNA and a final concentration of 300 nM HIV-1 IN-DNA complex was incubated with 100 µM of each compound for 30 minutes at 26°C with slight shaking. Compounds tested included the NCC-202 compounds, raltegravir, CRX05168, lovastatin acid, pravastatin, pravastatin lactone, atorvastatin, simvastatin and mevastatin. Raltegravir was used as the positive control.

Compounds displaying inhibition of above 50 % were subsequently rerun through the LEDGF/p75-IN-DNA AlphaScreen assay in ten serially diluted doses ranging from 0.39 µM to 200 µM to determine an IC_{50} for each compound. This was carried out in duplicate on the plate and in three separate experiments. Furthermore, the compounds displaying inhibition of above 50 % were also tested in the HIV-1 IN-LEDGF/p75 AlphaScreen assay and the PogZ- LEDGF/p75 binding AlphaScreen assay.

Material and methods

2.3.3.1.6 HIV-1 IN strand transfer assay

The donor DNA (Biotin 5'- ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCA-3' and 5'-ACTCCTAGAGATTTTCCACACTGACTAAAAG-3') (Inqaba Biotech SA) was annealed by heating to 95°C for five minutes and then allowed to cooled to room temperature.

A working concentration of donor DNA (0.15 μM) was made up in buffer F (10 mM Tris-HCl, 0.1 M NaCl, pH7.2). A volume of 100 μl was added to the wells of a streptavidin-coated micro ELISA plate (R&D Systems, USA). The plate was sealed and incubated for one hour at room temperature with shaking at 50 rpm. The wells were aspirated and washed three times with 300μl 0.01M PBS, after which 1 μM recombinant HIV-1 IN prepared in buffer G (20 mM Hepes, 75 mM NaCl, 10 mM MgCl$_2$, 2 μM ZnCl$_2$ (Sigma Aldrich, USA), 10 mM MnCl$_2$ (Sigma Aldrich, USA), 1% Glycerol pH 6.8) was added to each well and incubated for 30 minutes with shaking at 50 rpm. The wells were then washed two times with 200 μl buffer G. Compound samples were made up to 100 μM in buffer G and 10 μl added to each well in duplicate. The plate was then incubated at 37°C with shaking at 50rpm. A stock of 2.5 μM target DNA (5'-TGACCAAGGGCTAATTCACT-3; fluorescein and 5'-AGTGAATTAGCCCCTTGGTCA-3' fluorescein) (Inqaba Biotech, SA) was prepared and 10 μl added to each well and incubated at 37°C for one hour. The wells were then washed three times with 300 μl buffer H (0.03 M sodium citrate (Sigma Aldrich, USA), 0.3 M NaCl, pH 7.0) for 10 minutes. A working solution of 1:10000 monoclonal anti-FITC alkaline phosphatase (Sigma Aldrich, USA) solution was prepared in buffer I (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) and 200 μl was added to each well. The plate was then incubated for two hours at 25°C. Each well was then washed three times with 300 μl buffer J (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 10 minutes per wash. A BluePhos substrate (KPL Inc, USA) was prepared according to manufactures specifications and 200 μl was added to each well and incubated for 30 minutes at 37°C. The plate was then quantified using a multi plate reader at 620 nm (xMARK^{TM}, Bio-Rad, USA).

2.3.3.1.7 Reverse transcriptase assay

Lovastatin was tested in a direct HIV-1 RT colorimetric assay (Roche Diagnostics, Switzerland). All solutions were made up according to the manufacturer's specifications. The assay was performed according to

manufacturer's protocol. Briefly, 4-6 ng of HIV-1 RT was added to a micro tube followed by 100 µM final concentration inhibitor and 20 µl of reaction buffer. Each micro tube was incubated at 37°C for one hour after which the samples were transferred to a micro plate, covered in foil and incubated for a further one hour at 37°C. The solution was removed from the plate and wells washed five times with 250 µl of RT wash buffer for 30 seconds.

At this point 200 µl of a working dilution of anti-DIG-POD at 200 mU/ml was added per well, covered with foil and incubated for one hour at 37°C. The solution was removed and washed five times with RT wash buffer for 30 seconds. A final volume of 200 µl 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate solution was added to wells for 10-25 minutes until sufficient colour had developed. The plate was then read at 405 nm (xMARK^{TM}, Bio-Rad, USA) and data recorded.

2.3.3.1.8 Solubility assay

The aqueous solubility assay was adapted from the manufacturers protocol (E47 Committee, 2008). Briefly, stock solutions of 10 mg/ml for each compound were dissolved in DMSO. A standard curve for each compound was established using concentrations of 500 µM, 200 µM, 50 µM, 12.5 µM and 3.125 µM. Compounds were then made up in filtered 80:20 (PBS pH7.4: ACN) maintaining a DMSO concentration of 5%. Chloramphenicol was included as the control for aqueous solubility. A total of 100 µl of compounds were placed into the 96 well filter plate (Millipore, Merck, USA) and placed onto the shaker at 300 rpm overnight at room temperature. The plate was then vacuum filtered into a deep well master block and compounds transferred to a UV-Star^{TM} analysis plate. The spectrum of the compounds were analysed using the xMARK^{TM} (Bio-Rad, USA) and a standard curve was prepared from the data obtained.

Once the standard curve was determined compounds were made up to 200 µM and 100 µM samples in 100% PBS, pH 7.4 and placed into the filter plate and placed into the shaker at 300 rpm overnight at room temperature. The plate was then vacuum filtered and compounds transferred to a UV-Star^{TM} plate. A total of 40 µl ACN was added per sample and the compounds were quantified using the xMARK^{TM} (Bio-Rad, USA) at the optimum wavelength for each compound. The

optimal absorbance for each compound was divided by the slope from the standard curve for each compound and multiplied by the dilution factor of ACN as shown in the formula below:

Aqueous solubility = (A_{max} filtrate/slope) x 1.25

2.3.3.1.9 Permeability assay

The parallel artificial membrane permeability assay (PAMPA) was conducted as per manufacturers protocol (Avdeef et al., 2001). Briefly, the 96 well PAMPA plate was brought to room temperature 30 minutes prior to the assay. Original stock solutions of 10 mg/ml for each compound were prepared in DMSO. Compounds were then diluted to 100 µM in a filtered 0.01 M PBS pH7.4 solution keeping the DMSO concentration at 5%. Compounds were allowed to shake at 30 rpm for 30 minutes at room temperature and then 300 µl added to the receiver plate. A total of 200 µl 0.01 M PBS, pH7.4 was added to the filter plate and the filtered plate was then slowly lowered onto the receiver plate and incubated at room temperature for five hours. The plates were then separated and compounds from both filter and receiver plate were transferred to a UV-Star^{TM} analysis plate and quantified using the xMARK^{TM} (Bio-Rad, USA). The optimum wavelength was used as previously determined in the solubility assay. The permeability for each compound was calculated using the formula below.

$$P_e = \frac{-\ln [1-C_A\,(t)/C_{eq}]}{A^*(1/V_D + 1/V_A)^*t}$$

Permeability

$$R = 1-[C_D\,(t)^*V_D + C_A\,(t)^*V_A] / (C_0^* \; V_D)$$

Mass retention (%)

Where: C_0 = Initial compound concentration in the donor well (mM); C_D (t) = Compound concentration in donor well at time t (mM); C_A (t) = Compound concentration in acceptor well at time t (mM); V_D = Donor well volume (300 µl); V_A = Acceptor well volume (200µl); C_{eq} = [C_D (t)$^*V_D$ + C_A(t)$^*V_A$] / (V_D + V_A); A = Filter area (0.3 cm^2); t = incubation time (five hours = 18000 seconds).

2.3.4. Antiviral and cytotoxicity biological assays

2.3.4.1 Cell tissue culture maintenance

MT-4 cells (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: MT-4 catalogue number 120) were thawed from frozen stocks at a concentration of 1 x 10^7 cells per ml, in 10 ml of 20 % RPMI (Gibco, USA) (20% Foetal calf serum (Highveld Biologicals, SA), 100 µM Penicillin/ streptomycin (Life Technologies, USA), 100 µM Gentomycin (Life Technologies, USA), RPMI) media and placed into a 5 % CO_2 incubator at 37°C. The day after thawing the cells were pelleted at 360 xg for 10 minutes and media removed. The cells were resuspended in 1 ml 20 % RPMI media. A 1:10 dilution of trypan blue (Life Technologies, USA) and cells were placed onto a counting slide for the countess cell counter (Life Technologies, USA). The cells were counted and cell viability and concentration was determined. Cells were seeded at 1 x 10^6 cells per ml to a total volume of 10 ml in 10 % RPMI media (10% Foetal calf serum, 100 µM Penicillin/ streptomycin, 100 µM Gentamycin, RPMI). A constant supply of MT-4 cells were kept growing for experiments.

HeLa cells (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HeLa catalogue number 153) were thawed from frozen stocks at a concentration of 1 x 10^6 cells per ml, in 10 ml of 20 % DMEM (Gibco, USA) (20 % Foetal calf serum, 100 µM Penicillin/ streptomycin, 100 µM Gentamycin, DMEM) media and placed into a 5 % CO_2 incubator at 37°C. The day after thawing the cells were pelleted at 360 xg for 10 minutes and media removed. The cells were resuspended in 10 % DMEM (10% Foetal calf serum, 100 µM Penicillin/ streptomycin, 100 µM Gentamycin, DMEM) and placed into a 5 % CO_2 incubator at 37°C. The cells were monitored every day and once cells were 80 % confluent the DMEM media was aspirated off and the cells washed with 1x PBS and aspirated off. A total of 2 ml trypsin (Sigma Aldrich, USA) was added and cells were incubated for two minutes at 37°C after which 10 % DMEM solution was added to a total volume of 10 ml and cells were pelleted at 360 xg for 10 minutes and media removed. A 1:10 dilution of trypan blue (Life Technologies, USA) and cells were placed onto a counting slide for the countess cell counter (Life Technologies, USA). The cells were counted and cell viability and concentration was determined. Cells were seeded at 1 x 10^5 cells per ml to a total volume of 10 ml in 10 % DMEM media (10 % Foetal calf serum, 100µM Penicillin/

streptomycin, 100 µM Gentamycin, DMEM). A constant supply of HeLa cells were kept growing for experiments.

2.3.4.2 Antiviral inhibition assays

MT-4 cells (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: MT-4 catalogue number 120) were seeded the day before antiviral testing at 3×10^5 cells per ml. The following day the viability was checked and 2×10^5 cells per ml were placed into a 50 ml conical tube and HIV-1_{NL4-3} stock added. The cells were spinoculated (O'Doherty et al., 2000) at 3000 xg for 90 minutes. Cells were subsequently washed four times with 0.01 M PBS to remove any unbound virus. A control set of cells were spinoculated without virus and washed four times with 0.01 M PBS to replicate the test cells. A total of 10 ml 10 % RPMI media was then added to the cells and 100µl of cells were added to each well of a Corning® Costar® 96-Well Cell Culture Plates (Sigma Aldrich, USA). The plate was placed into the 37°C, 5 % CO_2 incubator to equilibrate for one hour. During the incubation compounds were made up in RPMI media containing 10 % heat inactivated FCS. The compounds were serially diluted from 100 µM to 1.56 µM. A total of 100 µl of compound solution was added to the wells containing cells and mixed to ensure they were homogeneous. The plate was placed into a 37°C, 5 % CO_2 incubator for five days.

A Biomerieux Vironostika HIV-1 Ab/Ag micro ELISA system was used to test for p24. All buffers were prepared according to manufacturer's specifications and the manufacturer's protocol was followed. Briefly, 145 µl of disruption buffer was added to each well including control wells and incubated at 37°C for one hour. A total volume of 5 µl of each test specimen was added to the disruption buffer and control samples were added. The plate was then incubated at 37°C for 60 minutes and wells were then washed with 1x wash buffer for 30 seconds. Washing was repeated six times. Once washed 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate mix (1:1 ratio of TMB substrate A and B) was added and incubated for 5-30 minutes in the dark until sufficient colour had developed. The reaction was stopped by the addition of 100 µl 1 M sulphuric acid. The plates were then read on a multiplate reader at 450nm (xMARK™, Bio-Rad, USA).

2.3.4.3 Cellular Toxicity

The day of cytotoxicity testing, cells were counted and seeded at 1×10^6 cells per ml. A total of 100 µl of cells were added to each well of a 96 well cell culture plate for testing. The plate was placed into the incubator to equilibrate to 37°C and 5 % CO_2. During this time, test compounds were made up in 10% RPMI solution in a serial dilution from 100 µM to 1.56 µM. A total of 100 µl compound was added to wells containing cells and mixed to ensure the solution was homogeneous with the cells. The plate was placed into the 37°C incubator for 5 days. On the 5th day 10 µl of MTS was added and mixed. The plates were then incubated for a further four hours, and read at 490 nm (xMARK[TM], Bio-Rad, USA.) The data analysis was completed on Origin 8.1 with the log value of the concentration plotted against the absorbance level to determine a dose curve. From the curve, a half maximal cytotoxicity CC_{50} for compounds was determined. Controls used included auranofin, raltegravir and CX05168.

2.3.4.4 XCELLigence

HeLa cells were seeded at 5×10^4 cells per well of the E plate (Roche Diagnostics, Switzerland) which was attached to the xCELLigence (Roche Diagnostics, Switzerland), and allowed to incubate overnight at 37°C and 5 % CO_2 concentration. Lovastatin was serially diluted in DMEM containing 10% FCS from 200 µM to 1.56 µM and 100 µl was added to the wells on the E plate. Control wells contained only 5×10^4 cells per well in media. The cells were allowed to grow for four days at 37°C and 5 % CO_2 concentration and underwent continuous evaluation for the four days. The data was then analyses using the Real Time Cell Analyser (RTCA) Software 1.2 (ACEA Biosciences, USA).

Chapter 3: Results

3.1 Protein expression, validation and modification
3.1.1 Protein expression

E. cloni EXPRESS® BL21 (DE3) (pLysS) and *E. cloni* 10G SOLOs competent cells (Lucigen Corporation, USA) were successfully transformed with plasmids carrying genes encoding for pLEDGF/p75, HIV-1 IN (pINSD.His.Sol) and pogo transposable element with ZNF domain (pPogZ). The bacterial colonies were successfully grown on LB agar plates containing the required antibiotics for each plasmid. Colonies were evenly spread with good separation allowing selection of a single colony. A single colony from each plate was placed in fresh LB broth with appropriate antibiotics and grown overnight at 37°C for HIV-1 IN and PogZ and 32°C for LEDGF/p75 followed by inoculation as described in section 2.2.1.2. Stocks were successfully produced for all plasmids transformed into *E cloni* BL21 (DE3) (pLysS) expression cells and *E cloni* 10G solo plasmid cells for future production of plasmid or recombinant protein.

Nucleotide sequencing of plasmid preparations of pLEDGF/p75, pINSD.His.Sol and pPogZ at Inqaba Biotechnical Industries (South Africa) confirmed the identity of the plasmids, and intact open reading frames (results not shown).

RE digests were successfully performed on all extracted plasmids, and further confirmed the identity of the constructs. An example of the RE digests of pLEDGF/p75 is shown in Figure 3.2.
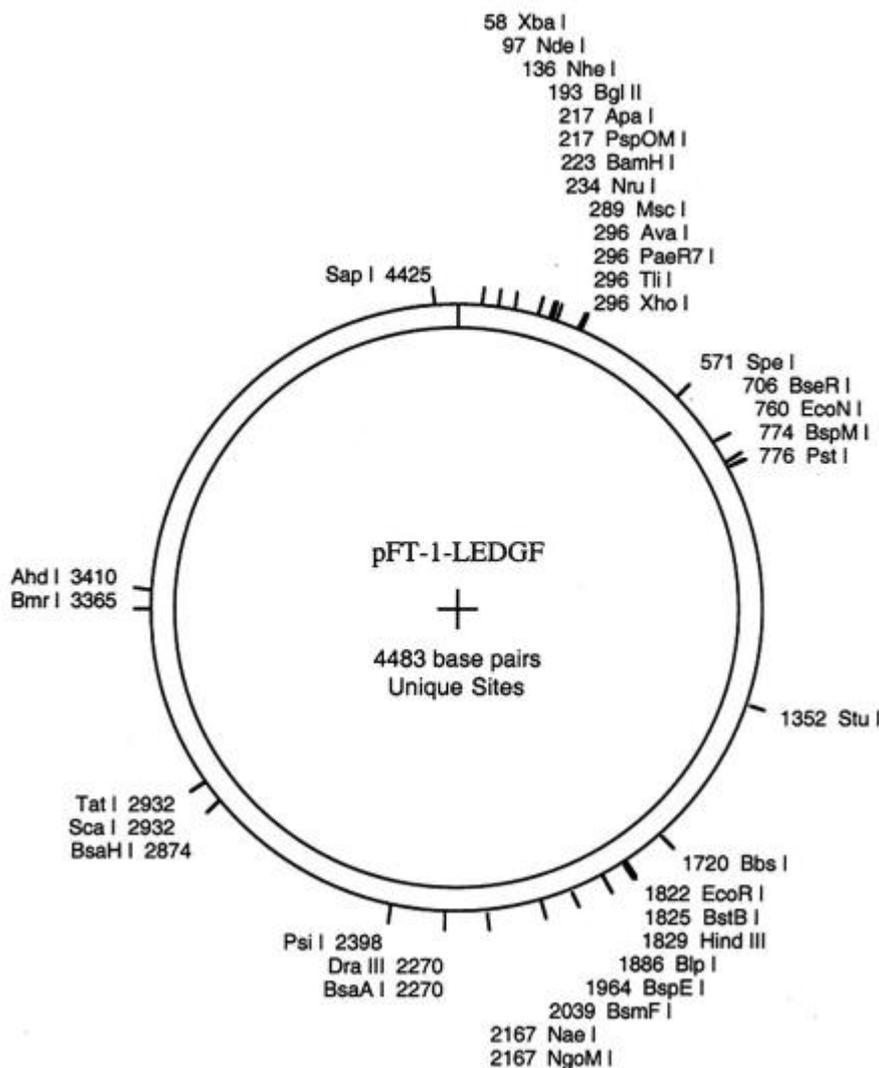
Figure 3.1 : Plasmid map of pFT-1-LEDGF, indicating restriction sites and their positions in base pairs. The following plasmid map was utilised to determine expected band sizes of products from restriction digest.

pFT-1-LEDGF was derived from pRSETB (Life Technologies, USA). Recombinant LEDGF/p75 was amplified with *Bgl*II and *EcoR*I using the *Pfu* Ultra DNA polymerase (Stratagene,USA) by Vandegraaff and co-workers (Vandegraaff et al., 2006). Restriction enzymes *Bgl*II and *EcoR*I were used to excise the LEDGF/p75 insert. *Bgl*II was expected to cut at positions 435 and 1702. Therefore bands of 1267bp and 2781bp are expected. *EcoR*I is expected to cut at positions 206 and 1822. Therefore band sizes of 1616bp and 2867bp are expected. LEDGF/p75 is 530 amino acids and is therefore expected to be 1590 nucleotides in length.
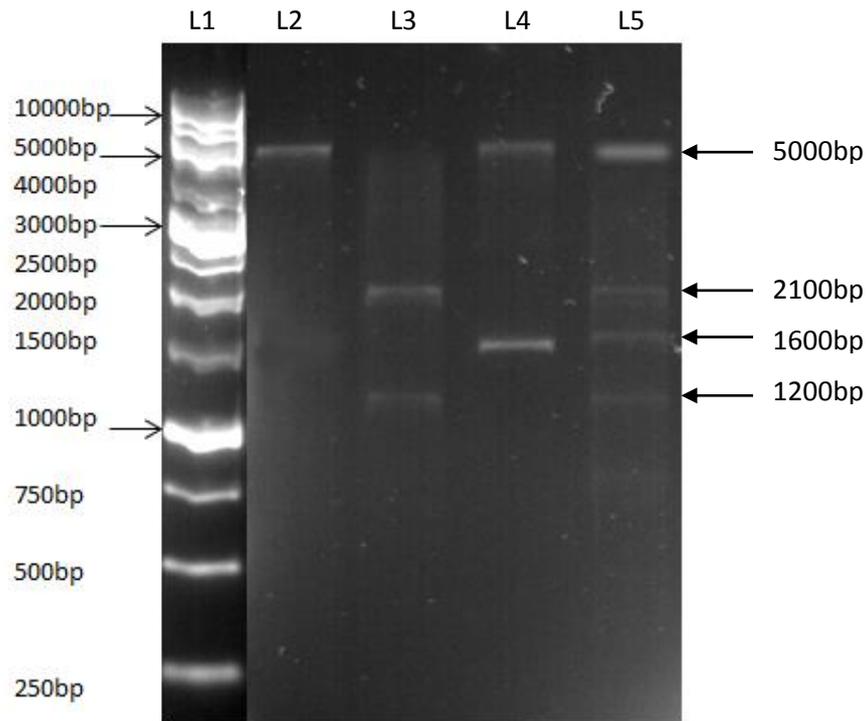
Figure 3.2: Agarose gel (1%) of pLEDGF/p75 with single and double restriction enzyme digests to confirm the presence of the gene insert in the vector. Lane 1, 1Kb DNA ladder (Fermentas, USA); Lane 2, undigested pLEDGF/p75; Lanes 3 and 4, single digest of pLEDGF/p75 with *Bgl*II and *Eco*RI respectively; Lane 5, double digest of pLEDGF/p75 with *Bgl*II and *Eco*RI. Molecular weight, in base pairs is indicated on the left.

Figure 3.2 show the agarose gel of pFT-1-LEDGF before and after restriction enzyme digest. Undigested plasmid can be seen in lane 2 with and approximation of 5000bp as expected. Lane 3 shows the digest with *Bgl*II showing band sizes of 2100bp and 1200bp which are within the expected sizes. Lane 4 shows the digest with *Eco*RI showing band sizes of 5000bp and 1600bp. The 5000bp band indicated incomplete digest and the 1600bp band was expected. Lane 5 shows the double digest with *Bgl*II and *Eco*RI confirming the single digest results. The LEDGF/p75 insert can be seen at 1600bp.

3.1.1.1 Optimization of recombinant protein expression
Studies were conducted on *E. cloni* expressing HIV-1 IN, LEDGF/p75 and PogZ to determine the optimal protein expression conditions as described in section 2.2.1.5. For HIV-1 IN, an IPTG concentration of 1 mM was found optimal for protein expression induction, and three hours was found to be an optimal time post induction to harvest cells for protein purification. For PogZ, an IPTG

concentration of 1mM was found to be best for expression and three hours was found to be an optimal time post induction to harvest cells for protein purification. Similarly, LEDGF/p75 was optimally expressed after 1mM IPTG induction, and allowed to express for three hours. Overall, for future bulk expressions it was found that glucose had no valuable effect on preventing expression prior to induction, and thus was excluded from further experiments. Figure 3.3 depicts expression of LEDGF/p75 (75 kDa) in *E. cloni* BL21 (DE3) (PlysS) for a total of three hours post induction with 1mM IPTG. Similar expression patterns were seen for HIV-1 IN (32 kDa), and PogZ (150 kDa) (results not shown).
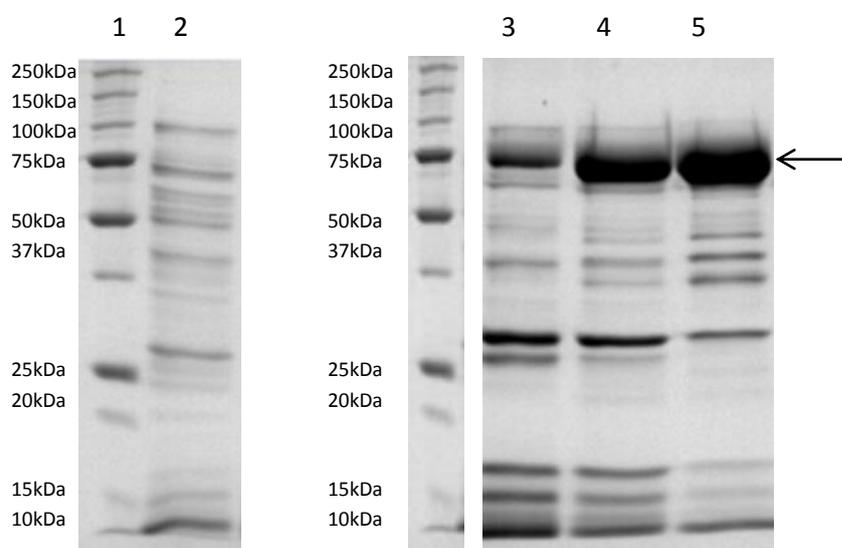
Figure 3.3: SDS PAGE gel indicating over expression of LEDGF/p75 after induction with 1mM IPTG. Lane 1, precision plus protein molecular weight marker (Bio-Rad, USA); Lane 2, uninduced culture; Lane 3, 1 hour post induction with IPTG; Lane 4, 2 hours post induction with IPTG; and Lane 5, three hours post induction with IPTG. Arrow indicated the expressed LEDGF/p75. Molecular weight, in kilo Daltons (kDa), is indicated on the left. The above two figures are from the same gel but have been separated for clarity.

