EFFECTIVENESS OF ENTRY INHIBITORS ON HIV-1 SUBTYPE C VIRUSES

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Abstract

The entry stage of the HIV-1 viral life cycle has become a prime target for preventing HIV-1 infection. This has led to the development of a new class of anti-retroviral agents termed entry inhibitors, which have proven effective *in vitro* and in the clinic. These new agents target three different stages of entry, namely CD4 binding, coreceptor interaction with either CCR5 or CXCR4 and the fusion process. Here we studied isolates from patients with HIV-1 subtype C infection and the effectiveness of different coreceptor and fusion inhibitors *in vitro*. Further we examined resistance profiles to the first licensed entry inhibitor, T-20.

In Chapter 2 we examined coreceptor usage of HIV-1 subtype C isolates and their sensitivity to CCR5 and CXCR4 inhibitors. Twenty-nine viral isolates with different coreceptor usage profiles were isolated from patients with advanced AIDS. The CCR5-specific agents, PRO140 an anti-CCR5 monoclonal antibody and RANTES, the natural ligand for CCR5 inhibited all 24 R5 isolates, while the two X4 and the three R5X4 viruses were sensitive to the CXCR4-specific inhibitor, AMD3100. The five X4 or R5X4 viruses were all able to replicate in peripheral blood mononuclear cells (PBMC) that did not express CCR5 confirming their ability to use CXCR4 on primary cells. When tested using coreceptor-transfected cell lines, one R5 virus was also able to use CXCR6, and another R5X4 virus could use CCR3, Bob/GPR15 and CXCR6. The R5X4 and X4 viruses contained more diverse V3 loop sequences with a higher overall positive charge, compared to the R5 viruses. Hence, HIV-1 subtype C viruses are able to use CCR5,

CXCR4 or both for entry, and they are sensitive to specific inhibitors of entry via these coreceptors.

In Chapter 3 we analyzed isolates from 10 acutely infected individuals, who were followed longitudinally for up to three years. Two of these individuals (Du151 and Du179) underwent a coreceptor switch and were studied more intensively. The other eight individuals retained CCR5 usage throughout the duration of the study. The initial 4 isolates from Du151 were able to utilize CCR5 but the later isolates were able to use both CCR5 and CXCR4 (R5X4). Du179 used both CCR5 and CXCR4 (R5X4) initially, but the later isolate was found to be monotropic and used CXCR4 (X4) exclusively. Viral isolates were tested for their sensitivity to small molecule inhibitors of CCR5, CXCR4 and the fusion process in a PBMC assay. All of the R5 isolates were sensitive to RANTES and PRO140 and insensitive to the two CXCR4 coreceptor inhibitors AMD3100 and T-140. There was a tendency for later isolates to become slightly less sensitive to the CCR5 inhibitors and more sensitive to the CXCR4 entry inhibitors. None of the R5X4 Du179 isolates were effectively inhibited by PRO140 and RANTES, but the X4 isolate of Du179 became sensitive to CXCR4 entry inhibitors. Both Du151 and Du179 underwent amino acid changes in their V3 sequences that included an increased charge associated with CXCR4 usage. This indicates that coreceptor switching can occur in subtype C infections and is associated with changes in the V3 loop. However, both Du151 and Du179 were subsequently found to be dually infected with another subtype C strain, which may account for some of the phenotypic and genotypic changes seen in these individuals including the appearance of CXCR4-virus variants.