3.1.1.3 Column chromatography

Proteins expressed during pLEDGF/p75 expression were eluted with a buffer containing a 200 mM imidazole concentration over a 50 ml gradient. Protein fractions at 14 ml through to 22 ml were run on a SDS PAGE gel (Figure 3.4). Fractions from the peaks containing an over expressed protein at 75 kDa for LEDGF/p75 were pooled and underwent ultrafiltration thereby concentrating the proteins.

Similarly, over expressed proteins at 32 kDa for HIV-1 IN and 100 kDa for PogZ were pooled and concentrated. In the case of HIV-1 IN, purity was observed after the ultrafiltration step, and was thus buffer exchanged into the IN storage buffer.
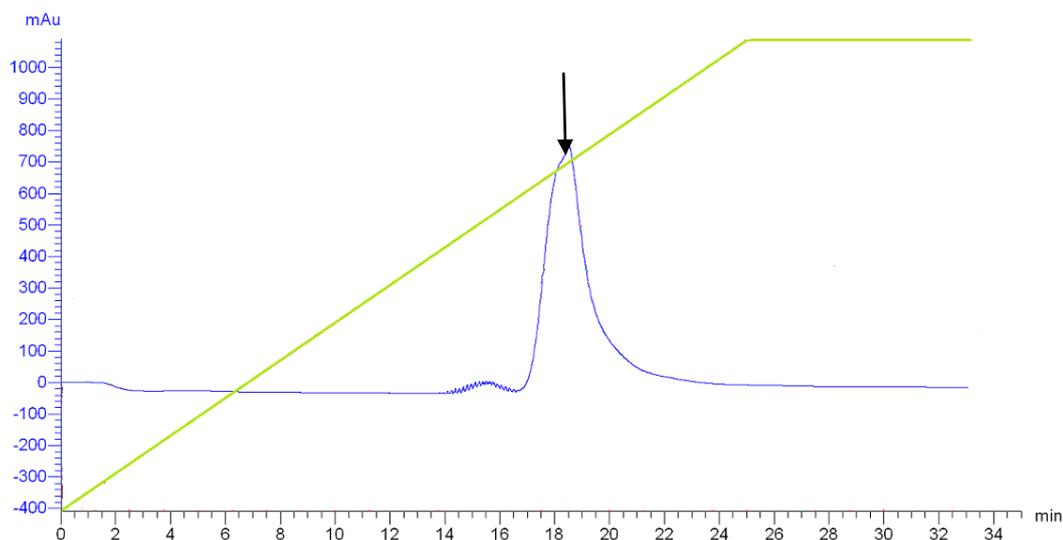
Figure 3.4: Representative elution chromatogram of HiTrap Nickel affinity chromatography used to purify recombinant LEDGF/p75. The blue line indicates the absorbance of each eluted fraction whereas the green line denotes the imidazole concentration gradient. The elution profile was obtained from the ÄKTAprime view software. Proteins were eluted at a flow rate of 1 ml per minute and collected in 2 ml fractions. The absorbance increases sharply at fraction 19 ml (as indicated by an arrow) denoting the elution of a HIS tagged protein.

However, LEDGF/p75 and PogZ required further purification as contaminating bands were visible. LEDGF/p75 was buffer exchanged to a sodium phosphate buffer and loaded onto a SP-Sepharose fast flow column (GE Healthcare, UK) that was attached to the ÄKTAprime plus. Proteins containing a negative charge were then eluted by an increase in the salt concentration to 1 M and eluting over a 50 ml gradient. The LEDGF/p75 SP-Sepharose elution profile can be seen in Figure 3.5.
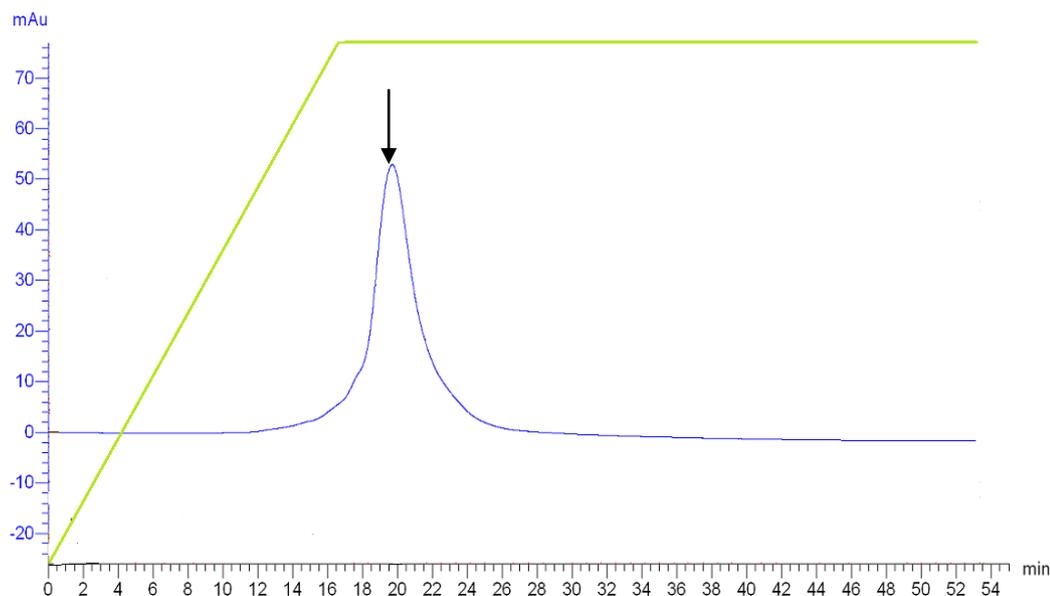
Figure 3.5: Representative elution profile of SP-Sepharose cation exchange chromatography used to purify LEDGF/p75. The blue line indicates the absorbance of each eluted fraction whereas the green line denotes salt concentration gradient. The elution profile was obtained using the AKTA prime view software. Proteins were eluted at a flow rate of 1 ml per minute and collected in 2 ml fractions. The absorbance increases sharply at 20 minutes denoting the elution of a negatively charged protein indicated by the arrow. Fractions collected between elution times of 16 to 26 minutes were run on an SDS PAGE gel.

Fractions 8 to 13 (correlating to 16 and 26 minutes; Figure 3.5) were run on a SDS PAGE gel alongside a protein molecular weight marker to determine the presence and purity of the protein of interest (data not shown). Fractions containing an over expressed protein at 75 kDa, were pooled and concentrated. Subsequently, concentrated protein was buffer exchanged into the LEDGF/p75 size exclusion buffer and loaded onto a Superdex 200 with use of the ÄKTAprime plus at a flow rate of 0.5 ml/min. The LEDGF/p75 Superdex 200 elution profile can be seen in Figure 3.6. Fractions 7 through to 11, correlating to 30 to 45 minute elution times, proved to be the 75 kDa LEDGF/p75.
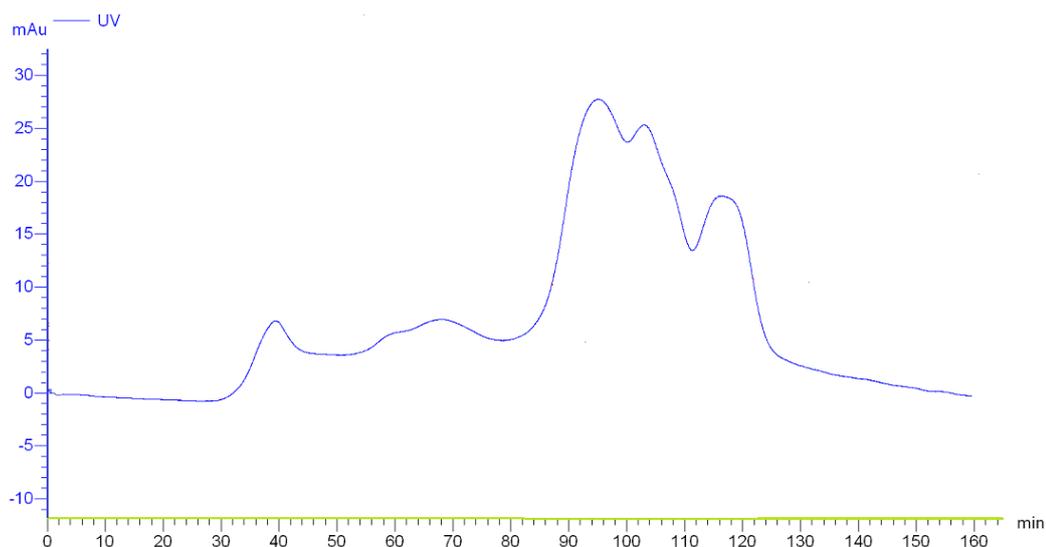
Figure 3.6: Representative elution profile of Superdex 200 size exclusion chromatography used to purify LEDGF/p75. The blue line indicates the absorbance of each eluted protein. The elution profile was obtained from the AKTA prime view software. Proteins were eluted at a flow rate of 0.5ml per minute and collected in 2 ml fractions. Fractions from each peak were run on a SDS PAGE gel.

Affinity purified PogZ was buffer exchanged into a size exclusion buffer and loaded onto Superdex 200 with use of the ÄKTAprime plus at a flow rate of 0.5 ml/min. Relevant PogZ containing fractions were pooled and concentrated (results not shown).

Samples at each stage of purification for each of the three recombinant proteins were run on SDS PAGE gels (Figures 3.7 to 3.9) and confirm the increased purity with each purification step. Purity of each sample as shown in the figure below was determined by using Gel Quant express (DNR Bio-Imaging technologies, Israel) software. Overall, purity of LEDGF/p75 increased from 44 % after affinity chromatography to 68 % after ion exchange chromatography to a final purity of over 95 % after size exclusion chromatography (Figure 3.7). HIV-1 IN was purified to 77 % after affinity chromatography to a final purity of 88 % after ultrafiltration (Figure 3.8). PogZ purity increased from 30 % after affinity chromatography to a final purity of 80 % by size exclusion chromatography (Figure 3.9), attributed to the presence of two additional bands, which despite further purification attempts could not be removed.
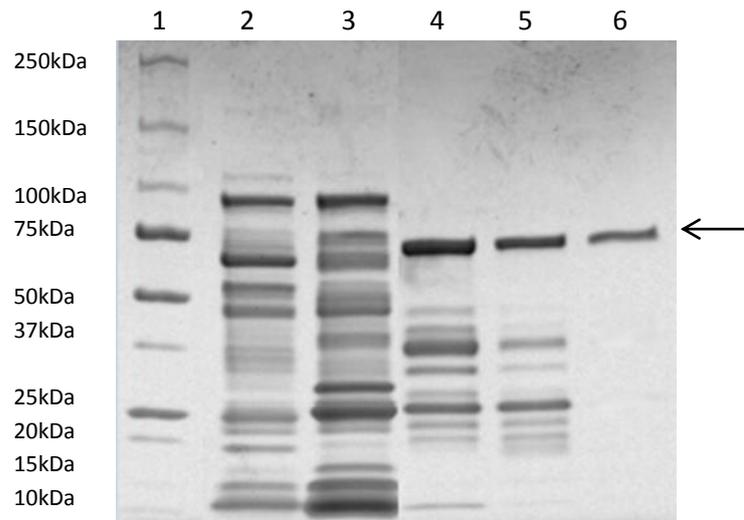
Figure 3.7: LEDGF/p75 SDS-PAGE gel analysis depicting purification. Lane 1, precision plus protein molecular weight marker (Bio-Rad, USA); Lane 2, supernatant prior to loading onto a Hi trap nickel affinity chromatography; Lane 3, sonicated pellet supernatant; Lane 4, concentrated fractions after Hi Trap nickel affinity chromatography; Lane 5, concentrated fractions after SP-Sepharose chromatography; and Lane 6, concentrated fractions from Superdex 200 size exclusion chromatography. The arrow indicates the 75 kDa protein of LEDGF/p75.
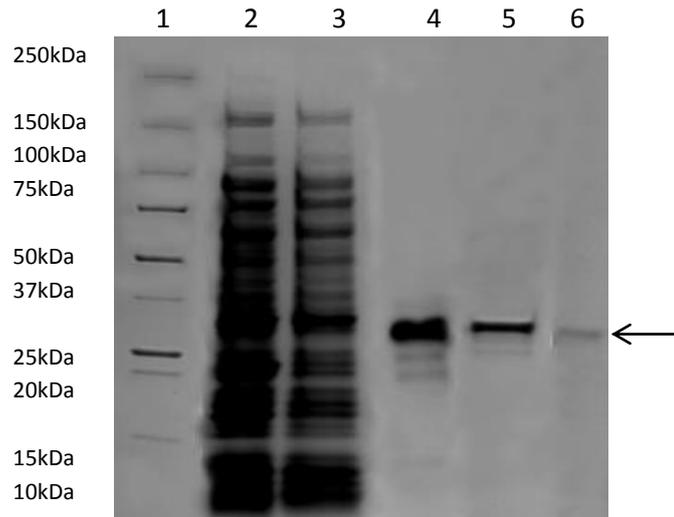
Figure 3.8: SDS-PAGE gel analysis depicting purification of HIV-1 IN. Lane 1, precision plus protein molecular weight marker (Bio-Rad, USA); Lane 2, sonicated pellet supernatant; Lane 3 sonicated cell pellet fraction; Lane 4, nickel affinity chromatography purified HIV-1 IN; Lane 5, concentrated HIV-1 IN; Lane 6, positive control HIV-1 IN (NIH AIDS Research and Reference Reagent Program). Recombinant IN at 32 kDa is denoted by the arrow.
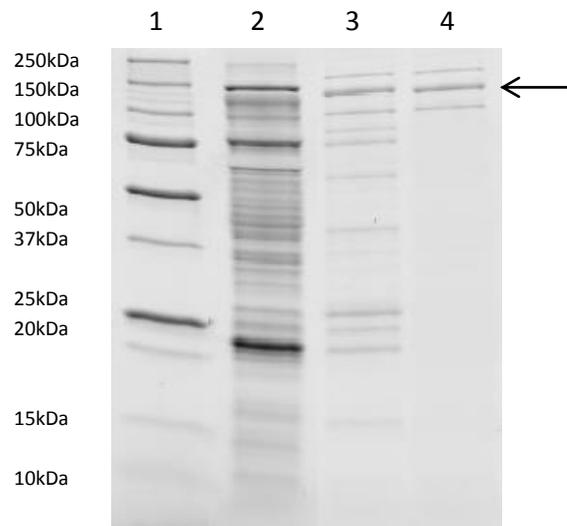
Figure 3.9: SDS PAGE gel depicting purification steps of PogZ. Lane 1, precision plus protein molecular weight marker (Bio-Rad, USA); Lane 2, sonicated pellet supernatant; Lane 3, nickel affinity chromatography purified PogZ; lane 4, Purified PogZ after size exclusion chromatography.

3.1.2 Validation of recombinant protein expression

3.1.2.1 Western blots

Western blots were run for each protein to confirm the expression of the desired protein. Purified recombinant LEDGF/p75 was run alongside an uniduced LEDGF/p75 bacterial culture fraction lacking a protein at 75 kDa and a Super Western C marker (Bio-Rad, USA). The Western blot displayed an absence of bands in the negative control lane and the presence of a 75 kDa band in lane 3, confirming the expression of LEDGF/p75 (Figure 3.10).

Similarly, bands were detected for the positive control recombinant HIV-1 IN and the newly purified recombinant HIV-1 IN (Figure 3.11, lanes 3 and 4, respectively), and recombinant PogZ at 100 kDa (Figure 3.12).
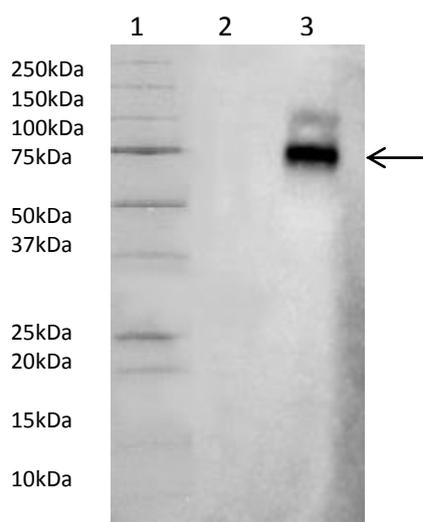
Figure 3.10: Western blot analysis confirming the expression of LEDGF/p75 with primary antibody anti-PSIP1. Lane 1, Super Western C marker (Bio-Rad, USA); Lane 2, negative control fraction from size exclusion chromatography; Lane 3, concentrated size exclusion chromatography purified LEDGF/p75.
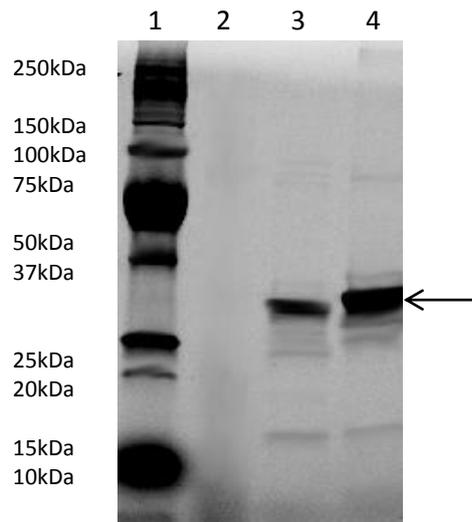
Figure 3.11: Western blot analysis confirming expression of recombinant HIV-1 IN. Lane 1, Super Western C marker (Bio-Rad, USA); Lane 2, negative control fraction from nickel affinity chromatography; Lane 3, positive control recombinant HIV-1 IN obtained from the NIH AIDS Research and Reference Reagent Program; Lane 4 concentrated nickel affinity chromatography purified HIV-1 IN.
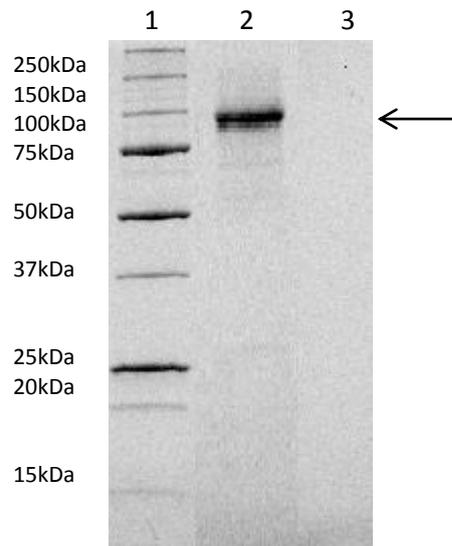
Figure 3.12: Western blot analysis confirming expression of recombinant PogZ. Lane 1, Super Western C marker (Bio-Rad, USA); Lane 2 concentrated protein following size exclusion chromatography; Lane 3, negative control fraction from size exclusion chromatography.

3.1.3 Modification of purified recombinant proteins

3.2.3.1 6XHIS tag cleavage

Thrombin cleavage was performed on both subtype B and subtype C HIV-1 IN for use in the AlphaScreen assay. Fractions containing the cleaved HIV-1 IN and uncleaved samples were analysed on an SDS-PAGE gel (Figure 3.13), and cleavage of the 6XHIS tag was confirmed by the absence of bands in the cleaved samples by Western blot analysis (Figure 3.14).
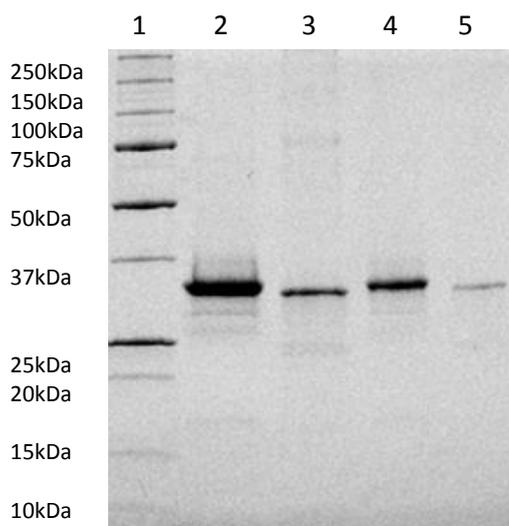
Figure 3.13: SDS PAGE gel following thrombin cleavage of recombinant HIV-1 IN to remove the 6xHIS tag. Lane 1, precision plus protein molecular weight marker (Bio-Rad, USA); Lane 2, uncleaved HIV-1 IN subtype B; Lane 3, thrombin cleaved HIV-1 subtype B IN; Lane 4, uncleaved HIV-1 subtype C IN; Lane 5, thrombin cleaved HIV-1 subtype C IN.

Figure 3.13 indicates an SDS PAGE gel of HIV-1 IN samples before undergoing thrombin cleavage and after thrombin cleavage. The removal of the HIS tag from the HIV-1 IN was needed to prevent cross interaction with the AlphaScreen acceptor beads as both LEDGF/p75 and HIV-1 IN contained a HIS tag. Lane 2 contains an uncleaved HIV-1 IN subtype B sample and lane 3 contains the sample after thrombin cleavage. A slight shift in migration on the gel can be seen between the uncleaved and cleaved samples, thus suggesting successful cleavage of the HIS tag. Lane 4 contains uncleaved HIV-1 IN subtype C and lane 5 contains cleaved HIV-1 IN subtype C. Also suggesting successful cleavage as indicated by the slight shift in migration.
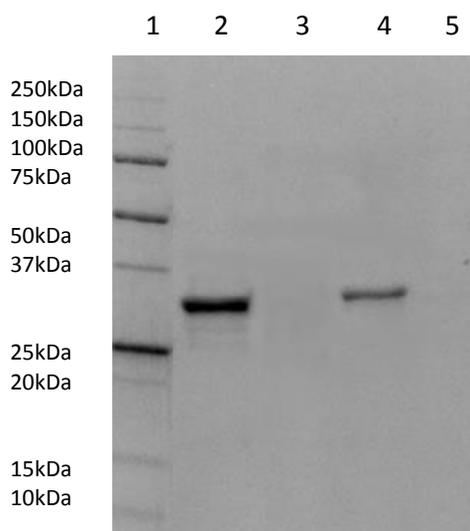
Figure 3.14: Western blot analysis of HIV-1 subtype B and C IN following thrombin cleavage of the 6XHIS tag. Lane 1, Super Western C marker (Bio-Rad, USA); Lane 2, HIV-1 subtype B IN 6xHIS tagged protein; Lane 3, cleaved HIV-1 subtype B IN; Lane 4, HIV-1 subtype C IN 6XHIS tagged protein; Lane 5, cleaved HIV-1 subtype C IN.

The SDS PAGE shown in Figure 3.13 was transferred to a PVDF membrane and a Western blot was conducted. A primary antibody of anti-HIS was used for first incubation. This would then indicate the presence of a HIS tag on the uncleaved HIV-1 and the absence of a HIS tag on the cleaved protein.

3.3.3.1 Biotinylation of recombinant proteins

Biotinylation of HIV-1 IN subtype B and PogZ was needed for use in the non DNA AlphaScreen. Following biotinylation of uncleaved HIV-1 IN subtype B and PogZ, proteins were bound to a streptavidin coated plate, and aspirate containing any unbound protein was removed. Absorbance readings of the removed aspirate, as compared to a control buffer (containing no biotinylated protein) confirmed that the HIV-1 IN and PogZ were successfully biotinylated (results not shown). Overall, the above results confirmed that HIV-1 IN, LEDGF/p75, and PogZ were all successfully expressed, purified and modified (where appropriate), for use in subsequent biochemical/biological assays.