In Chapter 4 we explored two HIV-1 isolates (CM4 and CM9) able to use alternate HIV-1 coreceptors for entry (i.e. coreceptors other than CCR5 or CXCR4) on transfected cell lines. These isolates were tested for their sensitivity to inhibitors of HIV-1 entry on primary cells. Both isolates were from patients with cryptococcal meningitis, a severe AIDS defining condition. CM4 was able to use CCR5 and Bob/GPR15 efficiently in transfected cells. This isolate grew in Δ32/Δ32 CCR5 PBMC in the presence of AMD3100, indicating that it used a receptor other than CCR5 or CXCR4 on primary cells. It was insensitive to the CCR5 entry inhibitors RANTES and PRO140, but was partially inhibited by vMIP-1, a chemokine that binds CCR3, CCR8, Bob/GPR15 and CXCR6. The coreceptor used by this isolate on primary cells is thus currently unknown. CM9 used CCR5, CXCR4, Bob/GPR15, CXCR6 and CCR3 on transfected cells and was able to replicate in the presence of AMD3100 in Δ32/Δ32 CCR5 PBMC. It was insensitive to vMIP-1, eotaxin and I309 used individually, but was inhibited completely when vMIP-1 or I309, the ligand for CCR8, were combined with AMD3100. These results strongly suggest that this isolate can use CCR8 on primary cells. Collectively these data suggest that some HIV-1 isolates can use alternate coreceptors on primary cells, which may have implications for strategies that aim to block viral entry using coreceptor inhibitors.

In Chapter 5 we examined the effectiveness of T-20 to inhibit HIV-1 subtype C isolates. T-20 blocks the fusion stage of the viral entry cycle and it is the first entry inhibitor to be licensed for clinical use. T-20 consists of 36 amino acids and was designed

based on the HR-2 region of HIV-1 subtype B. A total of 23 HIV-1 subtype C isolates were tested for their ability to replicate in the presence and absence of T-20. This included five isolates with multiple genotypic drug resistance mutations to reverse transcriptase and protease inhibitors. Among the 23 subtype C isolates there were 10-16 amino acid changes in the 36 amino acid region corresponding to T-20. However, all isolates were effectively inhibited by T-20 at 1 µg/ml, including the 5 isolates resistant to other anti-retroviral drugs. The gp41 region was sequenced and the HR-1 and HR-2 amino acids analyzed. All isolates had the amino acids GIV at positions 36-38 in gp41, which are associated with sensitivity to T-20. One X4 had a GVV motif but this did not affect its sensitivity. Thus, T-20 inhibited subtype C viruses despite significant genetic differences in the HR-2 regions of subtypes B and C. These data suggest that T-20 would be highly effective in patients with HIV-1 subtype C infection including those failing existing anti-retroviral drug regimens.

In Chapter 6 we examined the *in vitro* resistance patterns of HIV-1 subtype C to T-20. Resistance to T-20 is a consequence of persistent exposure to the antiretroviral peptide. To establish if patterns of resitance to T-20 were similar to resistance mutations occurring in subtype B viruses, 11 subtype C and 4 subtype B viruses were passaged in the presence of increasing concentrations of T-20. The subtype C isolates showed varying levels of replication at 1 µg/ml T-20 by day 18, but by day 29 all replicated efficiciently at 10 µg/ml T-20. All isolates showed evidence of genotypic changes in gp41 HR-1 following exposure to T-20 that included G36S/E/D, I37T, V38M/A/L/E, N42D, N43K/S, L45R/M and A50T/V. Five viruses had compensatory changes in the HR-2

region, which corresponds to the T-20 sequence, and two isolates had changes in the V3 region. Mutational patterns among the 4 subtype B viruses were similar to those for subtype C and those previously published in the literature. These data indicate that *in vitro* resistance to T-20 develops rapidly among HIV-1 subtype C isolates. In general, mutational patterns for subtype C were similar to those described for subtype B, suggesting that the mechanism of action for T-20 is similar for HIV-1 subtype B and C isolates.

Observations from these studies indicate that HIV-1 subtype C predominantly use the CCR5 coreceptor to enter cells. CXCR4 usage is rare compared to other subtypes, although such isolates are found in patients with advanced AIDS. The two cases of coreceptor switching reported here were dually infected. Subtype C isolates were sensitive to coreceptor and fusion inhibitors except for two isolates able to utilize alternate coreceptors. However, alternate coreceptor usage is very rare and unlikely to impact on the utility of coreceptor inhibitors. Given the propensity for CCR5 usage this may imply that CCR5 coreceptor inhibitors may be more effective in countries where HIV-1 subtype C predominate. Entry inhibitors may be useful for prevention and treatment strategies and have the potential to provide sterilizing immunity. These agents could be used as microbicides and as an adjunct to existing antiretroviral therapies for use in HIV-1 subtype C infected individuals. However resistance to entry inhibitors can emerge and should be used in combination with other antiretrovirals to minimize this outcome. While entry inhibitors provide a new line of defence against HIV-1, their cost may prevent their use in developing countries in the immediate future. Nevertheless, this

is the first comprehensive study of the sensitivity of HIV-1 subtype C isolates to entry inhibitors providing a data-driven rationale for their use in individuals infected with HIV-1 subtype C.

Declaration

I declare that this thesis is my own unaided work unless specified. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted for any other degree or examination at any other University.

_____

Reginald Anthony (Tonie) Cilliers

_____day of _____,_____

This thesis is dedicated to my personal Lord and saviour Jesus Christ.

To Him all the Glory.

In memory of my father Reginald Anthony Cilliers who passed away 17 July 2004

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ABBREVIATIONS

ag	Antigen
AIDS	Acquired immunodeficiency syndrome
AOP-RANTES	Aminooxypentane RANTES
ARV	Antiretroviral therapy
BMVEC's	Brain microvascular endothelial cells
bp	Base pair
C2	Second constant region
CC	Linker between first and second heptad repeat regions
CD4	Cluster differentiation four
CM	Cryptococcal meningitis
COOH	Carboxy terminus
CTL	Cytotoxic T-lymphocytes
CV-N	Cyanovirin-N
DNA	Deoxyribonucleic acid
EC	Effective concentration
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ENF	Enfuvirtide
Env	Envelope protein
env	Envelope gene
FCS	Fetal calf serum
FP	Fusion protein

gp	Glycoprotein
GPCR	G-protein coupled receptor
GPR1	G-protein receptor-1
GPR15	G-protein receptor-15
HAART	Highly active antiretroviral therapy
HHV-6	Human herpes virus type 6
HHV-8	Human herpes virus type 8
HIV-1	Human Immunodeficiency virus type 1
HMA	Heteroduplex mobility assay
HR-1	First heptad repeat region
HR-2	Second heptad repeat region
IC	Inhibitory concentration
IC_{50}	50% inhibitory concentration
IC_{90}	90% inhibitory concentration
IgG2a	Immunoglobulin group 2a
IL-2	Interleukin-2
KDa	Kilodalton
Mabs	Monoclonal antibodies
Mac	Mycobacterium avium cellulare
Met-RANTES	Methione-RANTES
mg	Milligram
MIP-1a	Macrophage inflammatory protein type 1 alpha
MIP-1ß	Macrophage inflammatory protein type 1 beta

ml	milliliter
MOTT	Mycobacterium other than tuberculosis
Mw	Molecular weight
ng	Nanogram
NH	Amino terminus
nM	Nanomolar
NSI	Non-syncytium inducing
^{o}C	Degree Celsius
PBMC	Peripheral blood mononuclear cells
PCP	*Pneumocystis carinii* pneumonia
PCR	Polymerase chain reaction
pg	picogram
PHA	Phytohemagglutinin
pol	Polymerase gene
R5	CCR5 using virus
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
sCD4	Soluble CD4
SI	Syncytium inducing
SIV	Simian immunodeficiency virus
TB	Tuberculosis
$TCID_{50}$	50% tissue culture infectious doses

TM	Transmembrane
TORO	T-20-versus-Optimized-Regimen only
V3	Third variable loop
V5	Fifth variable region
VL	Viral load
vMIP-1	Viral macrophage inflammatory protein type 1
wt	Wild type
X4	CXCR4-using virus
µg	Microgram
µM	Micromolar