3.2 Theoretical, biochemical and biological screening of test compounds

3.2.1 Theoretical screening of test compounds for HIV-1 IN-LEDGF/p75 inhibition

3.2.1.1 Molecular modelling of NCC collection

HIV-1 IN-LEDGF/p75 complex (PDB2B4J) was prepared by the removal of the LEDGF/p75 and analysed to determine the location of the LEDGF/p75 binding sites on the IN molecules. Four possible sites were identified as seen by yellow spheres in Figure 3.15. With further model optimisation, two of the four residues were confirmed as LEDGF/p75 binding sites (Figure 3.16).
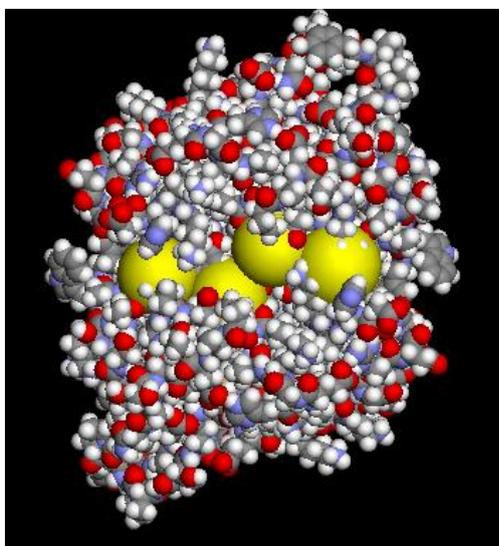
Figure 3.15: Space filled model of prepared HIV-1 IN-LEDGF/p75 complex with LEDGF/p75 removed (PDB2B4J) displaying four possible LEDGF/p75 interaction sites indicated by yellow spheres.
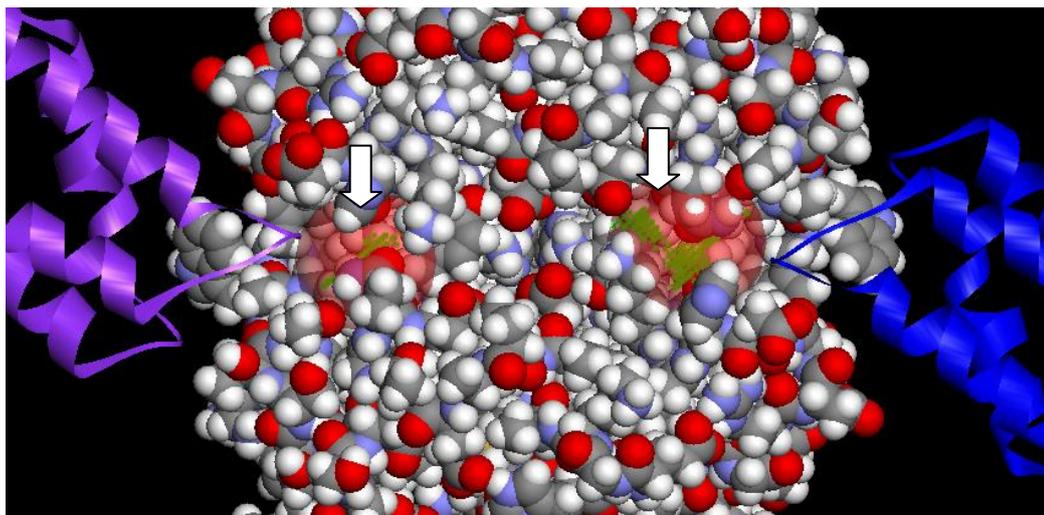
Figure 3.16: Space filled model of the prepared HIV-1 IN-LEDGF/p75 complex (PDB 2B4J) with the LEDGF/p75 removed showing the two confirmed LEDGF/p75 binding sites (arrows).

To ensure the model was accurate, it was trained using known HIV-1 IN-LEDGF/p75 inhibitors. A Boehringer ligand file was created based on data from Simoneau *et al.* including the IC_{50}, EC_{50}, CC_{50} and K_D provided (Simoneau et al., 2011), and used to validate the accuracy of the model. Of the various scoring functions utilised, the best fit scoring of the Boehringer compounds was seen to be *Jain* score as well as the *LibDockScore*.

Once the accuracy was confirmed, the NCC-202 files were obtained from the NIH Clinical Collection and prepared for docking. The entire NCC-202 library was screened through the model and docking scores and energies were recorded. The best one thousand *Jain* and *LibDock* scoring compounds (including tautomers of the compounds) were further subjected to *CDOCKER* docking and *In-Situ-Ligand-Minimisation*. Overall, the top one thousand scoring compounds were a combination of only 12 compounds in different tautomer structures.

The 12 compounds included prednisolone, quinidine hydrochloride, cefazolin A, naloxone, budesonide, amninocide, moban, methyl prednisolone, duanorubicin, naltrexone hydrochloride, betamethasone, and lovastatin. Because lovastatin was one of the 12 top scoring compounds, a further three statin compounds (pravastatin, simvastatin and mevastatin) were selected based on structural similarities to lovastatin, two derivatives (for lovastatin and pravastatin), as well

as atorvastatin. CX05168, a known HIV-1 IN-LEDGF/p75 inhibitor, and raltegravir, a known HIV-1 IN inhibitor, were included as positive controls. All structures of the 20 compounds selected for further screening are shown in Table 3.1.

Table 3.1: Chemical structures of the 20 selected compounds for theoretical, biochemical and biological screening.

Prednisolone	Quinidine hydrochloride
Cefazolin A	Naloxone
Budesonide	Amninocide

Results

Moban	Methyl prednisolone
Daunorubicin	CPD000058767
Betamethasone	CX05168
Lovastatin	Lovastatin Acid

Pravastatin	Pravastatin Lactone
Simvastatin	**Mevastatin**
Atorvastatin	**Raltegravir**

3.2.1.2 Theoretical analysis of all compounds used in this study

The 20 selected compounds were analysed in Osiris property explorer to evaluate their theoretical properties, including predictive analysis of compound lipophilicity, aqueous solubility, toxicity, drug likeness and drug score. Osiris property explorer also predicts if a compound will be a mutagen, turmorigen or an irritant. The data can be seen in Table 3.2 for the NCC compounds and Table 3.3 for statins.

Overall, of the twenty compounds screened only one (naloxone) was found to be an irritant, 38 % were lipophilic and 47 % were hydrophilic. Three statins were

found to be highly lipophilic. Solubility scores found that a total of 48 % of compounds had a solubility of less than -4 and all statins with the exception of pravastatin were found to be below -4.

Table 3.2: Osiris molecular property explorer data for the 12 NCC-202 compounds selected and 2 control compounds, showing lipophilicity (cLogP), solubility (LogS), molecular weight, drug likeness and drug score for each compound.

Compound name	cLogP	LogS	M.W. g/mol	Drug-likeness	Drug score	Mutagenic	Tumorigenic	Irritant	R.E
Raltegravir	-1.05	-1.18	444	5.95	0.81				
CX05168	5.68	-6.4	353	-2.05	0.21				
Prednisolone	1.4	-2.96	360	4.1	0.85				
Quinidine hydrochloride	2.84	-3.1	324	1.09	0.73				
CefazolinA	-0.77	-2.12	454	12.25	0.79				
Noloxone	0.6	-2.38	329	1.29	0.65			(red)	
Budisonide	2.15	-3.82	430	1.7	0.67				
Amninocide	2.77	-4.7	502	1.8	0.52				
Moban	1.84	-2.16	276	1.58	0.84				
Methyl Prednisolone	1.59	-3.12	374	4.17	0.83				
Daunorubicin	1.43	-5.01	527	6.72	0.52				
CPD000058767	1.81	-3.19	341	4.67	0.85				
Betamethazone	1.64	-3.25	392	3.18	0.8				
Lovastatin	4.28	-4.54	404	1.65	0.54				

■ (red) Negative effect
■ (green) No effect

Table 3.3: Osiris molecular property explorer data for the seven statin compounds selected, showing lipophilicity (cLogP), solubility (LogS), molecular weight, drug likeness and drug score for each compound.

Compound name	cLogP	LogS	M.W. g/mol	Drug-likeness	Drug score	Mutagen	Tumorig	Irritant	R.E
Lovastatin	4.28	-4.54	404	1.65	0.54				
Pravastatin	3.07	-3.53	424	3.14	0.71				
Simvastatin	4.79	-4.75	418	0.67	0.43				
Atorvastatin	5.55	-6.92	558	1.03	0.22				
Lovastatin acid	4.09	-4.09	422	3.25	0.62				
Prevastatin lactone	3.89	-4.53	418	1.37	0.55				
Mevastatin	4.08	-4.41	390	0.58	0.52				

■ (green) No effect

Results

The results for the Lipinski rule of five predictions of oral bioavailability of the 20 selected compounds are shown in Table 3.4 for the NCC library and Table 3.5 for statins.

Table 3.4: Lipinski rule of 5 for the 12 NCC-202 compounds selected for screening including 2 positive controls used, indicating adherence or aversion to the rules and a final score.

Compound name	Rule 1	Rule 2	Rule 3	Rule 4	Score
Raltegravir	√	√	√	√	4⁄4
CX05168	√	√	√	X	3⁄4
Prednisolone	√	√	√	√	4⁄4
Quinidine hydrochloridd	√	√	√	√	4⁄4
CefazolinA	X	√	√	√	3⁄4
Noloxone	√	√	√	√	4⁄4
Budisonide	√	√	√	√	4⁄4
Amninocide	√	√	X	√	3⁄4
Moban	√	√	√	√	4⁄4
Methyl Prednisolone	√	√	√	√	4⁄4
Daunorubicin	X	√	X	√	2⁄4
CPD000058767	√	√	√	√	4⁄4
Betamethazone	√	√	√	√	4⁄4
Lovastatin	√	√	√	√	4⁄4

Rule 1: Not more than 5 hydrogen bond acceptors
Rule 2: Not more than 10 hydrogen bond donors
Rule 3: A molecular weight under 500 g/mol
Rule 4: A partition coefficient log P of less than 5
X does not adhere to rule
√ adheres to the rule
Score: Lipinski's rule of 5 score out of 4

Results

Table 3.5: Lipinski's rule of five for the seven statins selected for screening, indicating adherence or aversion to the rules and a final score.

Compound name	Rule 1	Rule 2	Rule 3	Rule 4	Score
Lovastatin	√	√	√	√	4⁄4
Pravastatin	√	√	√	√	4⁄4
Simvastatin	√	√	√	√	4⁄4
Atorvastatin	√	√	X	X	2⁄4
Lovastatin acid	√	√	√	√	4⁄4
Prevastatin lactone	√	√	√	√	4⁄4
Mevastatin	√	√	√	√	4⁄4

Rule 1: Not more than 5 hydrogen bond acceptors
Rule 2: Not more than 10 hydrogen bond donors
Rule 3: A molecular weight under 500 g/mol
Rule 4: A partition coefficient log P of less than 5
X does not adhere to rule
√ adheres to the rule
Score: Lipinski's rule of 5 score out of 4

Of the 20 compounds selected, all but three (amninocide, daunorubicin and atorvastatin) were within the acceptable parameters for molecular weight. Overall, 76 % abided by all four of the Lipinski rule of five with 14 % failing one parameter and 9.5 % failing two parameters. Two compounds, CX05168 and atorvastatin, were found to be above the acceptable lipophilicity score of five (rule 4).

A summary of all properties for statins (excluding the two derivatives) using Accelrys Discovery Studio™, Chem Spider and Osiris molecular property explorer is shown in Table 3.6. In addition, Accelrys Discovery Studio™ and Osiris properties explorer ADMET screening data was obtained/ amalgamated for all NCC compounds (Table 3.7). This data was subsequently used to validate results from the biological testing.

Results

Table 3.6: Property analysis of statins

	Water solubility	logP	logS	pKa (strongest acidic)	pKa (strongest basic)	Physiological charge	Hydrogen acceptor count	Hydrogen donor count	Polar surface area	Rotatable bond count	Refractivity	Polarizability	Melting point
Lovastatin	2.43e-02 g/l	4.11	-4.2	14.91	-2.8	0	3	1	72.83	7	113.18	46.11	174.5 °C
Atorvastatin	6.30e-04 g/l	4.24	-6	4.33	-2.7	-1	5	4	111.79	12	158.2	59.25	159.2-160.7 °C
Simvastatin	1.22e-02 g/l	4.51	-4.5	14.91	-2.8	0	3	1	72.83	7	117.68	47.85	135-138 °C
Mevastatin	7.98e-02 g/l	4.03	-3.7	4.21	-2.7	-1	5	3	104.06	11	112.13	45.31	152 °C
Pravastatin	2.42e-01 g/l	2.23	-3.2	4.21	-2.7	-1	6	4	124.29	11	113.6	46.56	326 °C
Fluvastatin	4.41e-03 g/l	3.69	-5	4.56	-2.8	-1	4	3	82.69	8	114.86	44.31	194-197 °C

Results

Table 3.6 describes the property of analysis of statins studied in this research. Lovastatin, atorvastatin, simvastatin and mevastatin were similar in lipophilicity and were found to be more lipophilic than pravastatin and fluvastatin. There was no direct relationship between solubility and lipophilicity, with pravastatin being the most soluble and fluvastatin being the least soluble. Lovastatin and simvastatin had no physiological charge whereas the remaining statins were seen to be negatively physiological conditions.

Table 3.7: Accelrys Discovery Studio™ ADMET screening of compounds predicting molecular weight, acceptor and donor hydrogen atoms, rotatable bonds, solubility and lipophilicity (AlogP).

Compound name	Molecular weight	Aceptor	Donor	Rotatable bonds	Solubility	AlogP
Raltegravir	444.416	7	3	6	-2.193	-0.291
CX05168	353	2	1	5	-7.082	6.446
Prednisolone	360.452	5	3	2	-2.96	1.26
Quinidine hydrochloridd	324.424	4	1	4	-3.1	2.733
CefazolinA	476.479	11	1	7	-2.12	-2.807
Noloxone	363.842	5	2	2	-2.88	1.835
Budisonide	430.543	6	2	4	-3.82	2.198
Amninocide	502.573	7	1	4	-4.7	2.47
Moban	312.841	3	1	3	-2.15	2.522
Methyl Prednisolone	374.479	5	3	2	-3.12	1.512
Daunorubicin	527.53	11	5	4	-5.01	0.628
CPD000058767	377.869	5	2	2	-3.19	2.039
Betamethazone	392.469	5	3	2	-3.25	1.708
Lovastatin	404.549	5	1	7	-4.59	4.218
Pravastatin	424.528	7	4	11	-2.107	2.156
Simvastatin	418.566	5	1	7	-5.29	4.634
Atorvastatin	558.64	5	4	12	-5.091	5.555
Lovastatin acid	422.555	6	3	11	-3.332	3.634
Prevastatin lactone	406.512	6	2	7	-3.36	2.74
Mevastatin	390.513	5	1	7	-4.577	3.966

Table 3.7 displays the theoretical ADMET data for all compounds studied in this research. ADMET refers to the adsorption, distribution, metabolism excretion and toxicity of a compound. The tolerability of a drug can be predicted with use of these parameters. Amninocide, daunorubicin and atorvastatin all have molecular masses exceeding 500 g/mol. CX05168, lovastatin, simvastatin and atorvastatin were seen to be lipophilic and were subsequently seen to be the compounds with low solubility scores.

3.2.1.3 Preparation of compounds

The chemical structure of the extracted lovastatin, pravastatin, simvastatin, atorvastatin, synthesized lovastatin acid and pravastatin lactone, and purchased

mevastatin was analysed by NMR and mass spectroscopy and was found to be above 95 % pure in all cases (results not shown). In the case of lovastatin acid, the yield was 58 % showing a loss of 42 % from starting material. Pravastatin lactone also displayed a loss in starting material of 56 % leaving a yield of 44 %. The schematic representation of the hydrolysis reaction for lovastatin acid can be seen in Figure 3.17 and the condensation reaction for pravastatin lactone can be seen in Figure 3.18. Lovastatin was hydrolysed by the addition of 0.1 M NaOH resulting in the closed lactone ring opening as seen in Figure 3.17. Pravastatin was condensed by the addition of triflouroacetic acid thereby forcing the open chain closed into the lactone form as seen in Figure 3.18.
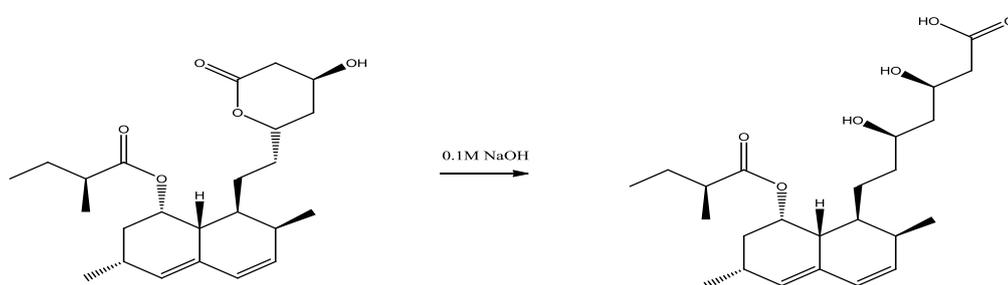
Figure 3.17: Schematic representation of the hydrolysis reaction of lovastatin to lovastatin acid by the addition of 0.1 M sodium hydroxide (NaOH). The closed lactone ring of lovastatin is forced open to form the β hydroxyl acid chain.
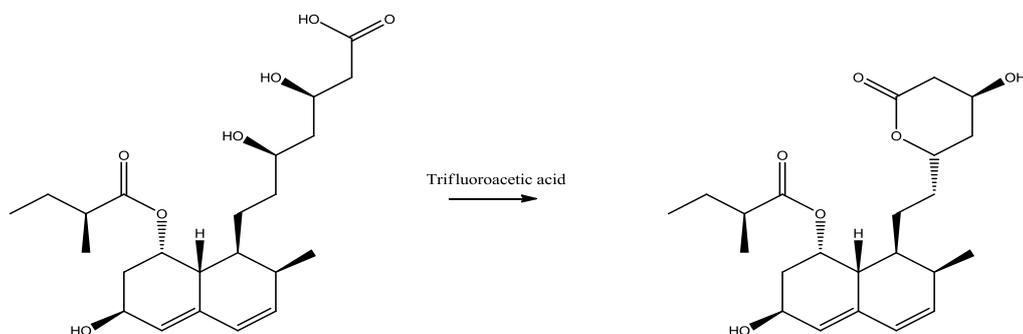
Figure 3.18: Schematic representation of the condensation reaction of pravastatin to pravastatin lactone by the addition of trifluoroacetic acid. The open β hydroxyl acid chain is condensed to a closed lactone ring.

Results

3.2.2 Biochemical and biological screening on HIV-1 IN-LEDGF/p75 inhibitors

3.2.2.1 Evaluation of the HIV-1 IN-LEDGF/p75 interaction by size exclusion chromatography

The standard curve generated for calibration of size exclusion chromatography is shown in Figure 3.19. Both high molecular weight and low molecular weight markers were loaded onto the equilibrated Superdex 200 column and allowed to elute at 0.5 ml/min. Blue dextran was used to determine the void volume. The Kav for each protein was calculated and plotted against its known molecular weight and a standard curve was obtained. The standard curve then allows the calculation of the molecular mass of unknown proteins and protein complexes. The elution profile of the HIV-1 IN-LEDGF/p75 complex showed minor peaks at 50 ml, 55 ml, 70 ml, 82.5 ml, 100 ml and a major peak at 115 ml (Figure 3.20).
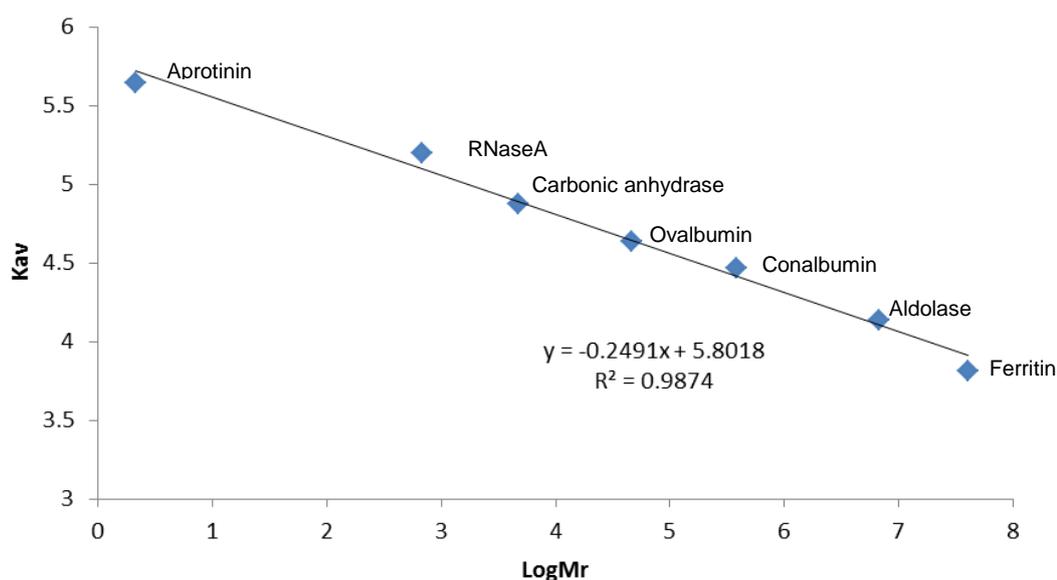
Figure 3.19: Standard curve drawn from the elution of protein standards on the size exclusion column, showing the calculated partition co-efficient (Kav) for each protein plotted against the log of the molecular weight (LogMr).

Results
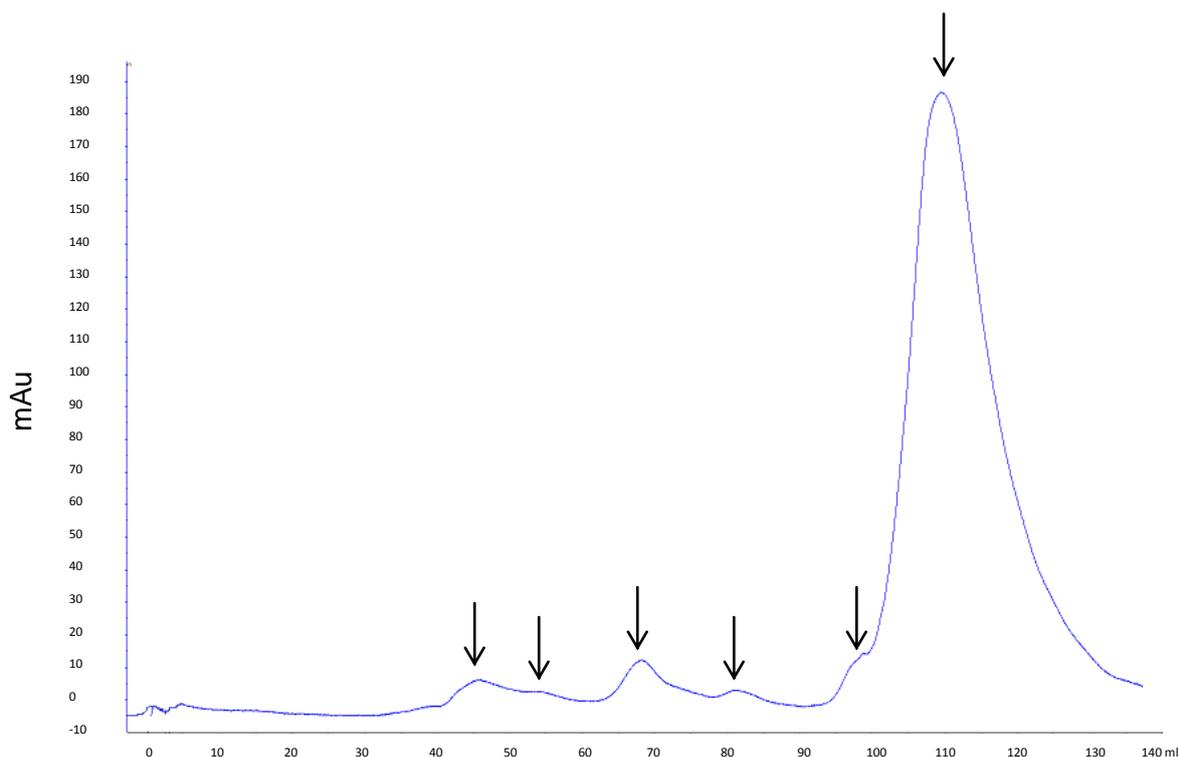
Figure 3.20: Size exclusion elution profile of HIV-1 IN-LEDGF/p75 complex and variants thereof. Arrows indicate the fractions used in subsequent analyses.

HIV-1 IN and LEDGF/p75 were allowed to incubate at room temperature and loaded onto the Superdex 200. The column was run at 0.5 µl/ml. The eluted peaks seen in Figure 3.20 were analysed using the standard curve seen in Figure 3.19, proteins eluted at sizes of 357026.0 kDa, 221368.9 kDa, 52767.5 kDa, 15973.8 kDa, 2998.2 kDa and 714.7 kDa. The peaks at 50 ml, 55 ml and 75 ml corresponding to the 357026.0 kDa , 221368.9 kDa and 52767.0 kDa peaks, respectively were run on an SDS-PAGE reducing gel to confirm the presence of both HIV-1 IN and LEDGF/p75 (Figure 3.21). Lanes 2 and 3 corresponding to the 50 ml peak contained a protein at 75 kDa and a protein at 32 kDa. Lanes 4 and 5 corresponding to the 55 ml peak similarly contained a protein at 75 kDa and 32 kDa. Lane 6 and 7 corresponding to the 75 ml peak only contained a protein at 32 kDa.

Western blot analysis, seen in Figure 22, was conducted using an anti-HIS primary antibody thereby confirming the HIV-1 IN-LEDGF/p75 complex (minimum 150 kDa), unbound IN dimers and monomers (50 kDa and 32 kDa, respectively), and degradation products (25 kDa) (Figure 3.22). The absence of unbound LEDGF/p75 implies it was all bound when generating the IN-LEDGF/p75 complex.