CHAPTER ONE

1. INTRODUCTION

The South African HIV-1 epidemic has reached explosive proportions. The total number of HIV-1 infected individuals is estimated to be 5.3 million [4.5million –6.2 million] (www.unaids.org). Over the next five years AIDS deaths will increase dramatically and urgent intervention is required (www.unaids.org). In April 2004 the antiretroviral rollout was initiated and 50,000 individuals are expected to be treated in the first year. It is foreseen that 500,000 people will be treated over the next 7 years. The criteria for treatment include patients with a CD4 count lower than 200 or a diagnosis of clinical AIDS. The provision of anti-retroviral therapy (ARV) in South Africa is expected to reduce morbidity and mortality as seen in other countries where treatment is available.

The predominant strain of HIV-1 circulating in South Africa is subtype C, which is the fastest spreading HIV-1 subtype worldwide (www.unaids.org). Subtype A/+CRF02_AG was estimated to be the second fastest spreading subtype followed by subtype B strains. Subtype A/+CRF02_AG is predominant in Africa together with subtype A and D and other circulating recombinant forms (CRF). Subtype B is most predominant in the developed world such as North America, Europe and Australia. Subtype C predominates in India, China, Ethiopia and other regions in southern Africa. Some evidence exists that subtype C is different from other subtypes in both phenotypic and genotypic characteristics. It was shown that subtype C was almost exclusively non-syncytium inducing (NSI) and did not switch to the syncytium inducing (SI) phenotype (Abebe *et*

al., 1999; Ping *et al.*, 1999; Tien *et al.*, 1999). Only recently have SI variants been identified but the frequency thereof is still low (Cilliers *et al.*, 2003; Janse Van Rensburg *et al.*, 2002; Johnston *et al.*, 2003; Pollakis *et al.*, 2004). More studies are needed on patient cohorts followed longitudinally to determine the frequency of a coreceptor switch in this subtype.

1.1 Natural history of HIV-1 infection in adults and children

HIV-1 is mainly transmitted through bodily fluids during sexual transmission (Haseltine, 1998). HIV-1 can also be transmitted through infected blood products as well as needle sharing amongst intravenous drug users. Sexual transmission of the virus occurs when it crosses the mucosal barrier and attaches to cells that express the necessary receptors to allow HIV-1 infection. After attachment the virus is transported to the local lymph nodes where it replicates and is later detected in the blood. An acute clinical syndrome occurs 2-4 weeks after the initial exposure to the virus. This syndrome presents as flu-like symptoms and it can also affect the central nervous system (Vanhems *et al.*, 2000). During the acute phase viral replication is very high and can be determined using a viral load assay (Mellors *et al.*, 1996). In the initial phases of HIV-1 infection there is a window period when patients are HIV-1 positive, but antibody negative. Antibodies can take up to 3 months to develop and are used to detect HIV-1 infection in an ELISA assay. HIV-1 is accompanied by a cellular as well as a humoral immune response (Fauci, 1996). The rate at which the initial infection is controlled is normally a good indicator of how quickly a patient will progress to develop AIDS (Fauci, 1996). The higher the viral load set point the higher the chances are the person will progress faster to develop AIDS. A

cytotoxic T-cell response in combination with an antibody response helps control the initial phase of infection and to reach a set point, which can remain relatively constant over a long time (Koup *et al*., 1994) (Figure 1.1). The acute phase is followed by the asymptomatic phase where the patient remains disease free. AIDS is the end stage of the disease and is associated with CD4 counts below 200 cells/µl, and the onset of opportunistic infections especially tuberculosis and meningitis.

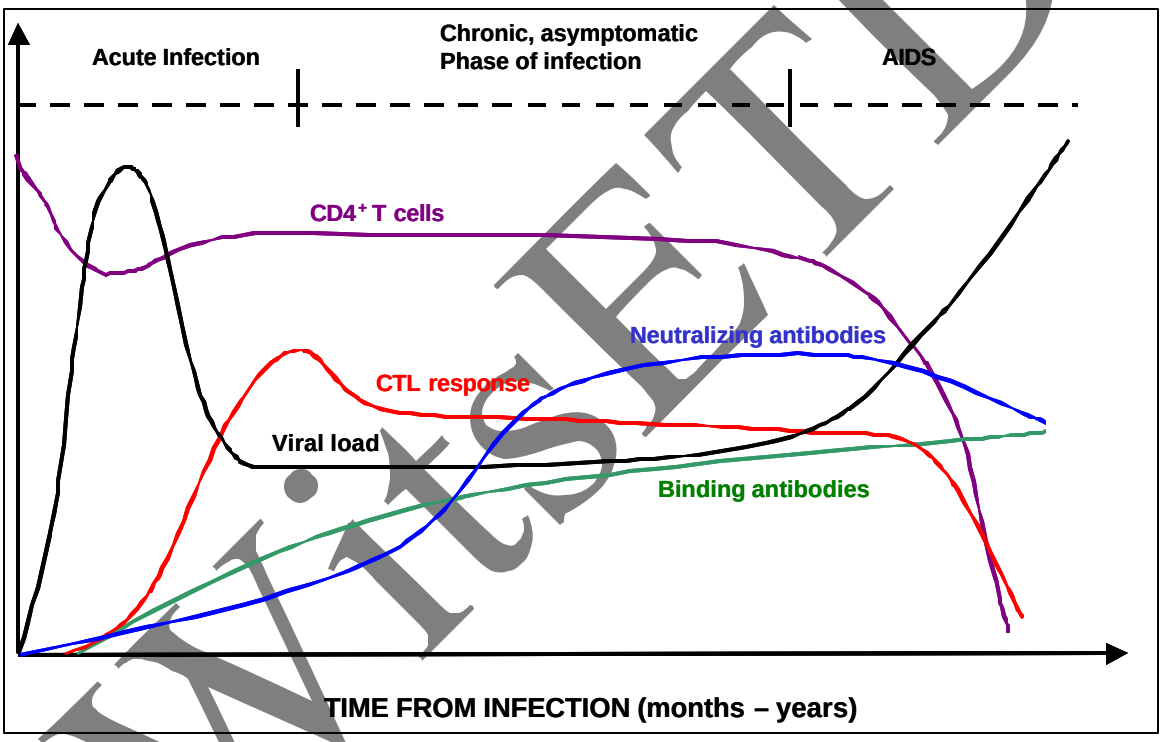


Figure 1.1: Schematic diagram showing the natural history of viral load and CD4 count. Three stages associated with disease progression are shown. HIV replicates and steadily depletes the immune system until the culmination of AIDS. The viral load increases dramatically in the acute phase and CD4 numbers decline. After a few weeks the immune system responds to HIV infection, which reduces the viral load and the CD4 number returns to near normal levels. The CTL response is responsible for control of viremia and to lesser extent the neutralizing antibody response. Neutralizing antibodies take much longer to develop and only play a role later in infection. In the AIDS phase viral load increases and CD4 counts drop. These events allow individuals to remain clinically well for many years. (Courtesy of Natasha Taylor)

In general, children progress at a faster rate than adults in developing AIDS (Powderly, 1997). This might be attributed to the timing of infection, as these infants could be infected *in utero* or as a result of their immature immune system. The highest incidence of AIDS occurs within the first year of life and accounts for over a third of all pediatric cases and are referred to as rapid progressors (RP) (Bobat *et al*., 1999; Powderly, 1997; Scarlatti *et al*., 1996). Some children do live longer and even into their adolescent years. These children are referred to as slow progressors (SP) (Powderly, 1997). Factors influencing this are mostly unexplained, but in some cases attenuation of the virus or defective genes or host genetic defects have been shown to be associated with slow disease progression (Greenough, Sullivan, and Desrosiers, 1999; Papathanasopoulos *et al*., 2003; Pilgrim *et al*., 1997).