Results
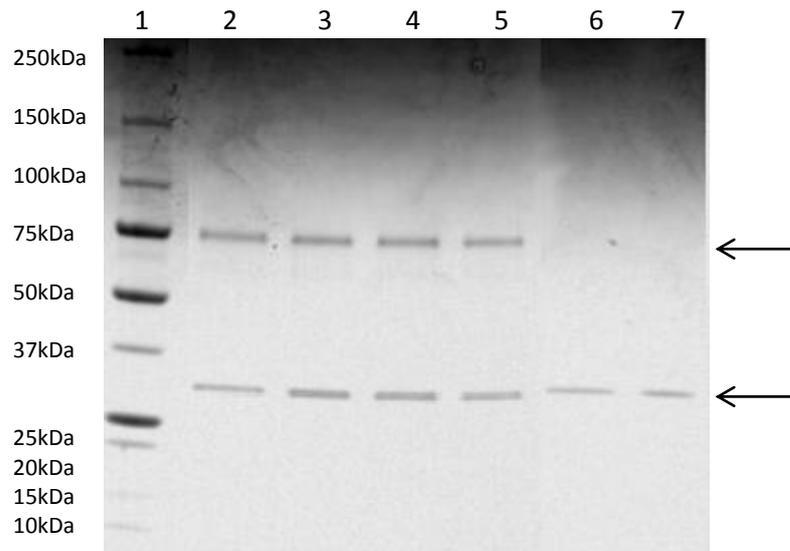
Figure 3.21: SDS PAGE reducing gel displaying peaks of possible interacting HIV-1 IN and LEDGF/p75. Lane 1, precision plus protein molecular weight marker (Bio-Rad, USA); Lanes 2 and 3, fractions from the 50ml peak, Lanes 4 and 5, fractions from the 55 ml peak and lanes 6 and 7, fractions from the peak at 75 ml. Molecular weights (in kDa) are indicated on the left. The arrows indicate LEDGF/p75 (75 kDa) and HIV-1 IN (32 kDa).
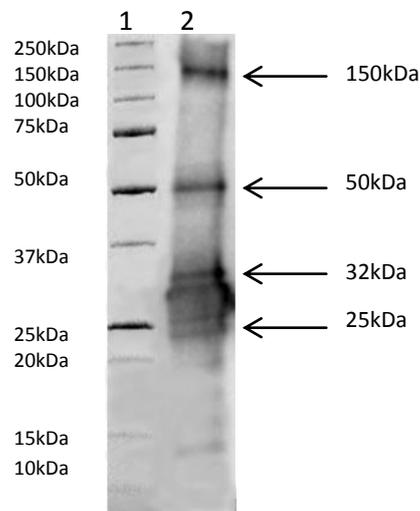
Figure 3.22: Western blot showing the interaction of HIV-1 IN-LEDGF/p75. Lane 1, Super Western C marker (Bio-Rad, USA); Lane 2, interacting proteins of LEDGF/p75 and HIV-1 IN.

The controls of LEDGF/p75 and HIV-1 IN through Western blot analysis can be seen in Figure 3.10 and Figure 3.11 respectively.

3.2.2.2 Evaluation of compound activity

The AlphaScreen assay was optimised as explained previously with DNA present in the assay. The assay was initially developed using the known HIV-1 IN-LEDGF/p75 inhibitor CX05168 in a 100 µl final volume assay on Opti-plates with subtype B HIV-1 IN. Once the assay was functional and an IC_{50} value was obtained for the control compound, the assay was scaled down to a 50 µl final volume. IC_{50} values were repeated for CX05168 to ensure the 50 µl assay was working accurately. A *p*-value of 0.83 was found showing no statistical difference between the CX05168 IC_{50} obtained in the 100 µl final volume assay in comparison with the scaled down 50 µl final assay volume.

Once the 50 µl AlphaScreen assay was optimized, all 20 compounds were tested (Table 3.8 for the NCC compound and Table 3.9 for the statins). A total of 8 test compounds (prednisolone, naloxone, budesonide, moban, amninocide, lovastatin, CX05168 and atorvastatin) showed inhibition of above 50 % at 100 µM. Screening all 20 compounds in the AlphaScreen assay using subtype C HIV-1 IN instead of subtype B HIV-1 IN showed no statistical difference.

Compounds were then run in the HIV-1 IN-LEDGF/p75 AlphaScreen without DNA to determine if the presence of DNA had any effect on the results. A statistically significant difference was seen for compounds daunorubicin and pravastatin lactone. In the case of daunorubicin, a *p*-value of 0.044 was seen when comparing the AlphaScreen results with the LEDGF/p75-IN-DNA assay. A *p*-value of 0.002 was seen in pravastatin lactone tests between HIV-1 IN-LEDGF/p75 and LEDGF/p75-IN-DNA assays. No other statistically significant differences could be seen (Tables 3.8 and 3.9). Graphic illustration of the data found in Tables 3.8 and 3.9 can be seen in Figure 3.23 and 3.24, respectively.

The LEDGF/p75-IN-DNA AlphaScreen assay was concurrently run with PogZ replacing HIV-1 IN, and confirmed that all compounds were interacting with HIV-1 IN and not LEDGF/p75 (results not shown).

Results

Table 3.8: Comparison of inhibition results obtained for 13 compounds by subtype B HIV-1 IN-LEDGF/p75 and LEDGF/p75-IN-DNA AlphaScreen assay. Subtype C HIV-1 IN was also tested in the LEDGF/p75-IN-DNA AlphaScreen assay.

Compound	LEDGF-IN		LEDGF-IN Subtype B with DNA		LEDGF-IN Subtype C with DNA	
	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
Prednisolone	46.93	2.75	49.03	0.11	44.09	1.21
Quinidine hydrochloride	30.28	4.11	31.71	5.40	33.26	2.79
Cefazolin A	33.68	7.08	37.15	2.80	31.46	2.05
Noloxone	53.51	2.29	53.67	5.69	52.35	3.00
Budisonide	53.91	3.08	58.95	9.03	51.42	1.85
Moban	51.62	0.88	53.79	1.52	52.80	5.77
Amninocide	53.63	1.72	54.76	6.78	53.02	2.02
Lovastatin	75.51	6.89	72.69	6.29	71.72	1.43
Methyl prednisolone	30.35	1.27	30.48	5.27	30.31	1.64
Daunorubicin	73.70	1.14	104.15	0.49	101.32	1.19
Betamethazone	30.81	1.57	32.97	6.97	30.75	2.06
Naltrexone hydrochloride	35.48	3.05	37.88	4.24	34.18	4.71
CX05168	91.37	6.85	85.76	2.96	88.00	1.30
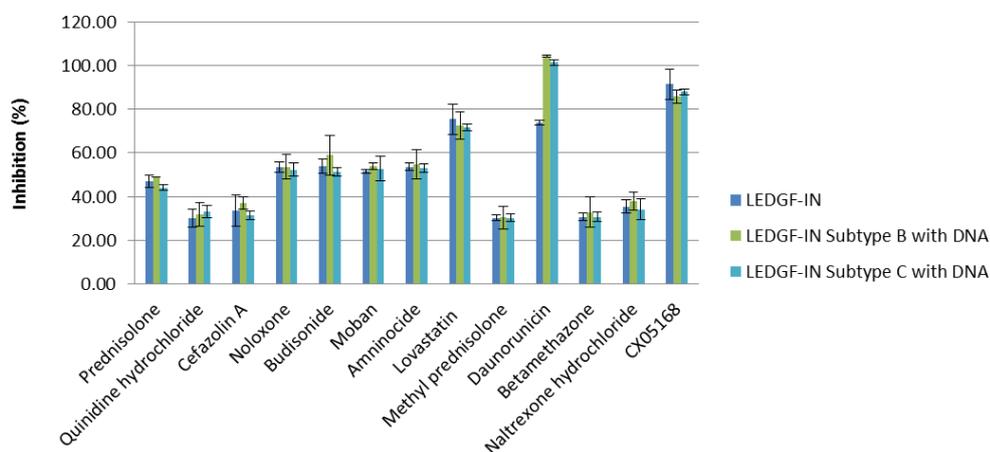
Figure 3.23: Graphic illustration of the data set in Table 3.8, showing the comparison of inhibition from the compounds between the HIV-1 IN-LEDGF/p75 interaction alone, the HIV-1 IN subtype B-LEDGF/p75 assay with DNA and the HIV-1 IN subtype C-LEDGF/p75 assay with DNA.

Figure 3.23 visually shows clear inhibition for lovastatin, daunorubicin and CX05168.

Results

Table 3.9: Comparison of inhibition results obtained for statins by subtype B HIV-1 IN-LEDGF/p75 and LEDGF/p75-IN-DNA AlphaScreen assay. Subtype C HIV-1 IN was also tested in the LEDGF/p75-IN-DNA AlphaScreen assay.

	LEDGF-IN		LEDGF-IN Subtype B with DNA		LEDGF-IN Subtype C with DNA	
Compound	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
Lovastatin	75.51	6.89	72.69	6.29	71.72	1.43
Prevastatin Lactone	25.77	5.53	48.52	1.13	48.30	0.96
Simvastatin	28.78	0.70	26.59	0.92	26.15	4.04
Pravastatin	8.55	3.22	3.21	3.70	8.07	1.59
Atorvastatin	54.26	2.03	54.76	1.70	52.38	1.95
Lovastatin Acid	42.48	1.69	34.24	3.10	41.25	1.78
Mevastatin	13.25	3.66	13.87	1.89	14.75	4.15
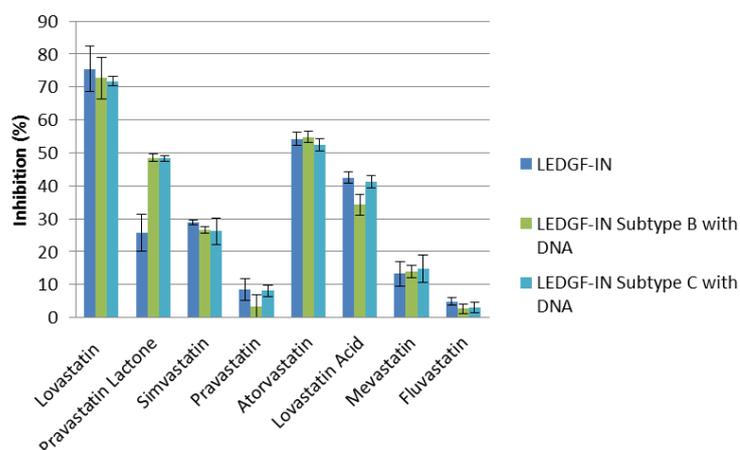
Figure 3. 24: Graphic representation of data points shown in Table 3.9, showing the inhibition results for statin compounds tested between the HIV-1 IN-LEDGF/p75 interaction alone, the HIV-1 IN subtype B- LEDGF/p75 assay with DNA and the HIV-1 IN subtype C-LEDGF/p75 assay with DNA.

Figure 3.24 illustrated the inhibition results for statins between the AlphaScreen assays. Clear inhibition can be seen for lovastatin, pravastatin lactone, atorvastatin and lovastatin acid.

Eight compounds displayed over 50 % inhibition at a single dose of 100 µM (Table 3.10), and were subjected to a dose response test to determine their IC_{50} values. The IC_{50} curves were run for a minimum of n=6 to confirm results obtained. Lovastatin had the lowest IC_{50} of 1.97 µM. Lovastatin inhibition dropped from 75.51 % down to 42.28 % when the lactone ring was opened to form lovastatin acid. Pravastatin lactone inhibition dropped from 25.77 % to 8.055 % when in open chain form. Pravastatin lactone differs from lovastatin in a hydroxyl group. Simvastatin inhibition was seen to be 28.78 % and contains an extra methyl group attached to the butyryl group found on lovastatin. Mevastatin

inhibition was 13.25 % and lacks a functional group on the naphthalene structure. A representative IC_{50} curve of the control inhibitor, CX05168 can be seen in Figure 3.26. All compounds where the IC_{50} was determined were plotted on the same graph to show differences in slopes (Figure 3.27).

Table 3.10: IC_{50} (in μM) of the eight compounds initially selected for showing above 50% inhibition, thereby preventing the HIV-1 IN-LEDGF/p75 interaction.

Compound	IC_{50} (μM)
Prednisolone	29.34 ± 0.61
Naloxone	16.53 ± 2.18
Budesonide	5.44 ± 0.57
Moban	27.80 ± 1.37
Amninocide	4.85 ± 0.76
Lovastatin	1.97 ± 0.45
CX05168	20.46 ± 0.76
Atorvastatin	10.22 ± 0.80
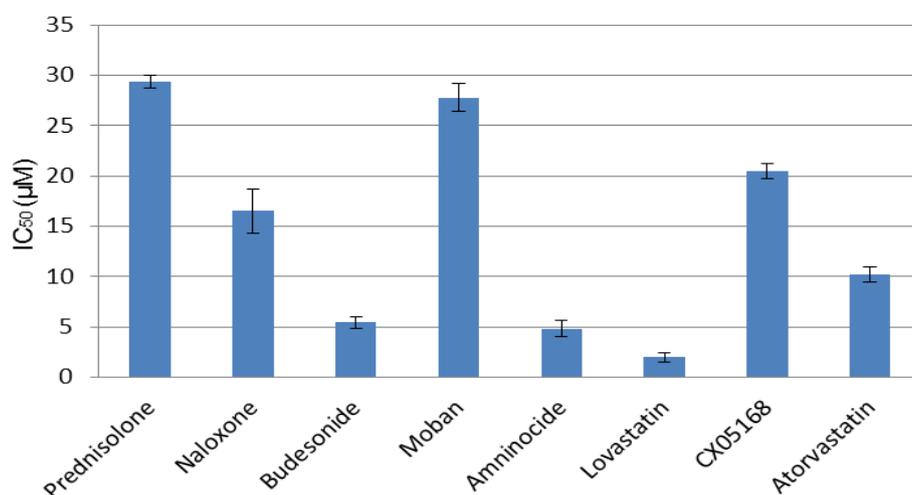
Figure 3.25: Graphic representation of data shown in Table 3.10 showing the IC_{50} values obtained in the HIV-1 IN-LEDGF/p75 AlphaScreen assay excluding DNA. The best IC_{50} values were seen for lovastatin, amninocide and budesonide.
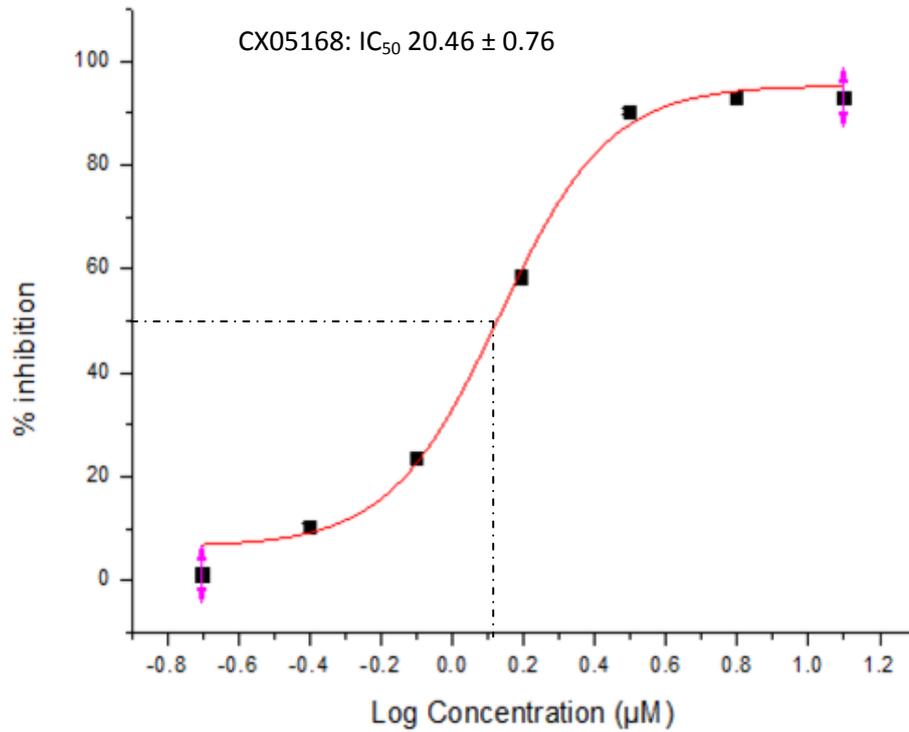
Figure 3.26: Representative IC_{50} curve of the control compound CX05168 within the LEDGF/p75-HIV 1 IN-DNA AlphaScreen assay. CX05168 was run in a dose response on the LEDGF/p75-HIV-1 IN AlphaScreen assay including DNA. The percentage inhibition was calculated for the experiment and was plotted against the log of the concentration. The IC_{50} was then calculated to be 20.46 ± 0.76 µM.
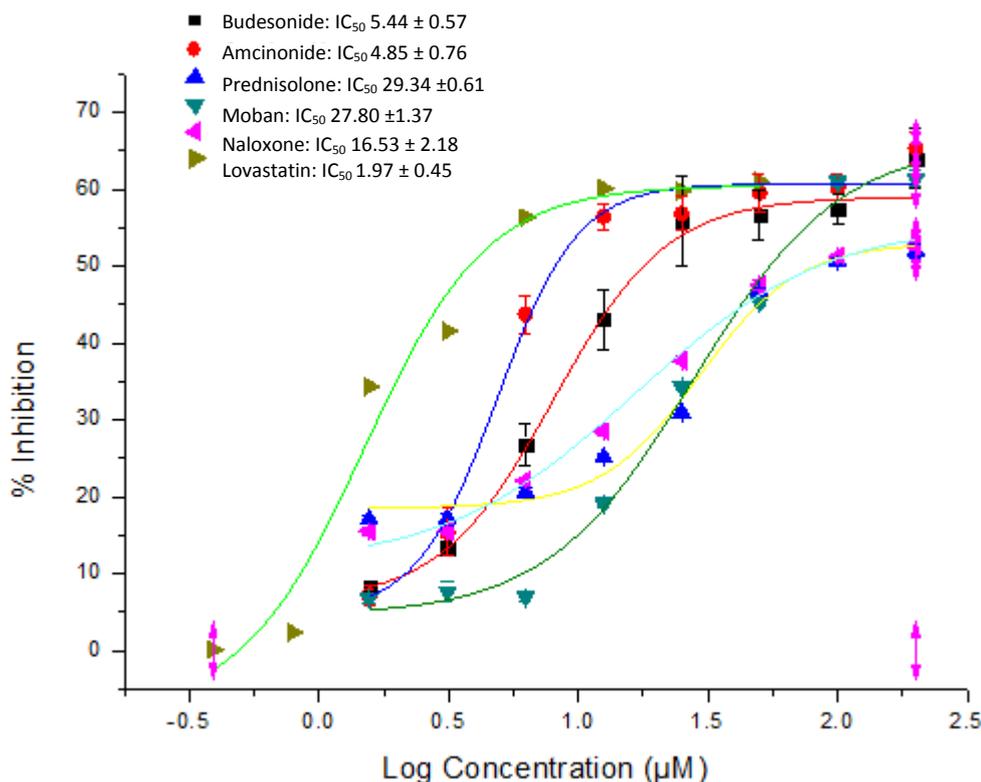
Figure 3.27: IC_{50} curves of compounds displaying inhibition in the LEDGF/p75-IN-DNA AlphaScreen assay. The curves for each compound were plotted on the same graph for comparative purposes.

Overall, because lovastatin exhibited the lowest IC_{50} of 1.97 µM, it was selected (together with structurally similar statins) for further screening. Strand transfer activity was conducted on all statins and CX05168 in the strand transfer assay (Table 3.11). Table 3.11 consists of data obtained through an ELISA based strand transfer inhibition assay, measured in percentage, and an ELISA based reverse transcriptase inhibition assay, measured in µM. The strand transfer ELISA assay was described in section 2.3.3.1.6. Minimal strand transfer inhibition was seen for lovastatin, simvastatin, lovastatin acid and mevastatin. A reduction of 18.54 % in inhibition of the LEDGF/p75-HIV-1 IN interaction was seen with pravastatin but the highest reduction was seen in CX05168 and pravastatin lactone. Enhanced activity was seen with atorvastatin. To ensure specificity, lovastatin and CX05168 were run through a reverse transcriptase assay. No inhibition was seen for either compound. The reverse transcriptase assay was a kit purchased from Roche Diagnostics and was carried out as described in section 2.3.3.1.7.

Table 3.11: The seven statins and CX05168 positive control compound tested in a strand transfer assay and reverse transcriptase assay.

Compound	[a]Strand transfer inhibition ± STD DEV (%)	[b]Reverse transcriptase inhibition ± STD DEV (µM)
CX05168	31.73 ± 4.89	0.12 ± 1.09
Lovastatin	7.82 ± 5.61	-1.85 ± 3.91
Atorvastatin	Enhanced activity	ND
Simvastatin	9.4 ± 1.2	ND
Lovastatin acid	8.6 ± 2.81	ND
Mevastatin	0.63 ± 14.12	ND
Pravastatin	18.54 ± 1.02	ND
Pravastatin lactone	31.65 ± 6.63	ND

[a]Strand transfer inhibition as calculated at single doses of 100µM represented in % with standard deviation(STD DEV) [b]Reverse transcriptase activity as calculated at single doses of 100µM represented by % with standard deviation (STD DEV), ND- not determined.

3.2.3 Solubility and permeability studies

3.2.3.1 Solubility

To determine aqueous solubility for lovastatin and CX05168, a standard curve was obtained for each compound. A representative curve for CX05168 can be seen in Figure 3.28. CX05168 was dissolved in 80:20 PBS and ACN respectively, the samples were allowed to dissolve overnight after which the samples were applied to a filter plate and filtered through by vacuum. The absorbance was read and plotted against the concentration to formulate a standard curve from which compound dissolved in PBS alone could be calculated. CX05168 was tested at 100 µM and 200 µM and found to be soluble at these concentrations. Lovastatin (tested at both 100 µM and 200 µM) was soluble at 100 µM but not 200 µM, and solubility was reduced to 125 µM.
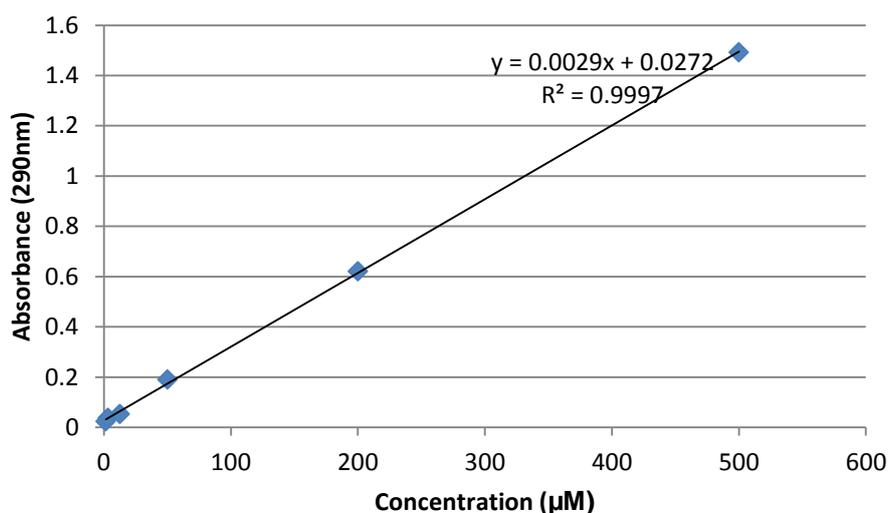
Figure 3.28: Representative standard curve for aqueous solubility of CX05168.

3.2.3.2 Permeability assay

Permeability was then tested with use of the PAMPA assay, using the standard curves obtained during the solubility testing. Lovastatin, lovastatin acid, pravastatin, pravastatin lactone and CX05168 were tested to determine permeability across a barrier, at physiological pH of 7.4. Lovastatin, pravastatin lactone and CX05168 were found to be highly permeable whereas, lovastatin acid and pravastatin were found to have low permeability.

Table 3.12: Permeability results for compounds tested in the PAMPA assay

Compound	Mean log P ± STD DEV
Lovastatin	-4.285 ± 0.075
Lovastatin acid	-5.815 ± 0.005
Pravastatin	-6.200 ± 0.040
Pravastatin lactone	-4.760 ± 0.090
CX05168	-4.105 ± 0.025

Mean log P as calculated by 100 µM single dose.

<-5.00 low permeablility,

>-5.00 high permeability.

3.2.4 Antiviral and cytotoxicity assay

All statin compounds and CX05168 were screened in MT-4 cells for cytotoxicity with auranofin and raltegravir used as controls (Table 3.13). MT-4 cells were

incubated with compound over 5 days and MTT was then added. The absorbance was read and CC_{50} were determined for each compound. All statins excluding pravastatin and pravastatin lactone were found to be highly toxic to cells, with mevastatin being the most toxic. Pravastatin was found to have no toxicity at concentrations of 100 µM and pravastatin lactone only being slightly toxic at 59.22 µM. Lovastatin and CX05168 were also tested in PBMCs but no statistical difference was seen between MT-4 cells and PBMC cytotoxicity (results not shown).

An antiviral assay was conducted with MT-4 cells (Table 3.13). The MT-4 cells were infected by spinoculation with HIV-1_{NL-43} and treated with compounds in a serially diluted dose response for CX05618, lovastatin, atorvastatin, mevastatin, pravastatin and pravastatin lactone. Notable antiviral activity was seen for all compounds, with the exception of pravastatin. A representative curve depicted in Figure 3.29 compares the antiviral activity of raltegravir, CX05168 and lovastatin.

Table 3.13: Compound table indicating antiviral activity and cytotoxicity levels in MT-4 cells.

Compound	[a]EC_{50}	Cytotoxicity (µM)
CX05168	1.78 ± 0.28	71.15 ± 2.33
Lovastatin	6.95 ± 0.21	5.31 ± 0.19
Atorvastatin	23.68 ± 0.21	13.36 ± 2.38
Simvastatin	ND	12.70 ± 0.66
Lovastatin acid	ND	6.53 ± 0.77
Mevastatin	6.70 ± 2.20	3.72 ± 0.25
Pravastatin	>100	>100
Pravastatin lactone	29.55 ± 0.56	59.22 ± 1.66

[a]EC_{50} correlating to antiviral activity in a dose response measures in µM
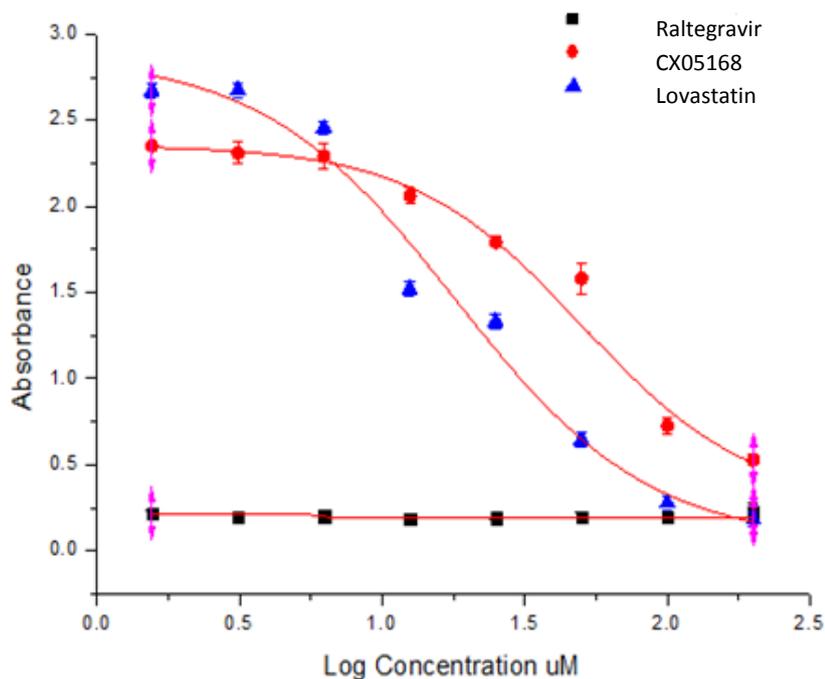
Figure 3.29: Comparative antiviral activity of raltegravir, CX05168 and lovastatin against HIV-1$_{NL4-3}$ in MT-4 cells.

The toxicity profile of lovastatin was further examined by use of the xCELLigence system to determine when HeLa cell death occurs. Cell index, refers to the number of living cells, was plotted against the time the assay was run. Cells were allowed to grow unhindered by compound for 24 hours after which compound was added in a dose response manner. Each line depicted in Figure 3.30 are indicative of a dose with dark green indicating the highest concentration and light green indicating the lowest dose. Cell death was detected as early as two hours after being treated with lovastatin (Figure 3.30).
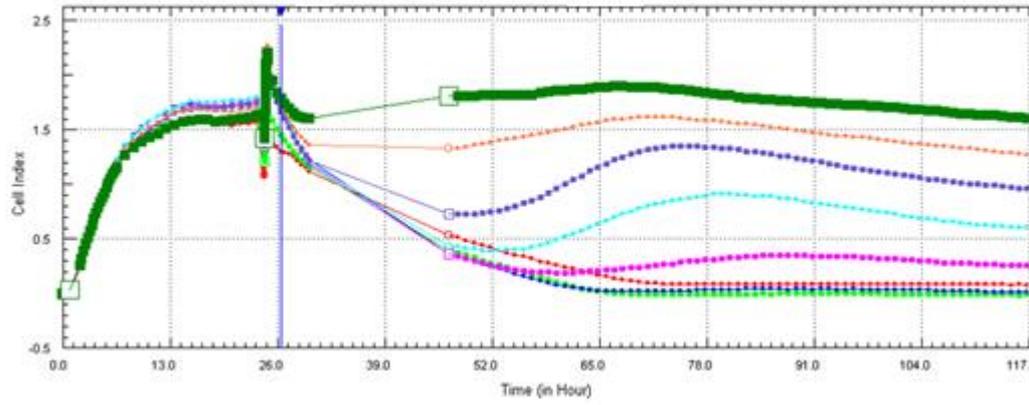
Figure 3.30: xCELLigence graph showing HeLa cell death initiated two hours after addition of lovastatin. Each line denotes concentration from 3.125 μM (dark green) to 100 μM (lime green).

Chapter 4: Discussion

Discussion

This study describes the successful identification of compounds targeting the HIV-1 IN-LEDGF/p75 interaction and further evaluation of the compounds identified. In addition, the following recombinant proteins: LEDGF/p75, HIV-1 IN subtype B and PogZ were successfully expressed, purified and modified for use in various HIV-1 IN-LEDGF/p75 assays.

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4.1 Repurposing of clinical compounds is a potential source of antiviral inhibitors

In the realm of drug discovery the main reason for drug failure is toxicity and efficacy. Safety involves favourable toxicological and pharmacokinetic profiles, whereas efficacy involves the precise protein target and biological pathway that is used for therapeutic treatment. Marketed old drugs that have already been optimised for safety and efficacy in a particular indication are a possible source of compounds that can be redirected toward new indications. This is commonly referred to as drug repurposing.

Computational methods, such as molecular modelling, have increased the amount of indications that can be investigated and thereby facilitate the identification of old drugs for new uses. An added benefit to repurposing drugs is that they have already been approved for human use, and there is no further need to conduct phase I and phase IIa clinical trials. Furthermore, the costs involved in the synthesis of the drug have already been addressed which makes the economic factors for repurposing attractive (Oprea et al., 2011; Sleigh and Barton, 2010). A disadvantage of repurposing is the level of patient compliance with reference to side effects. For example, side effects such as nausea experienced during cancer therapy is a side effected deemed acceptable to a life threatening condition, however this would not be considered acceptable to a person whose quality of life is of the utmost importance.

Several clinical compound collections are available for purchase such as the Screen-Well® FDA Approved Drug library, the John Hopkins Clinical Compound Collection, the Developmental Therapeutics Program Approved Oncology Drug Set and the National Institute of Health Clinical Collection (NCC). The NIH Clinical Collection (NCC) used in this study is a well-defined library of compounds with a history of prior use in human clinical trials, have known safety profiles and

are highly drug like. Moreover, the drugs contained within this library have not been represented in other arrayed collections ("NCC Clinical Collection,").

4.2 Molecular modelling is a useful tool in the identification of inhibitors

The Accelrys Discovery Studio 3.1 package was used for molecular modelling, and protein PDB code 2B4J was prepared using the CHARMM based force field function. The preparation included building missing loops, addition of hydrogen atoms, bond order assignment, and optimization of bond lengths; bond angels; torsion angels and non-bonded interactions. LEDGF/p75 was removed from the protein complex leaving the HIV-1 IN dimer protein complex. A ligand file was created with known HIV-1 IN-LEDGF/p75 inhibitors from Bruno Simoneau and co-workers which consisted of IC_{50}, $EC_{50,}$ CC_{50} and Kd (Simoneau et al., 2011). This file was used to test the accuracy of the model and for validation.

Once the model had been tested and found to be accurate the small molecules contained in the NIH Clinical Collection (NCC-202) were prepared utilizing a CHARMM based force field function contained in Small Molecules' Prepare and Filter Ligands function to add hydrogen atoms, assign bond orders, and optimize bond lengths, bond angles, torsion, change ionization and generate isomers and tautomer's of the ligands. Testing revealed that the top one thousand compounds was comprised of twelve compounds in different tautomer orientations and were selected for further biological screening. The twelve compounds selected for screening included prednisolone, quinidine hydrochloride, cefazolin A, naloxone, budesonide, amninocide, moban, methyl prednisolone, duanurubicin, naltrexone hydrochloride, betamethasone, and lovastatin.

Investigation of the abovementioned drug's mode of action revealed that there were five anti-inflammatory drugs, including prednisolone which is a corticosteroid (anti-inflammatory) used to control inflammatory conditions such as asthma rheumatoid arthritis and colitis; methylprednisolone, a glucocorticoid used to reduce inflammation; budesonide, a glucocorticoid steroidal anti-inflammatory used in the treatment of asthma, Crohn's disease, and non-infectious rhinitis; amninocide, a topical corticosteroid for the use in skin inflammation; and lastly, betamethasone, an anti-inflammatory ointment used for skin irritations. Two

drugs are opioid antagonists, including: naloxone, used to reverse the effects of opioid overdose, and naltrexone hydrochloride, primarily used in treatment of alcohol dependence. Quinidine hydrochloride is an antiarrhythmic heart agent and an antimalarial schizonticide. Moban is antipsychotic medication for the treatment of schizophrenia. Duanorubicin is an anthracycline that is most commonly used to treat acute leukemias. Cefazolin A is a cephalosporin antibiotic used to combat bacteria. Lastly, lovastatin is an anti-lipidaemia agent.

Because statins have been described to have anti HIV-1 activity (Amet et al., 2008; del Real, 2004; Ganesan et al., 2011; Giguère and Tremblay, 2004; Iyengar et al., 1998; Jain and Ridker, 2005; Mazière et al., 1994; Nabatov et al., 2007), the decision was made to perform further biochemical and biological testing of an additional four statins and two derivatives in addition to the above mentioned 12 compounds. The HIV-1 IN inhibitor, raltegravir (Summa et al., 2008), and LEDGF/p75 inhibitor CX05168 (Christ et al., 2010) were chosen as positive controls.

4.3 Theoretical analysis is a useful tool to predict drug likeness of selected inhibitors

Additional theoretical analysis and screening were performed using Osiris properties explorer as well as Accelrys Discovery Studio™. In addition, the Lipinski rule of five was used to assess the probability of compounds as orally bioavailable drugs. The selected 20 compounds were screened to gather information on the properties of compounds to be tested such as drug likeness and to identify any problems for future design of HIV-1 IN-LEDGF/p75 inhibitors.

Osiris property explorer takes six parameters into consideration, toxicity risks, cLogP, aqueous solubility, molecular weight, drug likeness and overall drug score. The toxicity risk assessment predictor relies on a set of structural fragments that give rise to toxicity alerts. These fragments are known to cause mutagenesis, tumourgenicity, irritancy and reproductive effects. The cLogP predictor is the logarithm of its partition coefficient between n octanol and water log and is a measure of compounds hydrophobicity. The desired hydrophobicity of a compound is below five to ensure reasonable absorption and permeation. Aqueous solubility (logS) is associated with the compound absorption ability and distribution. A high aqueous solubility is desired for systematic transportation.

Discussion

Molecular weight is always associated with efficacy of a drug, so optimization of molecular weight in drug design is always important. A high molecular weight is associated with decreased absorption and a reduction in reaching the target site. The fragment based drug likeness predictor quantifies the number of fragments within a compound. A positive value represents drug like qualities seen in traded drugs. The drug likeness score sums up all assessed parameters to judge a compounds overall potential to qualify as a drug (Lipinski, 2004; Lipinski et al., 2001; "Molecular Properties Prediction - Osiris Property Explorer,").

Findings from Osiris property explorer confirmed that all 20 compounds screened had a positive drug likeness, which was expected as most of the compounds are commercially available drugs. No compounds were predicted to be mutagenic, tumorigenic, irritants or have reproductive effects, with the exception of naloxone (predicted to be an irritant). Lipinski's rule of five predicts good oral absorption and drug bioavailability. The four rules include: not more than five H bond donors, not more than ten H bond acceptors, a molecular weight of not more than 500 g/mol and a logP of less than five. Utilizing the RO5 a score of two out of four would indicate that two parameters had not been met and poor absorption or permeability is possible. Interestingly, atorvastatin and duanorubicin had a score of two out of four, and CX05168, cefazolin A and amninocide had a three out of four score. The remainder compounds scored a perfect four out of four. From these results it was seen that 10 % of the compounds were predicted to have poor absorption and permeability.

Overall, the molecular modelling and screening allowed for the identification of 12 compounds as potential inhibitors of the HIV-1 IN-LEDGF/p75 interaction, and further theoretical screening of these compounds, four additional statins and two derivatives, raltegravir and CX05618 confirmed their drug likeness and thus warranted further biochemical and biological characterization for inhibition of HIV-1 IN-LEDGF/p75.

4.4 Effective recombinant protein production and modification for experimental use

The production of sufficient high yields and high purity recombinant protein was required for experimental use during the course of the project. Optimised

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protocols were available for recombinant subtype B HIV-1 IN production (Jenkins et al., 1996) however, optimisation was needed for the expression and purification of recombinant LEDGF/p75 and PogZ. All proteins were expressed in *E. cloni* BL21 (DE3) (pLysS) as these cells optimally express high levels of recombinant protein and are easily lysed by freeze/thaw cycles. In the *E. cloni* BL21 (DE3) cells, the T7 polymerase gene is under the control of the lacUV5 promoter which can never be fully turned off, and this results in the synthesis of some T7 polymerase (Studier and Moffatt, 1986). For this project we used pLysS instead of pLysE as it expresses low levels of the phage T7 lysozyme protein which are low enough that when the T7 polymerase gene is turned on normal expression of the target gene is observed. The lysozyme attaches to and inhibits the T7 RNA polymerase thereby inhibiting the polymerase activity (Studier and Moffatt, 1986). A concentration of 1 mM IPTG was found to be the optimum induction concentration throughout all the expression studies. Furthermore, the addition of glucose was tested to see if it aided in the prevention of protein expression prior to induction (Grossman et al., 1998), however there was no notable difference to justify the use of glucose in further expression studies.

The LEDGF/p75 expression protocol was modified from the protocol used by Vandegraaff and co-workers (Vandegraaff et al., 2006). Bacterial cells were grown until an optical density of 0.8-0.9, as compared to 0.6 recommended by most protocols. This allows bacterial cells to grow in abundance before induction, and requiring only a maximum of three hours incubation post-induction for optimal recombinant protein expression. The culture temperature was also altered from the normal 37°C down to 32°C before induction and further dropped to 28°C post induction to reduce protein degradation. This resulted in high expression of intact recombinant LEDGF/p75 protein (Miroux and Walker, 1996; Saïda et al., 2006; Vandegraaff et al., 2006). Affinity chromatography was used as the primary LEDGF/p75 purification step, followed by cation exchange chromatography to separate proteins based on their charge. Negatively charged proteins are washed through the column and positively charged proteins remain bound tightly. The negatively charged proteins are then eluted with use of a salt gradient that competes with the protein for column attachment (Marinsky et al., 1973). LEDGF/p75 has a pI of 9.15 and therefore a cation exchange column was used with buffers at pH7.6. Size exclusion chromatography was used as the final

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purification step where large proteins were eluted first and small proteins that were caught in the spaces between beads were eluted last (Lathe and Ruthven, 1956). The final column allowed for purification to levels of approximately 95% purity (Figure 3.7), and the identity of purified recombinant LEDGF/p75 (75 kDa) was confirmed by Western blot analysis (Figure 3.10).

The optimum parameters for subtype B HIV-1 IN soluble protein expression and purification have been previously optimised within our laboratory and these methodologies were successfully implemented in this study to purify subtype B HIV-1 IN (Figures 3.8 and Figure 3.11). The presence of additional bands in Figure 3.11 is a known phenomenon that occurs due to the antiserum having a degree of non-specificity (Pradidarcheep et al., 2008). In some instances, the 6XHIS tag on both Subtype B and C HIV-1 IN was cleaved to prevent HV-1 IN interacting with nickel chelating beads, since the AlphaScreen assay contained a nickel chelating bead to bind to LEDGF/p75. Thrombin cleaves the 6XHIS tag by recognising the consensus sequence Leu-Val-Pro-Arg-Gly-Ser and cleaves the peptide bond between Arg and Gly. This sequence is used by many vector systems encoding a protease cleavage site to allow the removal of an upstream domain (Jenny et al., 2003). In the case of subtype B HIV-1 IN, cleavage occurred with no aggregation of protein, since the protein was soluble. Usually, HIV-1 IN has a low solubility and tends to aggregate. Jenkins et al used site directed mutagenesis on subtype B HIV-1 IN and replaced the lysine on position 185 with phenylalanine which resulted in a highly soluble HIV-1 IN product (Jenkins et al., 1995), and this soluble subtype B HIV-1 IN was used in this study. However, the subtype C HIV-1 IN was wildtype (wt), and therefore a vast amount of aggregation occurred. For this reason, subtype B HIV-1 IN was incubated at room temperature and subtype C HIV-1 IN was incubated at 4°C to reduce the amount of aggregation. A minor change in migration can be seen in Figure 3.13 between uncleaved and cleaved HIV-1 IN for both subtype B and C, which is as expected due to the absence of the 6XHIS tag. Western blot analysis confirmed thrombin cleavage of the 6XHIS tag (Figure 3.14).

PogZ is known as a 155 kDa protein, however an over expressed protein was detected between 90-100 kDa, as well as at 155 kDa (Figure 3.9). Confirmation of the expressed protein was determined by a western blot with anti-PogZ

primary antibody, and bands were detected at 90-100 kDa (Figure 3.12). According to the anti-Pogz manufacturer's data sheet, a band at 90 kDa was expected. Thus, the overexpression of a 90-100 kDa protein could be attributed to alternative splicing variants of the same gene, resulting in expression of different sized proteins.

To this end, protocols for the successful bacterial expression, purification to sufficient yield and purity, and modifications as needed for experimental use during the course of this project were successfully established and implemented for recombinant HIV-1 IN, LEDGF/p75 and PogZ.

4.5 Biochemical and biological screening of test compounds

Once the recombinant proteins had been expressed, purified and modified for use it was important to confirm that they were conformationally intact, and functional. This was determined by monitoring the interaction between HIV-1 IN and LEDGF/p75 under physiological conditions. Size exclusion markers were used to calibrate the size exclusion column and establish a standard curve. Initially, LEDGF/p75 and HIV-1 IN were eluted separately (Figure 3.6). As LEDGF/p75 is a large molecule with an expected molecular mass of between 66-75 kDa, it was expected to elute in the first peak. A protein eluted at 66 kDa, and this fraction corresponded to LEDGF/p75, as confirmed on a non-reducing SDS PAGE gel. HIV-1 IN was eluted in peaks corresponding to 75 kDa, 50 kDa and 32 kDa, and analysed on a non-reducing SDS PAGE gel. HIV-1 IN dimerises at room temperature under non reducing conditions, explaining the bands at 75 kDa and 50 kDa as dimers (or oligomers) of HIV-1 IN (Bischerour et al., 2003).

Following establishment of the elution profiles of HIV-1 IN and LEDGF/p75, the subsequent HIV-1 IN-LEDGF/p75 interactions were visualised as a small peak at 357 kDa followed by peaks at 221 kDa, 52 kDa, 16 kDa and 3 kDa (Figure 3.20). The unique peaks found at 357 kDa and 221 kDa were attributed to an interaction between HIV-1 IN and LEDGF/p75 in different formations as these peaks are absent in the elution profiles of the single proteins. The peak found at 52 kDa, correlates with dimerised HIV-1 IN. From literature it is known that HIV-1 IN needs to dimerise prior to binding with LEDGF/p75 (Christ et al., 2010; Peat et al., 2012). It is possible the complex required a longer incubation time to form

dimers and then form a complex with LEDGF/p75 as a large peak can still be seen in dimeric form. However, longer incubation times caused degradation of proteins. Bands below 52 kDa were attributed to degradation of the proteins, possibly due to the extended room temperature incubation for four hours. Peaks pertaining to 357 kDa, 221 kDa and 53 kDa were run on a reducing SDS PAGE gel (Figure 3.21) and confirmed the 357 kDa and 221 kDa peaks contained both LEDGF/p75 and HIV-1 IN, and the 52 kDa peak contained only HIV-1 IN. Furthermore, the HIV-1 IN-LEDGF/p75 interaction was confirmed by non-reducing SDS-PAGE and western blot analysis, as a complex at 155 kDa (Figure 3.22). Although the analyses presented above confirm the IN-LEDGF/p75 interaction, size exclusion chromatograms and western blots describing these have not been published previously.

An AlphaScreen that was previously described by Christ and co-workers, was modified to incorporate DNA for use in this project (Christ et al., 2010). A definite interaction on the assay was confirmed with luminescence values in the range of 300 000 in comparison to control values of 2000-5000. At this stage the optimum concentration of LEDGF/p75 to HIV-1 IN was determined by means of a titration plate, cross titrating various concentrations from 30 nM to 1 µM LEDGF/p75 against various concentrations of HIV-1 IN of 30 nM to 1 µM. The optimum concentration ratio for IN to LEDGF/p75 was 100 nM LEDGF/p75 to 300 nM HIV-1 IN which correlated with the values obtained by Christ and co-workers. This concentration was chosen to minimise protein use with sufficient luminescence to show the interaction or the disruption thereof. The assay was then verified with the use of a known HIV-1 IN-LEDGF/p75 inhibitor CX05168. A final concentration of 100 µM CX05168 was used to show a disruption in the LEDGF/p75-HIV-1 IN-DNA interaction, with an inhibition of 85.76 %. Subsequently, a dose response curve was set up ranging from 200 µM to 0.395 µM. The IC_{50} for CX05168 was found to be 19.17 ± 1.13 µM for the 50 µl final assay volume. Despite repeated attempts, the IC_{50} for CX05168 reported in the literature (1.35 µM) was never achieved in the LEDGF/p75-HIV-1 IN-DNA AlphaScreen assay (Christ et al., 2010). The CX05168 compound was then tested in the HIV-1 IN-LEDGF/p75 AlphaScreen assay (no DNA) with ten serially diluted samples from 200 µM down to 0.195 µM, and an IC_{50} of 1.33 µM was determined, as expected. The differences in IC_{50} values noted between the two assays was attributed to recent findings showing that CX05168 is a dual inhibitor of HIV-1 IN multimerization and

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IN-LEDGF/p75 interaction (Le Rouzic et al., 2013). Thus, the prior addition of DNA in the IN-DNA-LEDGF/p75 AlphaScreen assay increasing multimerization of HIV-1 IN prior to LEDGF/p75 binding thus diminishes the inhibitory effect of CX05168.

Screening of all compounds at a single dose through the LEDGF/p75-HIV-1 IN-DNA AlphaScreen assay revealed that all compounds exhibited a degree of inhibition. However, only six compounds showed inhibition above 50 % (naloxone, budesonide, moban, amninocide, lovastatin and duanorubicin) with prednisolone falling just below at 49 % inhibition (n=7). Duanorubicin showed inhibition >100 %, and upon further inspection, it was found to be a DNA intercalator. The compounds' were further screened in the LEDGF/p75-HIV-1 IN-DNA AlphaScreen assay with subtype C HIV-1 IN, and no statistical difference could be seen between the results obtained with subtype B. In addition, the compounds were screened through the HIV-1 IN- LEDGF/p75 AlphaScreen assay (no DNA) at a single dose. Duanorubicin still showed an inhibition of 73.70 %, however, due to potential toxicity concerns it was not taken forward for further biological experimentation. Thus, only six compounds were studies further.

The IC_{50} values of prednisolone, naloxone, budesonide, moban, amninocide and lovastatin were tested in a dose response curve with ten serial dilutions from 200 µM to 0.39 µM. Prednisolone showed an IC_{50} of 29.34 µM, followed by moban at 27.80 µM, naloxone at 16.53 µM, budesonide at 5.44 µM, amninocide at 4.85 µM and finally 1.97 µM for lovastatin. Based on the low IC_{50} value obtained for lovastatin, the favourable theoretical profiles, and previously published literature on the anti HIV-1 activity of statins, it was decided to conduct further testing on lovastatin, and other statins with structurally similar components. Furthermore, lovastatin was found to specifically target the HIV-1 IN-LEDGF/p75 interaction, since no anti-RT or strand transfer activity was detected.

It should be noted that the dose response curves of lovastatin and CX05168 show differences. The slope of CX05168 reaches from 0 close to 100 % inhibition and follows a steep slope, indicative of a potent inhibitor and a full antagonist. By contrast, the slope of lovastatin is not as steep indicating a less potent inhibitor and only reaches 70-80 % inhibition, indicative of a partial antagonist. These

factors must all be considered when designing future inhibitors as it is desirable to have full antagonists and potent inhibitors (Shen et al., 2008).