1.2. HIV-1 life cycle

HIV-1 requires infection of a host cell to enable it to survive and to multiply. It enters the host cells by binding to CD4 and a cellular coreceptor. This enables the viral envelope glycoprotein to undergo conformational changes and for cell fusion to occur. Once it enters the cell the viral RNA is released into the host cell cytoplasm. The reverse transcriptase enzyme transcribes single stranded RNA into double stranded DNA, which then establishes itself via the enzyme integrase into the host DNA. This enables the virus to make use of the host cell machinery to produce more viral particles. Firstly strands of protein are synthesized which then assembles at the host cell membrane. These are cleaved into functional proteins by the viral protease enzyme. Once all the proteins are

cleaved viral particles bud out of the host cell membrane using parts of the host cell membrane to construct viral particles (Pope and Haase, 2003). Approximately 10^9 viral particles are produced daily (Zhang *et al*., 1999). Knowing how the virus enters and reproduces itself has presented novel approaches to inhibit parts of the viral life cycle. These opportunities to inhibit virus replication can be divided into distinct different phases. Entry inhibitors inhibit the first phase of the viral life cycle by preventing entry of HIV-1. The reverse transcriptase enzyme was one of the first therapeutic targets in preventing virus replication (Kilby and Eron, 2003). Targeting the integrase enzyme is another approach and agents preventing its function have entered clinical trials (Kilby and Eron, 2003). Protease inhibitors are already in use and play a major role in the clinical treatment of patients. Viral budding offers another potential target for inhibiting viral replication but no agent yet has been identified to inhibit this phase of replication.

1.3 Use of highly active antiretroviral therapy

Highly active antiretroviral therapy (HAART) is one of the major factors influencing morbidity and mortality among HIV-infected patients in industrialized countries (Gallant, 2000). The effectiveness of these drugs has been shown by the rapid decline in AIDS deaths in the USA since their introduction (Palella *et al*., 1998). Reverse transcriptase inhibitors can be grouped as nucleoside antagonists, which consist of zidovudine, didanosine, zalcitabine, lamivudine, stavudine, abacavir and tenofovir (Kilby and Eron, 2003). The non-nucleoside reverse transcriptase inhibitors consist of nevirapine, delavirdine, and efavirenz (Kilby and Eron, 2003). The protease inhibitors consist of ritonavir, indinavir, nelfinavir, amprenavir, lopinavir-ritonavir and two combinations of

saquinavir (Kilby and Eron, 2003). These drugs are usually used in combinations as triple drug therapies and prolong the asymptomatic phase of infection and can partially restore immune function. However, drug resistance and poor tolerability in some individuals has focused attention on the need for additional drug therapies (Hirschel and Opravil, 1999).

1.4. HIV-1 entry

In 1986, the CD4 molecule was shown to serve as the primary receptor used by HIV to enter cells (Maddon *et al*., 1986). However, it was soon realized that CD4 was not sufficient for entry. The discovery that chemokines secreted by CD8 cytotoxic T cells inhibit HIV-1 infection, as well as the existence of persons exposed to HIV who remained uninfected, provided clues to this second receptor (Cocchi *et al*., 1995; Paxton *et al*., 1996). Fusin was first identified as the receptor for viruses that caused syncytia (SI) in T-cells and was later renamed CXCR4 (Berger, 1997; Feng *et al*., 1996). A second coreceptor, termed CCR5 was shown to serve as the coreceptor for macrophage tropic or non-syncytium inducing (NSI) HIV-1 isolates (Deng *et al*., 1996; Dragic *et al*., 1996). Together, CD4 and CCR5 and/or CXCR4 serve as the major entry molecules for all subtypes of HIV-1 and the expression of these molecules on CD4 cells determines the cellular host range.