4.6 Statin class of drugs act as HIV-1 IN/LEDGF/p75 inhibitors

Lovastatin was identified as the "lead" HIV-1 IN-LEDGF/p75 inhibitor following screening of the twelve NCC-202 compounds chosen by molecular modelling. Overall, a 72.55 % inhibition was seen with a single dose of lovastatin on the AlphaScreen assay with an IC_{50} of 1.97 μM. Additionally, a DNA binding assay using cisplatin (Sigma Aldrich, USA) as a control, confirmed that lovastatin was not interacting with the DNA present on the assay (methods and results not shown). This then lead to utilizing various statin compounds with slight structural differences to elucidate which components contributed to the inhibitors profile. Atorvastatin was included as it was previously identified as a HIV-1 IN-LEDGF/p75 inhibitor (Hu et al., 2012). Additional statins chosen for screening included pravastatin, mevastatin, simvastatin and two pravastatin and lovastatin derivatives, namely pravastatin lactone and lovastatin acid. Three statins showed reasonable HIV-1 IN-LEDGF/p75 inhibition; lovastatin inhibited the interaction the most with 72.69 % inhibition in subtype B and 71.72 % inhibition with subtype C. Atorvastatin followed at 54.76 % inhibition with subtype B and 52.38 % inhibition in subtype C. Pravastatin lactone showed reasonable inhibition at 48.52 % in subtype B and 48.30 % in subtype C. Other statins showed inhibition but at lower levels such as simvastatin at 26.59 % and 26.15 % inhibition with subtype B and C respectively, lovastatin acid inhibited the interaction at 34.24 % and 41.25 % with subtype B and C respectively and mevastatin inhibited at 13.87 % and 14.75 % subtype B and C respectively. Other statins tested showed negligible inhibition.

Statins structures were compared with one another to determine which structural components contributed to inhibition. Lovastatin was derivatised to lovastatin acid by the hydrolysis of the lactone ring to an open chain (Deak et al., 2002; Huang et al., 2010b; Önal and Sagirli, 2006). This hydrolysis reduced the inhibition from 71.72 % to 41.25 %. Pravastatin showed little to no inhibition at 8% but once the open chain was condensed to pravastatin lactone (Deak et al., 2002; Önal and Sagirli, 2006; Tanaka and Terahara, 1982) the inhibition increased to 48.52 %. This indicates that the presence of the lactone form aids in the inhibitory profile. Pravastatin lactone and lovastatin differ by a single functional group, in the case

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of lovastatin a methyl group is present and in pravastatin lactone the methyl group is replaced by a hydroxyl group on the naphthalene structure. Mevastatin differs from lovastatin as there is no functional group on the mevastatin as opposed to the methyl group on lovastatin. Mevastatin inhibited by only 13 %. This indicated that the methyl group in combination with the closed lactone ring is needed for inhibition. Simvastatin contains an additional methyl group on the butyrl group in comparison to lovastatin and the inhibition dropped to 23 % in simvastatin. Overall, from the comparison of inhibition and structure of statins it appears the presence of a closed lactone ring in addition to a methyl group is involved in the inhibitory activity noted. Interestingly, alterations in the butyryl group appear to have an adverse effect on inhibition.

Comparison of results from the HIV-1 IN-LEDGF/p75 AlphaScreen assay, in the presence or absence of DNA showed no statistical differences, with the exception of pravastatin lactone. Inhibition in the absence of DNA showed a 22.75 % drop in inhibition, which was statistically significant (p=0.002), which is contrary to CX05168. This may be attributed to pravastatin lactone binding to/interfering with DNA thereby reducing the amount of HIV-1 IN in contact with the streptavidin donor bead thereby reducing signal detected. However, no literature could be found detailing the interaction of pravastatin lactone with DNA. Other possible factors include pH of the compound as triflouroacetic acid was used to condense the open chain. If the pH of the compound is extremely low it would cause the experimental pH to drop thereby giving inaccurate results.

Aqueous solubility testing was performed on lovastatin, lovastatin acid, pravastatin and pravastatin lactone to determine if the structural change had an effect on the solubility of the compounds. Aqueous solubility of a compound is a concentration (mol/l) of its saturated aqueous solution which is affected by molecular weight and the amount of energy required to dissociate from crystalline structure into solution. Lovastatin was soluble at levels of ≤100 µM, whereas lovastatin acid was soluble up to levels of 200 µM. Pravastatin and pravastatin lactone were soluble to levels of 200 µM. In this instance, solubility appears unaffected by structural changes.

The ability of compounds to permeate a membrane was tested for lovastatin, CX05168, pravastatin lactone, pravastatin, and lovastatin acid. Lovastatin,

CX05168 and pravastatin lactone were highly permeable, whereas pravastatin and lovastatin acid had low permeability with little compound crossing the membrane. Results of the solubility and permeability for statins reported here correlate with previous studies (Serajuddin et al., 1991), and confirm the hypothesis that the more hydrophilic a compound the less permeable a compound is.

4.7 Antiviral activity of statins overlaps with cytotoxicity

All statins were screened in a cell cytotoxicity assay as well as an antiviral assay. The EC_{50} for lovastatin was 6.95 ± 0.21 µM, with a CC_{50} of 5.31 µM in MT-4 cells and a CC_{50} of 6.50 ± 0.31 µM in PBMCs. Further testing of the cytotoxic effect of lovastatin on HeLa cells in the xCELLigence system showed cell death was initiated as early as two hours post lovastatin treatment. Thus, it was impossible to establish whether the compound exhibited any antiviral activity in MT-4 cells as a direct result of interfering with the HIV-1 IN-LEDGF/p75 interaction, or wholly due to cytotoxicity.

Lovastatin is a well-known anti-cholesterol drug marketed and used on a daily basis. This raises the question of how can lovastatin be cytotoxic, yet still be used on a daily basis. Lovastatin is a prodrug, and, when ingested the drug undergoes a structural change in the stomach acid to form the active drug which is then absorbed (Wu and Farrelly, 2007) the closed lactone ring opens to the non toxic open chain when ingested and is therefore not toxic to the body. As described earlier, we synthesized lovastatin hydroxyl acid which is the active drug form. However, lovastatin acid was also highly toxic, with a CC_{50} of 6.53 ± 0.77 µM. This was possibly attributed to the open hydroxy acid chain reverting back to the closed formed lactone ring of lovastatin during the five days the compound was incubated with cells at physiological pH of 7.4. Similarly, mevastatin activity was attributed to cytotoxic effects, since it had an antiviral EC_{50} of 6.70 ± 2.20 µM but had a CC_{50} of 3.72 ± 0.25 µM. Simvastatin and lovastatin acid were not tested in the antiviral assay as the high toxicity of the compounds excluded the possibility of an accurate antiviral EC_{50}.

Pravastatin was tested in both antiviral assays and cytotoxicity assay, but showed no toxicity at levels of 100 µM, nor antiviral effects at the same levels. As

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described earlier pravastatin was also condensed into the lactone form, and exhibited a CC_{50} of 59.22 ± 1.66 µM, and an EC_{50} of 29.55 ± 0.56 µM. An SI value of two implies the activity could not be completely attributed to cytotoxicity of the compound. SI is known as the therapeutic index and is a ratio of toxicity to effective dose of a compound, and the higher the SI the better this is for therapeutic use. An SI of two is found in some drugs such as digoxin which is a cardiac glucoside (Becker, 2007). However a SI of two is undesirable and would need constant monitoring to ensure correct circulating levels. Atorvastatin was also tested and was found to have a CC_{50} of 53.49 ± 1.03 µM and had an EC_{50} of 23.68 ± 0.21 µM. This gave us an SI of 2.26. With the SI taken into consideration and the lack of ability to test other statins in the structural formation needed for antiviral activity it was determined that future studies on statins for antiviral activity was not conducible to effective results.

Statin structures were further compared to try and decipher which structural components added to the cytotoxicity of the compound. The most toxic statin was mevastatin whose structure differs from that of lovastatin by the lack of a methyl group on the naphthalene structure. It was found to have a CC_{50} of 3.72 ± 0.25 µM; lovastatin was found to be the next most toxic with a CC_{50} value of 5.31 ± 0.19 µM. The addition of a methyl group on the naphthalene ring appears to have decreased the toxicity; however the effect is minimal. Simvastatin (CC_{50} of 12.70 ± 0.66 µM) is identical to lovastatin with an additional methyl group on the butryl chain. Pravastatin lactone differs from lovastatin in the replacement of the methyl group on the naphthalene ring by a hydroxyl group and toxicity was seen at levels of 59.22 ± 1.66 µM, further decreasing the toxicity. Lovastatin acid was found to be toxic at levels of 6.95 ± 0.21 µM but we could not guarantee the hydroxyl acid form as we hypothesised that the lactone ring was reforming during the five day incubation. Pravastatin (no toxicity at 100 µM) differs from lovastatin by the lactone ring, which is open in the hydroxyl acid form and the methyl group on the naphthalene structure which is replaced with a hydroxyl group. From these findings it was determined that the open hydroxyl acid form decreased the toxicity of a compound. Furthermore, the addition of a methyl group on the naphthalene reduced toxicity which was further reduced by the replacement of the methyl group with a hydroxyl group. The addition of a methyl group on the butyryl chain appeared to improve tolerance of the drug.

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Overall, structural attributes of statins were investigated for both toxicity and inhibitory effects. The closed lactone ring increased the inhibition of a compound with the need for a functional group on the naphthalene structure. Weighing both toxicity and inhibitory effect *in vitro* we determined that the presence of a closed lactone ring is needed with a functional group on the naphthalene ring. However, combining all the results, we found that the hydroxyl group on the naphthalene ring made the compound too hydrophilic and prevented it from staying bound in the LEDGF/p75 binding pocket.

From the molecular modelling, the LEDGF/p75 binding pocket was found to be mostly hydrophobic and therefore hydrophilic compounds would be unable to remain in the binding pocket. Compounds that are hydrophobic are expected to bind in the pocket for longer. However, if the lipophilicity of a compound is too high, it cannot remain in the pocket, due to the lipophilic compound repelling water, thereby preventing it from forming a protective barrier over the compound-LEDGF/p75 binding site complex. A balance between lipophilicity and hyrdophilicity needs to be struck to allow the binding of the compound in the cleft and the formation of a water barrier to keep the complex in place. This phenomenon can partially explain the binding of statins such as lovastatin, pravastatin lactone, and atorvastatin, and the lack of binding of the highly lipophilic pravastatin.

4.8. Conclusions and future work

In summary, twelve potential HIV-1 IN-LEDGF/p75 inhibitors were identified by molecular modelling, of which six significantly interfered with the HIV-1 IN-LEDGF/p75 interaction. The subsequent work focused on a class of compounds known as statins. Future work should investigate the five remaining NCC-202 compounds identified as potential HIV-1 IN-LEDGF/p75 inhibitors.

Statins were found to be HIV-1 IN-LEDGF/p75 inhibitors and the study of structurally similar statins enabled us to identify structural components contributing to inhibition activity and cytotoxicity. A closed lactone ring in addition to a methyl group had a favourable effect on inhibition; however the lactone ring appeared to infer toxicity. A delicate balance between the lipophilicity of a compound and hydrophilicity is needed for future design and/or optimization of

effective HIV-1 IN-LEDGF/p75 inhibitors. Statins were found to be highly toxic in their prodrug form which appears necessary for inhibition of the HIV-1 IN-LEDGF/p75 interaction. The toxicity of the compounds made accurate antiviral levels unattainable with the exception of pravastatin lactone and atorvastatin.

Based on the results of this study, the statin class of drugs appears to have no or little value as antiretroviral drugs, primarily due to toxicity concerns. Future work on the design of novel compounds targeting the HIV-1 IN-LEDGF/p75 interaction must consider the structural insights gained from this study.

Chapter 5: References

References

Abbink, T.E.M., Berkhout, B., 2008. HIV-1 reverse transcription initiation: A potential target for novel antivirals? Virus Res. 134, 4–18. doi:10.1016/j.virusres.2007.12.009

Adessi, C., Soto, C., 2002. Converting a peptide into a drug: strategies to improve stability and bioavailability. Curr. Med. Chem. 9, 963–978.

Al-Mawsawi, L.Q., Christ, F., Dayam, R., Debyser, Z., Neamati, N., 2008. Inhibitory profile of a LEDGF/p75 peptide against HIV-1 integrase: Insight into integrase–DNA complex formation and catalysis. FEBS Lett. 582, 1425–1430. doi:10.1016/j.febslet.2008.02.076

Alarcón, J., Aguila, S., Arancibia-Avila, P., Fuentes, O., Zamorano-Ponce, E., Hernández, M., 2003. Production and purification of statins from Pleurotus ostreatus (Basidiomycetes) strains. Z. Für Naturforschung C J. Biosci. 58, 62–64.

Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., Springer, J., 1980. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proc. Natl. Acad. Sci. 77, 3957–3961.

AlphaScreen® for Protein-Protein Interaction Assays | PerkinElmer [WWW Document], n.d. PerkinElmer Inc. URL http://www.perkinelmer.com/Catalog/Category/ID/AlphaScreen Assays and Reagents (accessed 9.16.13).

Amemiya, F., Maekawa, S., Itakura, Y., Kanayama, A., Matsui, A., Takano, S., Yamaguchi, T., Itakura, J., Kitamura, T., Inoue, T., Sakamoto, M., Yamauchi, K., Okada, S., Yamashita, A., Sakamoto, N., Itoh, M., Enomoto, N., 2008. Targeting lipid metabolism in the treatment of hepatitis C virus infection. J. Infect. Dis. 197, 361–370. doi:10.1086/525287

Amet, T., Nonaka, M., Dewan, M.Z., Saitoh, Y., Qi, X., Ichinose, S., Yamamoto, N., Yamaoka, S., 2008. Statin-induced inhibition of HIV-1 release from latently infected U1 cells reveals a critical role for protein prenylation in HIV-1 replication. Microbes Infect. 10, 471–480. doi:10.1016/j.micinf.2008.01.009

References

Arts, E.J., Hazuda, D.J., 2012. HIV-1 Antiretroviral Drug Therapy. Cold Spring Harb. Perspect. Med. 2. doi:10.1101/cshperspect.a007161

Avdeef, A., Strafford, M., Block, E., Balogh, M.P., Chambliss, W., Khan, I., 2001. Drug absorption in vitro model: filter-immobilized artificial membranes. 2. Studies of the permeability properties of lactones in Piper methysticum Forst. Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci. 14, 271–280.

Bally, F., Martinez, R., Peters, S., Sudre, P., Telenti, A., 2000. Polymorphism of HIV type 1 gag p7/p1 and p1/p6 cleavage sites: clinical significance and implications for resistance to protease inhibitors. AIDS Res. Hum. Retroviruses 16, 1209–1213. doi:10.1089/08892220050116970

Barré-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, W., Montagnier, L., 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220, 868–871.

Bartholomeeusen, K., Christ, F., Hendrix, J., Rain, J.-C., Emiliani, S., Benarous, R., Debyser, Z., Gijsbers, R., De Rijck, J., 2008. Lens Epithelium-derived Growth Factor/p75 Interacts with the Transposase-derived DDE Domain of PogZ. J. Biol. Chem. 284, 11467–11477. doi:10.1074/jbc.M807781200

Bartholomeeusen, K., De Rijck, J., Busschots, K., Desender, L., Gijsbers, R., Emiliani, S., Benarous, R., Debyser, Z., Christ, F., 2007. Differential Interaction of HIV-1 Integrase and JPO2 with the C Terminus of LEDGF/p75. J. Mol. Biol. 372, 407–421. doi:10.1016/j.jmb.2007.06.090

Beaudet, L., Rodriguez-Suarez, R., Venne, M.-H., Caron, M., Bédard, J., Brechler, V., Parent, S., Bielefeld-Sévigny, M., 2008. AlphaLISA immunoassays: the no-wash alternative to ELISAs for research and drug discovery. Nat. Methods 5.

Becker, D.E., 2007. Drug therapy in dental practice: general principles. Part 2 - pharmacodynamic considerations. Anesth. Prog. 54, 19–23; quiz 24–25. doi:10.2344/0003-3006(2007)54[19:DTIDPG]2.0.CO;2

Bernstein, H.B., Tucker, S.P., Kar, S.R., McPherson, S.A., McPherson, D.T., Dubay, J.W., Lebowitz, J., Compans, R.W., Hunter, E., 1995. Oligomerization of the hydrophobic heptad repeat of gp41. J. Virol. 69, 2745–2750.

References

Berthoux, L., Sebastian, S., Muesing, M.A., Luban, J., 2007. The role of lysine 186 in HIV-1 integrase multimerization. Virology 364, 227–236. doi:10.1016/j.virol.2007.02.029

Bischerour, J., Leh, H., Deprez, E., Brochon, J.-C., Mouscadet, J.-F., 2003. Disulfide-Linked Integrase Oligomers Involving C280 Residues Are Formed In Vitro and In Vivo but Are Not Essential for Human Immunodeficiency Virus Replication. J. Virol. 77, 135–141. doi:10.1128/JVI.77.1.135-141.2003

Bobkov, A.F., Kazennova, E.V., Selimova, L.M., Khanina, T.A., Ryabov, G.S., Bobkova, M.R., Sukhanova, A.L., Kravchenko, A.V., Ladnaya, N.N., Weber, J.N., Pokrovsky, V.V., 2004. Temporal trends in the HIV-1 epidemic in Russia: predominance of subtype A. J. Med. Virol. 74, 191–196. doi:10.1002/jmv.20177

Botbol, Y., Raghavendra, N.K., Rahman, S., Engelman, A., Lavigne, M., 2008. Chromatinized templates reveal the requirement for the LEDGF/p75 PWWP domain during HIV-1 integration in vitro. Nucleic Acids Res. 36, 1237–1246. doi:10.1093/nar/gkm1127

Bouyac-Bertoia, M., Dvorin, J.D., Fouchier, R.A., Jenkins, Y., Meyer, B.E., Wu, L.I., Emerman, M., Malim, M.H., 2001. HIV-1 infection requires a functional integrase NLS. Mol. Cell 7, 1025–1035.

Brown, P., 1997. Integration, in: Coffin, J.M., Hughes, S.H., Varmus, H.E. (Eds.), Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).

Brown, P.O., Bowerman, B., Varmus, H.E., Bishop, J.M., 1987. Correct integration of retroviral DNA in vitro. Cell 49, 347–356.

Bukrinsky, M.I., Sharova, N., Dempsey, M.P., Stanwick, T.L., Bukrinskaya, A.G., Haggerty, S., Stevenson, M., 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc. Natl. Acad. Sci. 89, 6580–6584.

Bukrinsky, M.I., Sharova, N., McDonald, T.L., Pushkarskaya, T., Tarpley, W.G., Stevenson, M., 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. Proc. Natl. Acad. Sci. 90, 6125–6129.

References

Bushman, F.D., Fujiwara, T., Craigie, R., 1990. Retroviral DNA integration directed by HIV integration protein in vitro. Science 249, 1555–1558.

Busschots, K., 2004. The Interaction of LEDGF/p75 with Integrase Is Lentivirus-specific and Promotes DNA Binding. J. Biol. Chem. 280, 17841–17847. doi:10.1074/jbc.M411681200

Busschots, K., Voet, A., De Maeyer, M., Rain, J.-C., Emiliani, S., Benarous, R., Desender, L., Debyser, Z., Christ, F., 2007. Identification of the LEDGF/p75 Binding Site in HIV-1 Integrase. J. Mol. Biol. 365, 1480–1492. doi:10.1016/j.jmb.2006.10.094

Capon, D.J., Ward, R.H., 1991. The CD4-gp120 interaction and AIDS pathogenesis. Annu. Rev. Immunol. 9, 649–678. doi:10.1146/annurev.iy.09.040191.003245

Chan, D.C., Fass, D., Berger, J.M., Kim, P.S., 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell 89, 263–273.

Chandler, D., 2005. Interfaces and the driving force of hydrophobic assembly. Nature 437, 640–647. doi:10.1038/nature04162

Charton, M., Charton, B.I., 1982. The structural dependence of amino acid hydrophobicity parameters. J. Theor. Biol. 99, 629–644.

Chaudhari, B., Patel, N., Shah, P., 2007. Determination of simvastatin, pravastatin sodium and rosuvastatin calcium in tablet dosage forms by HPTLC. Indian J. Pharm. Sci. 69, 130. doi:10.4103/0250-474X.32127

Chen, Z., Luckay, A., Sodora, D.L., Telfer, P., Reed, P., Gettie, A., Kanu, J.M., Sadek, R.F., Yee, J., Ho, D.D., Zhang, L., Marx, P.A., 1997. Human immunodeficiency virus type 2 (HIV-2) seroprevalence and characterization of a distinct HIV-2 genetic subtype from the natural range of simian immunodeficiency virus-infected sooty mangabeys. J. Virol. 71, 3953–3960.

Cherepanov, P., 2002. HIV-1 Integrase Forms Stable Tetramers and Associates with LEDGF/p75 Protein in Human Cells. J. Biol. Chem. 278, 372–381. doi:10.1074/jbc.M209278200

Cherepanov, P., Ambrosio, A.L., Rahman, S., Ellenberger, T., Engelman, A., 2005a. Structural basis for the recognition between HIV-1 integrase and transcriptional coactivator p75. Proc. Natl. Acad. Sci. U. S. A. 102, 17308–17313.

References

Cherepanov, P., Devroe, E., Silver, P.A., Engelman, A., 2004. Identification of an evolutionarily conserved domain in human lens epithelium-derived growth factor/transcriptional co-activator p75 (LEDGF/p75) that binds HIV-1 integrase. J. Biol. Chem. 279, 48883–48892. doi:10.1074/jbc.M406307200

Cherepanov, P., Sun, Z.-Y.J., Rahman, S., Maertens, G., Wagner, G., Engelman, A., 2005b. Solution structure of the HIV-1 integrase-binding domain in LEDGF/p75. Nat. Struct. Mol. Biol. 12, 526–532. doi:10.1038/nsmb937

Chiu, I.-M., Yaniv, A., Dahlberg, J.E., Gazit, A., Skuntz, S.F., Tronick, S.R., Aaronson, S.A., 1985. Nucleotide sequence evidence for relationship of AIDS retrovirus to lentiviruses. Nature 317, 366–368. doi:10.1038/317366a0

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., Sodroski, J., 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 85, 1135–1148.

Christ, F., Shaw, S., Demeulemeester, J., Desimmie, B.A., Marchand, A., Butler, S., Smets, W., Chaltin, P., Westby, M., Debyser, Z., Pickford, C., 2012. Small-Molecule Inhibitors of the LEDGF/p75 Binding Site of Integrase Block HIV Replication and Modulate Integrase Multimerization. Antimicrob. Agents Chemother. 56, 4365–4374. doi:10.1128/AAC.00717-12

Christ, F., Voet, A., Marchand, A., Nicolet, S., Desimmie, B.A., Marchand, D., Bardiot, D., Van der Veken, N.J., Van Remoortel, B., Strelkov, S.V., De Maeyer, M., Chaltin, P., Debyser, Z., 2010. Rational design of small-molecule inhibitors of the LEDGF/p75-integrase interaction and HIV replication. Nat. Chem. Biol. 6, 442–448. doi:10.1038/nchembio.370

Christians, U., Jacobsen, W., Floren, L.C., 1998. Metabolism and drug interactions of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in transplant patients: are the statins mechanistically similar? Pharmacol. Ther. 80, 1–34.

Ciuffi, A., Bushman, F., 2006. Retroviral DNA integration: HIV and the role of LEDGF/p75. Trends Genet. 22, 388–395. doi:10.1016/j.tig.2006.05.006

Coffin, J.M., 1986. Genetic variation in AIDS viruses. Cell 46, 1–4.

References

Coffin, J.M., Hughes, S.H., Varmus, H.E. (Eds.), 1997. Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).

Craigie, R., 2001. HIV Integrase, a Brief Overview from Chemistry to Therapeutics. J. Biol. Chem. 276, 23213–23216. doi:10.1074/jbc.R100027200

Craigie, R., Fujiwara, T., Bushman, F., 1990. The IN protein of Moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration in vitro. Cell 62, 829–837.

Das, K., Arnold, E., 2013. HIV-1 reverse transcriptase and antiviral drug resistance. Part 1. Curr. Opin. Virol. 3, 111–118. doi:10.1016/j.coviro.2013.03.012

De Luca, L., Ferro, S., Gitto, R., Barreca, M.L., Agnello, S., Christ, F., Debyser, Z., Chimirri, A., 2010. Small molecules targeting the interaction between HIV-1 integrase and LEDGF/p75 cofactor. Bioorg. Med. Chem. 18, 7515–7521. doi:10.1016/j.bmc.2010.08.051

De Martino, I., Visone, R., Fedele, M., Petrocca, F., Palmieri, D., Hoyos, J.M., Forzati, F., Croce, C.M., Fusco, A., 2009. Regulation of microRNA expression by HMGA1 proteins. Oncogene 28, 1432–1442. doi:10.1038/onc.2008.495

De Rijck, J., Bartholomeeusen, K., Ceulemans, H., Debyser, Z., Gijsbers, R., 2010. High-resolution profiling of the LEDGF/p75 chromatin interaction in the ENCODE region. Nucleic Acids Res. 38, 6135–6147. doi:10.1093/nar/gkq410

Deak, L., Forgacs, I., Keri, V., Nagyne, A.E., Szabo, C., 2002. Pravastatin sodium substantially free of pravastatin lactone and epi-pravastatin, and compositions containing same. WO2002030415 A1.

Del Real, G., 2004. Statins Inhibit HIV-1 Infection by Down-regulating Rho Activity. J. Exp. Med. 200, 541–547. doi:10.1084/jem.20040061

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R., Landau, N.R., 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature 381, 661–666. doi:10.1038/381661a0

Desimmie, B.A., Humbert, M., Lescrinier, E., Hendrix, J., Vets, S., Gijsbers, R., Ruprecht, R.M., Dietrich, U., Debyser, Z., Christ, F., 2012. Phage Display-

References

directed Discovery of LEDGF/p75 Binding Cyclic Peptide Inhibitors of HIV Replication. Mol. Ther. 20, 2064–2075. doi:10.1038/mt.2012.132

Devroe, E., Engelman, A., Silver, P.A., 2003. Intracellular transport of human immunodeficiency virus type 1 integrase. J. Cell Sci. 116, 4401–4408. doi:10.1242/jcs.00747

Dietz, F., Franken, S., Yoshida, K., Nakamura, H., Kappler, J., Gieselmann, V., 2002. The family of hepatoma-derived growth factor proteins: characterization of a new member HRP-4 and classification of its subfamilies. Biochem. J. 366, 491–500. doi:10.1042/BJ20011811

Dill, K.A., 1990. Dominant forces in protein folding. Biochemistry (Mosc.) 29, 7133–7155.

Doranz, B.J., Rucker, J., Yi, Y., Smyth, R.J., Samson, M., Peiper, S.C., Parmentier, M., Collman, R.G., Doms, R.W., 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell 85, 1149–1158.

Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P., Paxton, W.A., 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 381, 667–673. doi:10.1038/381667a0

Du, L., Zhao, Y., Chen, J., Yang, L., Zheng, Y., Tang, Y., Shen, X., Jiang, H., 2008. D77, one benzoic acid derivative, functions as a novel anti-HIV-1 inhibitor targeting the interaction between integrase and cellular LEDGF/p75. Biochem. Biophys. Res. Commun. 375, 139–144. doi:10.1016/j.bbrc.2008.07.139

E47 Committee, 2008. Test Method for Measurements of Aqueous Solubility. ASTM International.

Eckert, D.M., Kim, P.S., 2001. Mechanisms of viral membrane fusion and its inhibition. Annu. Rev. Biochem. 70, 777–810. doi:10.1146/annurev.biochem.70.1.777

Engelman, A., Mizuuchi, K., Craigie, R., 1991. HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. Cell 67, 1211–1221.

Esposito, D., Craigie, R., 1998. Sequence specificity of viral end DNA binding by HIV-1 integrase reveals critical regions for protein-DNA interaction. EMBO J. 17, 5832–5843. doi:10.1093/emboj/17.19.5832

References

Esposito, D., Craigie, R., 1999. HIV integrase structure and function. Adv. Virus Res. 52, 319–333.

Fan, X., Zhang, F.-H., Al-Safi, R.I., Zeng, L.-F., Shabaik, Y., Debnath, B., Sanchez, T.W., Odde, S., Neamati, N., Long, Y.-Q., 2011. Design of HIV-1 integrase inhibitors targeting the catalytic domain as well as its interaction with LEDGF/p75: a scaffold hopping approach using salicylate and catechol groups. Bioorg. Med. Chem. 19, 4935–4952. doi:10.1016/j.bmc.2011.06.058

Farnet, C.M., Bushman, F.D., 1997. HIV-1 cDNA integration: Requirement of HMG I(Y) protein for function of preintegration complexes in vitro. Cell 88, 483–492.

Fatma, N., Kubo, E., Chylack, L.T., Jr, Shinohara, T., Akagi, Y., Singh, D.P., 2004. LEDGF regulation of alcohol and aldehyde dehydrogenases in lens epithelial cells: stimulation of retinoic acid production and protection from ethanol toxicity. Am. J. Physiol. Cell Physiol. 287, C508–516. doi:10.1152/ajpcell.00076.2004

Fatma, N., Singh, D.P., Shinohara, T., Chylack, L.T., Jr, 2001. Transcriptional regulation of the antioxidant protein 2 gene, a thiol-specific antioxidant, by lens epithelium-derived growth factor to protect cells from oxidative stress. J. Biol. Chem. 276, 48899–48907. doi:10.1074/jbc.M100733200

Fauquet, C.M., Fargette, D., 2005. International Committee on Taxonomy of Viruses and the 3,142 unassigned species. Virol J 2, 64.

FDA notifications. Accelerated approval for raltegravir tablets, 2007. . AIDS Alert 22, 143–144.

Feng, L., Sharma, A., Slaughter, A., Jena, N., Koh, Y., Shkriabai, N., Larue, R.C., Patel, P.A., Mitsuya, H., Kessl, J.J., Engelman, A., Fuchs, J.R., Kvaratskhelia, M., 2013. The A128T Resistance Mutation Reveals Aberrant Protein Multimerization as the Primary Mechanism of Action of Allosteric HIV-1 Integrase Inhibitors. J. Biol. Chem. 288, 15813–15820. doi:10.1074/jbc.M112.443390

Fenwick, C., Bethell, R., Cordingley, M., Edwards, P., Quinson, A., Robinson, P., Simoneau, B., Yoakim, C., 2011. BI 224436, a Non-Catalytic Site Integrase Inhibitor, is a potent inhibitor of the replication of treatment-naïve and raltegravir-resistant clinical isolates of HIV-1.

References

Fisher, A.G., Ensoli, B., Ivanoff, L., Chamberlain, M., Petteway, S., Ratner, L., Gallo, R.C., Wong-Staal, F., 1987. The sor gene of HIV-1 is required for efficient virus transmission in vitro. Science 237, 888–893.

Foley, B., Apetrei, C., Mizrach, I., Rambaut, A., Kober, B., 2013. HIV Sequence Compedium.

Fouchier, R.A., Malim, M.H., 1999. Nuclear import of human immunodeficiency virus type-1 preintegration complexes. Adv. Virus Res. 52, 275–299.

Gallay, P., Hope, T., Chin, D., Trono, D., 1997. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc. Natl. Acad. Sci. U. S. A. 94, 9825–9830.

Gallo, R.C., Salahuddin, S.Z., Popovic, M., Shearer, G.M., Kaplan, M., Haynes, B.F., Palker, T.J., Redfield, R., Oleske, J., Safai, B., Et, A., 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224, 500–503. doi:10.1126/science.6200936

Ganesan, A., Crum-Cianflone, N., Higgins, J., Qin, J., Rehm, C., Metcalf, J., Brandt, C., Vita, J., Decker, C.F., Sklar, P., Bavaro, M., Tasker, S., Follmann, D., Maldarelli, F., 2011. High Dose Atorvastatin Decreases Cellular Markers of Immune Activation Without Affecting HIV-1 RNA Levels: Results of a Double-blind Randomized Placebo Controlled Clinical Trial. J. Infect. Dis. 203, 756–764. doi:10.1093/infdis/jiq115

Gao, F., Bailes, E., Robertson, D.L., Chen, Y., Rodenburg, C.M., Michael, S.F., Cummins, L.B., Arthur, L.O., Peeters, M., Shaw, G.M., Sharp, P.M., Hahn, B.H., 1999. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature 397, 436–441. doi:10.1038/17130

Ge, H., Si, Y., Roeder, R.G., 1998. Isolation of cDNAs encoding novel transcription coactivators p52 and p75 reveals an alternate regulatory mechanism of transcriptional activation. EMBO J. 17, 6723–6729.

Ge, H., Si, Y., Wolffe, A.P., 1998. A novel transcriptional coactivator, p52, functionally interacts with the essential splicing factor ASF/SF2. Mol. Cell 2, 751–759.

Gelderblom, H.R., 1991. Assembly and morphology of HIV: potential effect of structure on viral function. AIDS Lond. Engl. 5, 617–637.

Giguère, J.-F., Tremblay, M.J., 2004. Statin compounds reduce human immunodeficiency virus type 1 replication by preventing the interaction

References

between virion-associated host intercellular adhesion molecule 1 and its natural cell surface ligand LFA-1. J. Virol. 78, 12062–12065.

Gijsbers, R., Ronen, K., Vets, S., Malani, N., De Rijck, J., McNeely, M., Bushman, F.D., Debyser, Z., 2010. LEDGF hybrids efficiently retarget lentiviral integration into heterochromatin. Mol. Ther. J. Am. Soc. Gene Ther. 18, 552–560. doi:10.1038/mt.2010.36

Glushakova, S., Münch, J., Carl, S., Greenough, T.C., Sullivan, J.L., Margolis, L., Kirchhoff, F., 2001. CD4 down-modulation by human immunodeficiency virus type 1 Nef correlates with the efficiency of viral replication and with CD4(+) T-cell depletion in human lymphoid tissue ex vivo. J. Virol. 75, 10113–10117. doi:10.1128/JVI.75.21.10113-10117.2001

Goto, T., Nakai, M., Ikuta, K., 1998. The life-cycle of human immunodeficiency virus type 1. Micron Oxf. Engl. 1993 29, 123–138.

Gottlieb, M.S., Schroff, R., Schanker, H.M., Weisman, J.D., Fan, P.T., Wolf, R.A., Saxon, A., 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N. Engl. J. Med. 305, 1425–1431. doi:10.1056/NEJM198112103052401

Gower, T.L., Graham, B.S., 2001. Antiviral activity of lovastatin against respiratory syncytial virus in vivo and in vitro. Antimicrob. Agents Chemother. 45, 1231–1237. doi:10.1128/AAC.45.4.1231-1237.2001

Grossman, T.H., Kawasaki, E.S., Punreddy, S.R., Osburne, M.S., 1998. Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability. Gene 209, 95–103.

Gulizia, J., Dempsey, M.P., Sharova, N., Bukrinsky, M.I., Spitz, L., Goldfarb, D., Stevenson, M., 1994. Reduced nuclear import of human immunodeficiency virus type 1 preintegration complexes in the presence of a prototypic nuclear targeting signal. J. Virol. 68, 2021–2025.

Gupta, K., Diamond, T., Hwang, Y., Bushman, F., Van Duyne, G.D., 2010. Structural properties of HIV integrase. Lens epithelium-derived growth factor oligomers. J. Biol. Chem. 285, 20303–20315. doi:10.1074/jbc.M110.114413

References

Habermann, S.M., Murphy, K.P., 1996. Energetics of hydrogen bonding in proteins: a model compound study. Protein Sci. Publ. Protein Soc. 5, 1229–1239.

Hamelin, B.A., Turgeon, J., 1998. Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors. Trends Pharmacol. Sci. 19, 26–37.

Hare, S., Cherepanov, P., 2009. The Interaction Between Lentiviral Integrase and LEDGF: Structural and Functional Insights. Viruses 1, 780–801. doi:10.3390/v1030780

Hayouka, Z., Hurevich, M., Levin, A., Benyamini, H., Iosub, A., Maes, M., Shalev, D.E., Loyter, A., Gilon, C., Friedler, A., 2010. Cyclic peptide inhibitors of HIV-1 integrase derived from the LEDGF/p75 protein. Bioorg. Med. Chem. 18, 8388–8395. doi:10.1016/j.bmc.2010.09.046

Hayouka, Z., Rosenbluh, J., Levin, A., Loya, S., Lebendiker, M., Veprintsev, D., Kotler, M., Hizi, A., Loyter, A., Friedler, A., 2007. Inhibiting HIV-1 integrase by shifting its oligomerization equilibrium. Proc. Natl. Acad. Sci. 104, 8316–8321.

Heinzinger, N.K., Bukinsky, M.I., Haggerty, S.A., Ragland, A.M., Kewalramani, V., Lee, M.A., Gendelman, H.E., Ratner, L., Stevenson, M., Emerman, M., 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. U. S. A. 91, 7311–7315.

Henrich, T.J., Kuritzkes, D.R., 2013. HIV-1 entry inhibitors: recent development and clinical use. Curr. Opin. Virol. 3, 51–57. doi:10.1016/j.coviro.2012.12.002

Hirsch, V.M., Olmsted, R.A., Murphey-Corb, M., Purcell, R.H., Johnson, P.R., 1989. An African primate lentivirus (SIVsm) closely related to HIV-2. Nature 339, 389–392. doi:10.1038/339389a0

HIV databases [WWW Document], n.d. URL http://www.hiv.lanl.gov (accessed 1.9.14).

Hoffman, T.L., Stephens, E.B., Narayan, O., Doms, R.W., 1998. HIV type I envelope determinants for use of the CCR2b, CCR3, STRL33, and APJ coreceptors. Proc. Natl. Acad. Sci. U. S. A. 95, 11360–11365.

Hombrouck, A., De Rijck, J., Hendrix, J., Vandekerckhove, L., Voet, A., Maeyer, M.D., Witvrouw, M., Engelborghs, Y., Christ, F., Gijsbers, R., Debyser, Z.,

References

2007. Virus Evolution Reveals an Exclusive Role for LEDGF/p75 in Chromosomal Tethering of HIV. PLoS Pathog. 3. doi:10.1371/journal.ppat.0030047

Hu, G., Li, X., Sun, X., Lu, W., Liu, G., Huang, J., Shen, X., Tang, Y., 2012. Identification of old drugs as potential inhibitors of HIV-1 integrase – human LEDGF/p75 interaction via molecular docking. J. Mol. Model. 18, 4995–5003. doi:10.1007/s00894-012-1494-0

Huang, Z., Xu, Y., Li, Y., Wang, Y., 2010a. Conversion Investigation for Lovastatin and Its Derivatives By HPLC. J. Chromatogr. Sci. 48, 631–636.

Huang, Z., Xu, Y., Li, Y., Wang, Y., 2010b. Conversion investigation for lovastatin and its derivatives by HPLC. J. Chromatogr. Sci. 48, 631–636.

Huet, T., Cheynier, R., Meyerhans, A., Roelants, G., Wain-Hobson, S., 1990. Genetic organization of a chimpanzee lentivirus related to HIV-1. Nature 345, 356–359. doi:10.1038/345356a0

Ikeda, M., Abe, K., Yamada, M., Dansako, H., Naka, K., Kato, N., 2006. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatol. Baltim. Md 44, 117–125. doi:10.1002/hep.21232

Iordanskiy, S., Berro, R., Altieri, M., Kashanchi, F., Bukrinsky, M., 2006. Intracytoplasmic maturation of the human immunodeficiency virus type 1 reverse transcription complexes determines their capacity to integrate into chromatin. Retrovirology 3, 4. doi:10.1186/1742-4690-3-4

Istvan, E.S., Deisenhofer, J., 2001. Structural Mechanism for Statin Inhibition of HMG-CoA Reductase. Science 292, 1160–1164. doi:10.1126/science.1059344

Iyengar, S., Hildreth, J.E., Schwartz, D.H., 1998. Actin-dependent receptor colocalization required for human immunodeficiency virus entry into host cells. J. Virol. 72, 5251–5255.

Jain, M.K., Ridker, P.M., 2005. Anti-Inflammatory Effects of Statins: Clinical Evidence and Basic Mechanisms. Nat. Rev. Drug Discov. 4, 977–987. doi:10.1038/nrd1901

Jenkins, T.M., Engelman, A., Ghirlando, R., Craigie, R., 1996. A soluble active mutant of HIV-1 integrase: involvement of both the core and carboxyl-terminal domains in multimerization. J. Biol. Chem. 271, 7712–7718.

Jenkins, T.M., Hickman, A.B., Dyda, F., Ghirlando, R., Davies, D.R., Craigie, R., 1995. Catalytic domain of human immunodeficiency virus type 1

integrase: identification of a soluble mutant by systematic replacement of hydrophobic residues. Proc. Natl. Acad. Sci. 92, 6057–6061.

Jenny, R.J., Mann, K.G., Lundblad, R.L., 2003. A critical review of the methods for cleavage of fusion proteins with thrombin and factor Xa. Protein Expr. Purif. 31, 1–11.

Jiang, L., Lai, L., 2002. CH...O hydrogen bonds at protein-protein interfaces. J. Biol. Chem. 277, 37732–37740. doi:10.1074/jbc.M204514200

Joint United Nations Program, United Nations, 2013. Global Report: UNAIDS Report on the Global AIDS epidemic:2013. UNAIDS.

Joint United Nations Programme on HIV/AIDS, United Nations, 2012. Global Report: UNAIDS Report on the Global AIDS epidemic: 2012. UNAIDS, [Geneva].

KAUZMANN, W., 1959. Some factors in the interpretation of protein denaturation. Adv. Protein Chem. 14, 1–63.

Keele, B.F., Van Heuverswyn, F., Li, Y., Bailes, E., Takehisa, J., Santiago, M.L., Bibollet-Ruche, F., Chen, Y., Wain, L.V., Liegeois, F., Loul, S., Ngole, E.M., Bienvenue, Y., Delaporte, E., Brookfield, J.F.Y., Sharp, P.M., Shaw, G.M., Peeters, M., Hahn, B.H., 2006. Chimpanzee Reservoirs of Pandemic and Nonpandemic HIV-1. Science 313, 523–526. doi:10.1126/science.1126531

Kessl, J.J., Jena, N., Koh, Y., Taskent-Sezgin, H., Slaughter, A., Feng, L., de Silva, S., Wu, L., Le Grice, S.F.J., Engelman, A., Fuchs, J.R., Kvaratskhelia, M., 2012. Multimode, Cooperative Mechanism of Action of Allosteric HIV-1 Integrase Inhibitors. J. Biol. Chem. 287, 16801–16811. doi:10.1074/jbc.M112.354373

Kilby, J.M., Lalezari, J.P., Eron, J.J., Carlson, M., Cohen, C., Arduino, R.C., Goodgame, J.C., Gallant, J.E., Volberding, P., Murphy, R.L., Valentine, F., Saag, M.S., Nelson, E.L., Sista, P.R., Dusek, A., 2002. The Safety, Plasma Pharmacokinetics, and Antiviral Activity of Subcutaneous Enfuvirtide (T-20), a Peptide Inhibitor of gp41-Mediated Virus Fusion, in HIV-Infected Adults. AIDS Res. Hum. Retroviruses 18, 685–693. doi:10.1089/088922202760072294

Korber, B., Kuiken, C., Foley, B., Hahn, B.H., McCutchan, F.E., Mellors, J.., Sodroski, J. (Eds.), n.d. A complilation and analysis of nucleic acids and

References

amino acid sequences, in: Human Retroviruses and AIDS Research. p. 1999.

Korber, B., Muldoon, M., Theiler, J., Gao, F., Gupta, R., Lapedes, A., Hahn, B.H., Wolinsky, S., Bhattacharya, T., 2000. Timing the Ancestor of the HIV-1 Pandemic Strains. Science 288, 1789–1796. doi:10.1126/science.288.5472.1789

Kubo, E., Fatma, N., Sharma, P., Shinohara, T., Chylack, L.T., Jr, Akagi, Y., Singh, D.P., 2002. Transactivation of involucrin, a marker of differentiation in keratinocytes, by lens epithelium-derived growth factor (LEDGF). J. Mol. Biol. 320, 1053–1063.

Lalezari, J.P., Eron, J.J., Carlson, M., Cohen, C., DeJesus, E., Arduino, R.C., Gallant, J.E., Volberding, P., Murphy, R.L., Valentine, F., Nelson, E.L., Sista, P.R., Dusek, A., Kilby, J.M., 2003. A phase II clinical study of the long-term safety and antiviral activity of enfuvirtide-based antiretroviral therapy. AIDS Lond. Engl. 17, 691–698. doi:10.1097/01.aids.0000050825.06065.84

Lathe, G.H., Ruthven, C.R.J., 1956. The separation of substances and estimation of their relative molecular sizes by the use of columns of starch in water. Biochem. J. 62, 665–674.

Le Rouzic, E., Bonnard, D., Chasset, S., Bruneau, J.-M., Chevreuil, F., Le Strat, F., Nguyen, J., Beauvoir, R., Amadori, C., Brias, J., 2013. Dual inhibition of HIV-1 replication by integrase-LEDGF allosteric inhibitors is predominant at the post-integration stage. Retrovirology 10, 144.

Lee, M.S., Craigie, R., 1998. A previously unidentified host protein protects retroviral DNA from autointegration. Proc. Natl. Acad. Sci. U. S. A. 95, 1528–1533. doi:10.1073/pnas.95.4.1528

Lieberman-Blum, S.S., Fung, H.B., Bandres, J.C., 2008. Maraviroc: a CCR5-receptor antagonist for the treatment of HIV-1 infection. Clin. Ther. 30, 1228–1250.

Lipinski, C.A., 2004. Lead- and drug-like compounds: the rule-of-five revolution. Drug Discov. Today Technol. 1, 337–341. doi:10.1016/j.ddtec.2004.11.007

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. 46, 3–26.

References

Liu, S., Rodriguez, A.V., Tosteson, M.T., 2006. Role of simvastatin and methyl-beta-cyclodextrin [corrected] on inhibition of poliovirus infection. Biochem. Biophys. Res. Commun. 347, 51–59. doi:10.1016/j.bbrc.2006.06.107

Llano, M., Saenz, D.T., Meehan, A., Wongthida, P., Peretz, M., Walker, W.H., Teo, W., Poeschla, E.M., 2006a. An essential role for LEDGF/p75 in HIV integration. Science 314, 461–464. doi:10.1126/science.1132319

Llano, M., Vanegas, M., Fregoso, O., Saenz, D., Chung, S., Peretz, M., Poeschla, E.M., 2004. LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes. J. Virol. 78, 9524–9537.

Llano, M., Vanegas, M., Hutchins, N., Thompson, D., Delgado, S., Poeschla, E.M., 2006b. Identification and Characterization of the Chromatin-binding Domains of the HIV-1 Integrase Interactor LEDGF/p75. J. Mol. Biol. 360, 760–773. doi:10.1016/j.jmb.2006.04.073

Lodi, P.J., Ernst, J.A., Kuszewski, J., Hickman, A.B., Engelman, A., Craigie, R., Clore, G.M., Gronenborn, A.M., 1995. Solution structure of the DNA binding domain of HIV-1 integrase. Biochemistry (Mosc.) 34, 9826–9833.

Maertens, G., 2003. LEDGF/p75 Is Essential for Nuclear and Chromosomal Targeting of HIV-1 Integrase in Human Cells. J. Biol. Chem. 278, 33528–33539. doi:10.1074/jbc.M303594200

Maertens, G., 2004. Identification and Characterization of a Functional Nuclear Localization Signal in the HIV-1 Integrase Interactor LEDGF/p75. J. Biol. Chem. 279, 33421–33429. doi:10.1074/jbc.M404700200

Maertens, G.N., Cherepanov, P., Engelman, A., 2006. Transcriptional co-activator p75 binds and tethers the Myc-interacting protein JPO2 to chromatin. J. Cell Sci. 119, 2563–2571. doi:10.1242/jcs.02995

Makhatadze, G.I., Privalov, P.L., 1995. Energetics of protein structure. Adv. Protein Chem. 47, 307–425.

Marchand, C., Johnson, A.A., Karki, R.G., Pais, G.C.G., Zhang, X., Cowansage, K., Patel, T.A., Nicklaus, M.C., Burke, T.R., Jr, Pommier, Y., 2003. Metal-dependent inhibition of HIV-1 integrase by beta-diketo acids and resistance of the soluble double-mutant (F185K/C280S). Mol. Pharmacol. 64, 600–609. doi:10.1124/mol.64.3.600

References

Marinsky, J.A., Reddy, M.M., Amdur, S., 1973. Prediction of ion-exchange selectivity. J. Phys. Chem. 77, 2128–2132. doi:10.1021/j100636a019

Marshall, H.M., Ronen, K., Berry, C., Llano, M., Sutherland, H., Saenz, D., Bickmore, W., Poeschla, E., Bushman, F.D., 2007. Role of PSIP1/LEDGF/p75 in lentiviral infectivity and integration targeting. PloS One 2, e1340. doi:10.1371/journal.pone.0001340

Marx, J.L., 1986. Trophic factor aids synapse formation. Science 232, 1501.

Matthews, B.W., 2001. Hydrophobic interactions in proteins. eLS.

Mazière, J.C., Landureau, J.C., Giral, P., Auclair, M., Fall, L., Lachgar, A., Achour, A., Zagury, D., 1994. Lovastatin inhibits HIV-1 expression in H9 human T lymphocytes cultured in cholesterol-poor medium. Biomed. Pharmacother. Bioméd. Pharmacothérapie 48, 63–67.

McCormack, G.P., Glynn, J.R., Crampin, A.C., Sibande, F., Mulawa, D., Bliss, L., Broadbent, P., Abarca, K., Pönnighaus, J.M., Fine, P.E.M., Clewley, J.P., 2002. Early evolution of the human immunodeficiency virus type 1 subtype C epidemic in rural Malawi. J. Virol. 76, 12890–12899.

McKee, C.J., Kessl, J.J., Shkriabai, N., Dar, M.J., Engelman, A., Kvaratskhelia, M., 2008. Dynamic modulation of HIV-1 integrase structure and function by cellular lens epithelium-derived growth factor (LEDGF) protein. J. Biol. Chem. 283, 31802–31812. doi:10.1074/jbc.M805843200

Miroux, B., Walker, J.E., 1996. Over-production of Proteins in< i> Escherichia coli</i>: Mutant Hosts that Allow Synthesis of some Membrane Proteins and Globular Proteins at High Levels. J. Mol. Biol. 260, 289–298.

Mitsuya, H., Broder, S., 1987. Strategies for antiviral therapy in AIDS. Nature 325, 773–778. doi:10.1038/325773a0

Moghadasian, M.H., 1999. Clinical pharmacology of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. Life Sci. 65, 1329–1337.

Molecular Properties Prediction - Osiris Property Explorer [WWW Document], n.d. URL http://www.organic-chemistry.org/prog/peo/ (accessed 8.4.12).

Moncunill, G., Negredo, E., Bosch, L., Vilarrasa, J., Witvrouw, M., Llano, A., Clotet, B., Esté, J.A., 2005. Evaluation of the anti-HIV activity of statins. AIDS Lond. Engl. 19, 1697–1700.

Mondor, I., Ugolini, S., Sattentau, Q.J., 1998. Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans. J. Virol. 72, 3623–3634.

References

Montal, M., 2003. Structure-function correlates of Vpu, a membrane protein of HIV-1. FEBS Lett. 552, 47–53.

Moulard, M., Lortat-Jacob, H., Mondor, I., Roca, G., Wyatt, R., Sodroski, J., Zhao, L., Olson, W., Kwong, P.D., Sattentau, Q.J., 2000. Selective interactions of polyanions with basic surfaces on human immunodeficiency virus type 1 gp120. J. Virol. 74, 1948–1960.

Nabatov, A.A., Pollakis, G., Linnemann, T., Paxton, W.A., de Baar, M.P., 2007. Statins Disrupt CCR5 and RANTES Expression Levels in CD4+ T Lymphocytes In Vitro and Preferentially Decrease Infection of R5 Versus X4 HIV-1. PLoS ONE 2, e470. doi:10.1371/journal.pone.0000470

NCC Clinical Collection, n.d.

Neilson, J.R., John, G.C., Carr, J.K., Lewis, P., Kreiss, J.K., Jackson, S., Nduati, R.W., Mbori-Ngacha, D., Panteleeff, D.D., Bodrug, S., Giachetti, C., Bott, M.A., Richardson, B.A., Bwayo, J., Ndinya-Achola, J., Overbaugh, J., 1999. Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya. J. Virol. 73, 4393–4403.

Nekhai, S., Jeang, K.-T., 2006. Transcriptional and post-transcriptional regulation of HIV-1 gene expression: role of cellular factors for Tat and Rev. Future Microbiol. 1, 417–426. doi:10.2217/17460913.1.4.417

Nishizawa, Y., Usukura, J., Singh, D.P., Chylack, L.T., Jr, Shinohara, T., 2001. Spatial and temporal dynamics of two alternatively spliced regulatory factors, lens epithelium-derived growth factor (ledgf/p75) and p52, in the nucleus. Cell Tissue Res. 305, 107–114.

O'Doherty, U., Swiggard, W.J., Malim, M.H., 2000. Human Immunodeficiency Virus Type 1 Spinoculation Enhances Infection through Virus Binding. J. Virol. 74, 10074–10080.

Önal, A., Sagirli, O., 2006. Development of a Selective LC Method for the Determination of Pravastatin Sodium. Chromatographia 64, 1–6. doi:10.1365/s10337-006-0843-5

Oprea, T.I., Bauman, J.E., Bologa, C.G., Buranda, T., Chigaev, A., Edwards, B.S., Jarvik, J.W., Gresham, H.D., Haynes, M.K., Hjelle, B., Hromas, R., Hudson, L., Mackenzie, D.A., Muller, C.Y., Reed, J.C., Simons, P.C., Smagley, Y., Strouse, J., Surviladze, Z., Thompson, T., Ursu, O., Waller, A., Wandinger-Ness, A., Winter, S.S., Wu, Y., Young, S.M., Larson, R.S., Willman, C., Sklar, L.A., 2011. Drug repurposing from an academic

References

perspective. Drug Discov. Today Ther. Strateg. 8, 61–69. doi:10.1016/j.ddstr.2011.10.002

Peat, T.S., Rhodes, D.I., Vandegraaff, N., Le, G., Smith, J.A., Clark, L.J., Jones, E.D., Coates, J.A.V., Thienthong, N., Newman, J., Dolezal, O., Mulder, R., Ryan, J.H., Savage, G.P., Francis, C.L., Deadman, J.J., 2012. Small Molecule Inhibitors of the LEDGF Site of Human Immunodeficiency Virus Integrase Identified by Fragment Screening and Structure Based Design. PLoS ONE 7, e40147. doi:10.1371/journal.pone.0040147

Perelson, A.S., Neumann, A.U., Markowitz, M., Leonard, J.M., Ho, D.D., 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. Science 271, 1582–1586.

Plantier, J.-C., Leoz, M., Dickerson, J.E., De Oliveira, F., Cordonnier, F., Lemée, V., Damond, F., Robertson, D.L., Simon, F., 2009. A new human immunodeficiency virus derived from gorillas. Nat. Med. 15, 871–872. doi:10.1038/nm.2016

Popovic, M., Sarngadharan, M.G., Read, E., Gallo, R.C., 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224, 497–500.

Potena, L., Frascaroli, G., Grigioni, F., Lazzarotto, T., Magnani, G., Tomasi, L., Coccolo, F., Gabrielli, L., Magelli, C., Landini, M.P., Branzi, A., 2004. Hydroxymethyl-glutaryl coenzyme a reductase inhibition limits cytomegalovirus infection in human endothelial cells. Circulation 109, 532–536. doi:10.1161/01.CIR.0000109485.79183.81

Pradidarcheep, W., Labruyere, W.T., Dabhoiwala, N.F., Lamers, W.H., 2008. Lack of Specificity of Commercially Available Antisera: Better Specifications Needed. J. Histochem. Cytochem. 56, 1099–1111. doi:10.1369/jhc.2008.952101

Probasco, J.C., Spudich, S.S., Critchfield, J., Lee, E., Lollo, N., Deeks, S.G., Price, R.W., 2008. Failure of atorvastatin to modulate CSF HIV-1 infection: results of a pilot study. Neurology 71, 521–524. doi:10.1212/01.wnl.0000325006.84658.e7

Rahman, S., Lu, R., Vandegraaff, N., Cherepanov, P., Engelman, A., 2007. Structure-based mutagenesis of the integrase-LEDGF/p75 interface uncouples a strict correlation between in vitro protein binding and HIV-1 fitness. Virology 357, 79–90. doi:10.1016/j.virol.2006.08.011

References

Renjifo, B., Chaplin, B., Mwakagile, D., Shah, P., Vannberg, F., Msamanga, G., Hunter, D., Fawzi, W., Essex, M., 1998. Epidemic expansion of HIV type 1 subtype C and recombinant genotypes in Tanzania. AIDS Res. Hum. Retroviruses 14, 635–638.

Rhodes, D.I., Peat, T.S., Vandegraaff, N., Jeevarajah, D., Newman, J., Martyn, J., Coates, J.A.V., Ede, N.J., Rea, P., Deadman, J.J., 2011. Crystal structures of novel allosteric peptide inhibitors of HIV integrase identify new interactions at the LEDGF binding site. Chembiochem Eur. J. Chem. Biol. 12, 2311–2315. doi:10.1002/cbic.201100350

Rice, P., Craigie, R., Davies, D.R., 1996. Retroviral integrases and their cousins. Curr. Opin. Struct. Biol. 6, 76–83.

Saïda, F., Uzan, M., Odaert, B., Bontems, F., 2006. Expression of highly toxic genes in E. coli: special strategies and genetic tools. Curr. Protein Pept. Sci. 7, 47–56.

Salminen, M.O., Johansson, B., Sönnerborg, A., Ayehunie, S., Gotte, D., Leinikki, P., Burke, D.S., McCutchan, F.E., 1996. Full-length sequence of an ethiopian human immunodeficiency virus type 1 (HIV-1) isolate of genetic subtype C. AIDS Res. Hum. Retroviruses 12, 1329–1339.

Santiago, M.L., Rodenburg, C.M., Kamenya, S., Bibollet-Ruche, F., Gao, F., Bailes, E., Meleth, S., Soong, S.-J., Kilby, J.M., Moldoveanu, Z., Fahey, B., Muller, M.N., Ayouba, A., Nerrienet, E., McClure, H.M., Heeney, J.L., Pusey, A.E., Collins, D.A., Boesch, C., Wrangham, R.W., Goodall, J., Sharp, P.M., Shaw, G.M., Hahn, B.H., 2002. SIVcpz in wild chimpanzees. Science 295, 465. doi:10.1126/science.295.5554.465

Schrijvers, R., De Rijck, J., Demeulemeester, J., Adachi, N., Vets, S., Ronen, K., Christ, F., Bushman, F.D., Debyser, Z., Gijsbers, R., 2012. LEDGF/p75-Independent HIV-1 Replication Demonstrates a Role for HRP-2 and Remains Sensitive to Inhibition by LEDGINs. PLoS Pathog. 8, e1002558. doi:10.1371/journal.ppat.1002558

Seelamgari, A., Maddukuri, A., Berro, R., de la Fuente, C., Kehn, K., Deng, L., Dadgar, S., Bottazzi, M.E., Ghedin, E., Pumfery, A., Kashanchi, F., 2004. Role of viral regulatory and accessory proteins in HIV-1 replication. Front. Biosci. J. Virtual Libr. 9, 2388–2413.

Serajuddin, A.T., Ranadive, S.A., Mahoney, E.M., 1991. Relative lipophilicities, solubilities, and structure-pharmacological considerations of 3-hydroxy-3-

References

methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors pravastatin, lovastatin, mevastatin, and simvastatin. J. Pharm. Sci. 80, 830–834.

Shaheduzzaman, S., Krishnan, V., Petrovic, A., Bittner, M., Meltzer, P., Trent, J., Venkatesan, S., Zeichner, S., 2002. Effects of HIV-1 Nef on cellular gene expression profiles. J. Biomed. Sci. 9, 82–96.

Sharma, P., Singh, D.P., Fatma, N., Chylack, L.T., Jr, Shinohara, T., 2000. Activation of LEDGF gene by thermal-and oxidative-stresses. Biochem. Biophys. Res. Commun. 276, 1320–1324. doi:10.1006/bbrc.2000.3606

Sharp, P.M., Bailes, E., Chaudhuri, R.R., Rodenburg, C.M., Santiago, M.O., Hahn, B.H., 2001. The origins of acquired immune deficiency syndrome viruses: where and when? Philos. Trans. R. Soc. Lond. B. Biol. Sci. 356, 867–876. doi:10.1098/rstb.2001.0863

Sharp, P.M., Hahn, B.H., 2010. The evolution of HIV-1 and the origin of AIDS. Philos. Trans. R. Soc. B Biol. Sci. 365, 2487–2494. doi:10.1098/rstb.2010.0031

Sharp, P.M., Robertson, D., Gao, F., Hahn, B.H., 1994. Origins and diversity of human immunodefeciency viruses. AIDS 8, S27–42.

Sheehy, A.M., Gaddis, N.C., Malim, M.H., 2003. The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. Nat. Med. 9, 1404–1407. doi:10.1038/nm945

Shehu-Xhilaga, M., Oelrichs, R., 2009. Basic HIV virology. HIV Manag. Australas. 9–18.

Shen, L., Peterson, S., Sedaghat, A.R., McMahon, M.A., Callender, M., Zhang, H., Zhou, Y., Pitt, E., Anderson, K.S., Acosta, E.P., Siliciano, R.F., 2008. Dose-response curve slope sets class-specific limits on inhibitory potential of anti-HIV drugs. Nat. Med. 14, 762–766. doi:10.1038/nm1777

Sherman, M.P., Greene, W.C., 2002. Slipping through the door: HIV entry into the nucleus. Microbes Infect. Inst. Pasteur 4, 67–73.

Shin, J.-H., Piao, C.-S., Lim, C.-M., Lee, J.-K., 2008. LEDGF binding to stress response element increases alphaB-crystallin expression in astrocytes with oxidative stress. Neurosci. Lett. 435, 131–136. doi:10.1016/j.neulet.2008.02.029

Shinohara, T., Singh, D.P., Fatma, N., 2002. LEDGF, a survival factor, activates stress-related genes. Prog. Retin. Eye Res. 21, 341–358.

References

Shun, M.-C., Raghavendra, N.K., Vandegraaff, N., Daigle, J.E., Hughes, S., Kellam, P., Cherepanov, P., Engelman, A., 2007. LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. Genes Dev. 21, 1767–1778. doi:10.1101/gad.1565107

Simoneau, B., Amad, M., Bailey, M., Bethell, R., Bhardwaj, P., Bilodeau, F., Bonneau, P., Bos, M., Bousquet, Y., Brochu, C., Carson, R., Chabot, C., Cordingley, M., Duan, J., Edwards, P., Fader, L., Faucher, A., Fenwick, C., Garneau, M., Halmos, T., Jakalian, A., Kawai, S., Landry, S., LaPlante, S., Malenfant, E., Mason, S., Morin, S., Parisien, M., Pesant, M., Poupart, M., Tsantrizos, Y., Wernic, D., Yoakim, C., Coulombe, R., 2011. Identification of novel non-catalytic site integrase inhibitors (NCINIs) of LTR DNA 3' processing. Presented at the 24th International Conference on Antiviral Research (ICAR).

Singh, D.P., Kimura, A., Chylack Jr, L.T., Shinohara, T., 2000. Lens epithelium-derived growth factor (LEDGF/p75) and p52 are derived from a single gene by alternative splicing. Gene 242, 265–273.

Singh, D.P., Ohguro, N., Chylack, L.T., Jr, Shinohara, T., 1999. Lens epithelium-derived growth factor: increased resistance to thermal and oxidative stresses. Invest. Ophthalmol. Vis. Sci. 40, 1444–1451.

Sklar, P.A., Masur, H., Grubb, J.R., Voell, J., Witek, J., Ono, A., Freed, E.O., Maldarelli, F., 2005. Pravastatin does not have a consistent antiviral effect in chronically HIV-infected individuals on antiretroviral therapy. Aids 19, 1109–1111.

Sleigh, S.H., Barton, D.C.L., 2010. Repurposing Strategies for Therapeutics. Pharm. Med. 24, 151–159. doi:10.1007/BF03256811

Stoddart, C.A., Geleziunas, R., Ferrell, S., Linquist-Stepps, V., Moreno, M.E., Bare, C., Xu, W., Yonemoto, W., Bresnahan, P.A., McCune, J.M., Greene, W.C., 2003. Human immunodeficiency virus type 1 Nef-mediated downregulation of CD4 correlates with Nef enhancement of viral pathogenesis. J. Virol. 77, 2124–2133.

Strebel, K., Klimkait, T., Martin, M.A., 1988. A novel gene of HIV-1, vpu, and its 16-kilodalton product. Science 241, 1221–1223.

Studier, F.W., Moffatt, B.A., 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189, 113–130.

References

Summa, V., Petrocchi, A., Bonelli, F., Crescenzi, B., Donghi, M., Ferrara, M., Fiore, F., Gardelli, C., Gonzalez Paz, O., Hazuda, D.J., Jones, P., Kinzel, O., Laufer, R., Monteagudo, E., Muraglia, E., Nizi, E., Orvieto, F., Pace, P., Pescatore, G., Scarpelli, R., Stillmock, K., Witmer, M.V., Rowley, M., 2008. Discovery of raltegravir, a potent, selective orally bioavailable HIV-integrase inhibitor for the treatment of HIV-AIDS infection. J. Med. Chem. 51, 5843–5855. doi:10.1021/jm800245z

Tanaka, M., Terahara, A., 1982. ML-236B Derivatives and their preparation. US4346227 A.

Tsiang, M., Jones, G.S., Niedziela-Majka, A., Kan, E., Lansdon, E.B., Huang, W., Hung, M., Samuel, D., Novikov, N., Xu, Y., Mitchell, M., Guo, H., Babaoglu, K., Liu, X., Geleziunas, R., Sakowicz, R., 2012. New Class of HIV-1 Integrase (IN) Inhibitors with a Dual Mode of Action. J. Biol. Chem. 287, 21189–21203. doi:10.1074/jbc.M112.347534

Tungaturthi, P.K., Sawaya, B.E., Singh, S.P., Tomkowicz, B., Ayyavoo, V., Khalili, K., Collman, R.G., Amini, S., Srinivasan, A., 2003. Role of HIV-1 Vpr in AIDS pathogenesis: relevance and implications of intravirion, intracellular and free Vpr. Biomed. Pharmacother. Bioméd. Pharmacothérapie 57, 20–24.

Turlure, F., Maertens, G., Rahman, S., Cherepanov, P., Engelman, A., 2006. A tripartite DNA-binding element, comprised of the nuclear localization signal and two AT-hook motifs, mediates the association of LEDGF/p75 with chromatin in vivo. Nucleic Acids Res. 34, 1653–1665. doi:10.1093/nar/gkl052

Vallari, A., Holzmayer, V., Harris, B., Yamaguchi, J., Ngansop, C., Makamche, F., Mbanya, D., Kaptué, L., Ndembi, N., Gürtler, L., Devare, S., Brennan, C.A., 2011. Confirmation of Putative HIV-1 Group P in Cameroon. J. Virol. 85, 1403–1407. doi:10.1128/JVI.02005-10

Vandegraaff, N., Devroe, E., Turlure, F., Silver, P.A., Engelman, A., 2006. Biochemical and genetic analyses of integrase-interacting proteins lens epithelium-derived growth factor (LEDGF)/p75 and hepatoma-derived growth factor related protein 2 (HRP2) in preintegration complex function and HIV-1 replication. Virology 346, 415–426. doi:10.1016/j.virol.2005.11.022

References

Vandekerckhove, L., Christ, F., Van Maele, B., De Rijck, J., Gijsbers, R., Van den Haute, C., Witvrouw, M., Debyser, Z., 2006. Transient and Stable Knockdown of the Integrase Cofactor LEDGF/p75 Reveals Its Role in the Replication Cycle of Human Immunodeficiency Virus. J. Virol. 80, 1886–1896. doi:10.1128/JVI.80.4.1886-1896.2006

Vanegas, M., Llano, M., Delgado, S., Thompson, D., Peretz, M., Poeschla, E., 2005. Identification of the LEDGF/p75 HIV-1 integrase-interaction domain and NLS reveals NLS-independent chromatin tethering. J. Cell Sci. 118, 1733–1743. doi:10.1242/jcs.02299

Vogt, P., 1997. Historical Introduction to the General Properties of Retroviruses, in: Coffin, J.M., Hughes, S.H., Varmus, H.E. (Eds.), Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).

Wain-Hobson, S., Alizon, M., Montagnier, L., 1985. Relationship of AIDS to other retroviruses. Nature 313, 743–743. doi:10.1038/313743a0

Weissenhorn, W., Dessen, A., Harrison, S.C., Skehel, J.J., Wiley, D.C., 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature 387, 426–430. doi:10.1038/387426a0

Wensing, A.M.J., van Maarseveen, N.M., Nijhuis, M., 2010. Fifteen years of HIV Protease Inhibitors: raising the barrier to resistance. Antiviral Res. 85, 59–74. doi:10.1016/j.antiviral.2009.10.003

Worobey, M., Gemmel, M., Teuwen, D.E., Haselkorn, T., Kunstman, K., Bunce, M., Muyembe, J.-J., Kabongo, J.-M.M., Kalengayi, R.M., Van Marck, E., Gilbert, M.T.P., Wolinsky, S.M., 2008. Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. Nature 455, 661–664. doi:10.1038/nature07390

Wu, K.-M., Farrelly, J.G., 2007. Regulatory perspectives of Type II prodrug development and time-dependent toxicity management: Nonclinical Pharm/Tox analysis and the role of comparative toxicology. Toxicology 236, 1–6. doi:10.1016/j.tox.2007.04.005

Xu, Z., Zheng, Y., Ao, Z., Clement, M., Mouland, A.J., Kalpana, G.V., Belhumeur, P., Cohen, E.A., Yao, X., 2008. Contribution of the C-terminal region within the catalytic core domain of HIV-1 integrase to yeast lethality, chromatin binding and viral replication. Retrovirology 5, 102. doi:10.1186/1742-4690-5-102

References

Yao, X.-J., Lemay, J., Rougeau, N., Clément, M., Kurtz, S., Belhumeur, P., Cohen, E.A., 2002. Genetic selection of peptide inhibitors of human immunodeficiency virus type 1 Vpr. J. Biol. Chem. 277, 48816–48826. doi:10.1074/jbc.M207982200

Yoakim, C., Amad, M., Bailey, M., Bethell, R., Bos, M., Cordingley, M., Coulombe, R., Duan, J., Edwards, P., Fader, L., Faucher, A., Garneau, M., Jakalian, A., Kawai, S., Lamarte, L., LaPlante, S., Luo, L., Mason, S., Paupart, M., Rioux, N., Simoneau, B., Tsantrizos, Y., Witvrouw, M., Fenwick, C., 2011. Preclinical Profile of BI 224436, a Novel HIV-1 Non-Catalytic Site Integrase Inhibitor.

Yokoyama, A., Cleary, M.L., 2008. Menin critically links MLL proteins with LEDGF on cancer-associated target genes. Cancer Cell 14, 36–46. doi:10.1016/j.ccr.2008.05.003

Yoshino, G., Kazumi, T., Kasama, T., Iwatani, I., Iwai, M., Inui, A., Otsuki, M., Baba, S., 1986. Effect of CS-514, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, on lipoprotein and apolipoprotein in plasma of hypercholesterolemic diabetics. Diabetes Res. Clin. Pract. 2, 179–181.

Yu, F., Jones, G.S., Hung, M., Wagner, A.H., MacArthur, H.L., Liu, X., Leavitt, S., McDermott, M.J., Tsiang, M., 2007. HIV-1 integrase preassembled on donor DNA is refractory to activity stimulation by LEDGF/p75. Biochemistry (Mosc.) 46, 2899–2908. doi:10.1021/bi602387u

Zeldin, R.K., Petruschke, R.A., 2004. Pharmacological and therapeutic properties of ritonavir-boosted protease inhibitor therapy in HIV-infected patients. J. Antimicrob. Chemother. 53, 4–9. doi:10.1093/jac/dkh029

Zhang, L., He, T., Huang, Y., Chen, Z., Guo, Y., Wu, S., Kunstman, K.J., Brown, R.C., Phair, J.P., Neumann, A.U., Ho, D.D., Wolinsky, S.M., 1998. Chemokine coreceptor usage by diverse primary isolates of human immunodeficiency virus type 1. J. Virol. 72, 9307–9312.

Zhang, S., Feng, Y., Narayan, O., Zhao, L.J., 2001. Cytoplasmic retention of HIV-1 regulatory protein Vpr by protein-protein interaction with a novel human cytoplasmic protein VprBP. Gene 263, 131–140.

Zheng, R., Jenkins, T.M., Craigie, R., 1996. Zinc folds the N-terminal domain of HIV-1 integrase, promotes multimerization, and enhances catalytic activity. Proc. Natl. Acad. Sci. U. S. A. 93, 13659–13664.

References

Zheng, Y., Ao, Z., Jayappa, K.D., Yao, X., 2010. Characterization of the HIV-1 integrase chromatin-and LEDGF/p75-binding abilities by mutagenic analysis within the catalytic core domain of integrase. Virol J 7, 422X–7.

Zhu, T., Korber, B.T., Nahmias, A.J., Hooper, E., Sharp, P.M., Ho, D.D., 1998. An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. Nature 391, 594–597. doi:10.1038/35400

Appendix A - Human Ethics Waiver

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical))

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

Ref: W-CJ-150213-3   15/02/2013

TO WHOM IT MAY CONCERN:

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

Investigator: **Ms A Harrison (student no 690739).**

Project title: **Investigation of the LEDGF-integrase interaction as a target for HIV-
                     1 drug intervention.**

Reason: This is a wholly laboratory study one objective of which is to develop a
                     novel biological assay. No humans are involved.
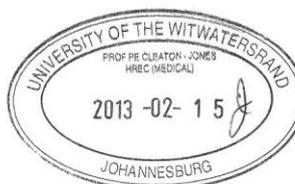
Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav / Zanele Ndlovu Research Office, Senate House, Wits