1.4.1 The envelope glycoprotein

The entry of HIV-1 into cells is a multi-step process that is mediated by the viral envelope (Env) glycoprotein (Wyatt and Sodroski, 1998). Env originates as a single chain glycoprotein precursor, gp160, which dissociates to yield the functional glycoproteins

gp120 and gp41. These two subunits assemble non-covalently on the virion surface as trimers (Chan *et al*., 1997; Wyatt and Sodroski, 1998). The envelope region comprises of five variable domains (V1-V5), in which nucleotide substitutions, duplications and deletions produce extensive amino acid diversity and five constant regions (C1-C5), which is responsible for the more conserved functions of the envelope (Lamers *et al*., 1993). The third variale loop (V3 loop) is associated with coreceptor binding and cellular tropism (Shimizu *et al*., 1999; Chan *et al*., 1999). The V3 loop is located between amino acids 296 and 331 of gp120 and has a type 2ß-turn conserved secondary structure and usually consists of 35 amino acids (De Jong *et al*., 1992; Jansson *et al*., 1994). The V3 loop shows a high degree of genetic diversity including substitutions, deletions and insertions (Lamers *et al*., 1993). A minimum of three amino acid substitutions in V3 can confer macrophage tropism and alter T-cell line tropism (De Jong *et al*., 1992). The tip of the V3 loop is very conserved in HIV-1 subtype C isolates with a GPGQ motif in almost all cases whereas for subtype B it is GPGR (Milich *et al*., 1993). Subtype E isolates in Asia also has the GPGQ motif for non-syncytium inducing (NSI) isolates, whereas for syncytium inducing (SI) isolates it is GPGR or GPGH (Menu *et al*., 1999).

A high net charge of V3, due to additional basic amino acids substituting negatively charged amino acids, is correlated with the SI phenotype. SI and NSI variants can be predicted by studying positively charged, or neutral charged amino acid changes at positions 11, 25, 13, 19, 23, 24 and 32 (De Wolf *et al*., 1994; Hwang *et al*., 1991; Milich, Margolin, and Swanstrom, 1997; Shioda, Levy, and Cheng-Mayer, 1991; Yoshimura *et al*., 1996). Determination of the total number of positively charged amino acids in the V3 loop revealed that SI variants have the highest positive charge compared to the NSI

variants (De Wolf *et al*., 1994; Fauci *et al*., 1996).

The crystal structure of the gp120 subtype B strain HXBc2 was elucidated in 1998 in complex with a two-domain fragment of the CD4 receptor and a neutralizing human antibody that blocks chemokine receptor binding (Kwong *et al*., 1998). From this data it was shown that the CD4 binding site on gp120 is fairly well conserved and is located within a cavity at the interface of the outer domain, inner domain and the bridging sheet. A total of 22 CD4 and 26 gp120 amino-acid residues are required for the gp120-CD4 structure to interact (Kwong *et al*., 1998). As a result of the binding to CD4 the Env molecule undergoes conformational changes enabling the virus to bind to the coreceptor. The gp120 molecule requires approximately three CD4 molecules to enable this conformational change to occur (Doms, 2000). An approximately ninety degree angle is formed between the chemokine receptor-binding site and the CD4 binding site and consists of the bridging sheet and the base of the V1/V2 loop (Kwong *et al*., 1998; Parren *et al*., 1999; Wyatt and Sodroski, 1998). Experimental data has shown that the V1/V2 loops partially cover the CD4 and chemokine receptor binding sites (Wyatt and Sodroski, 1998). When gp120 binds CD4 further displacement of the V1/V2 and V3 loop occurs, which allows chemokine receptor binding (Parren *et al*., 1999; Rizutto and Sodroski, 2000). About four to six CCR5 coreceptors are required for viral fusion to occur (Kuhmann *et al*., 2000). Binding to CCR5 induces further conformational changes that activate the fusion machinery of gp41. These changes include the formation of a coiled coil structure domain of gp41 (Chan *et al*., 1997; Weissenhorn *et al*., 1997). Fusion with the cell membrane takes place by insertion of the gp41 subunit into the lipid bilayer of the target cell, thus rendering Env an integral component of the viral and the target cell

membranes (Chan *et al*., 1997; Sattentau, Zolla-Pazner, and Poignard, 1995).

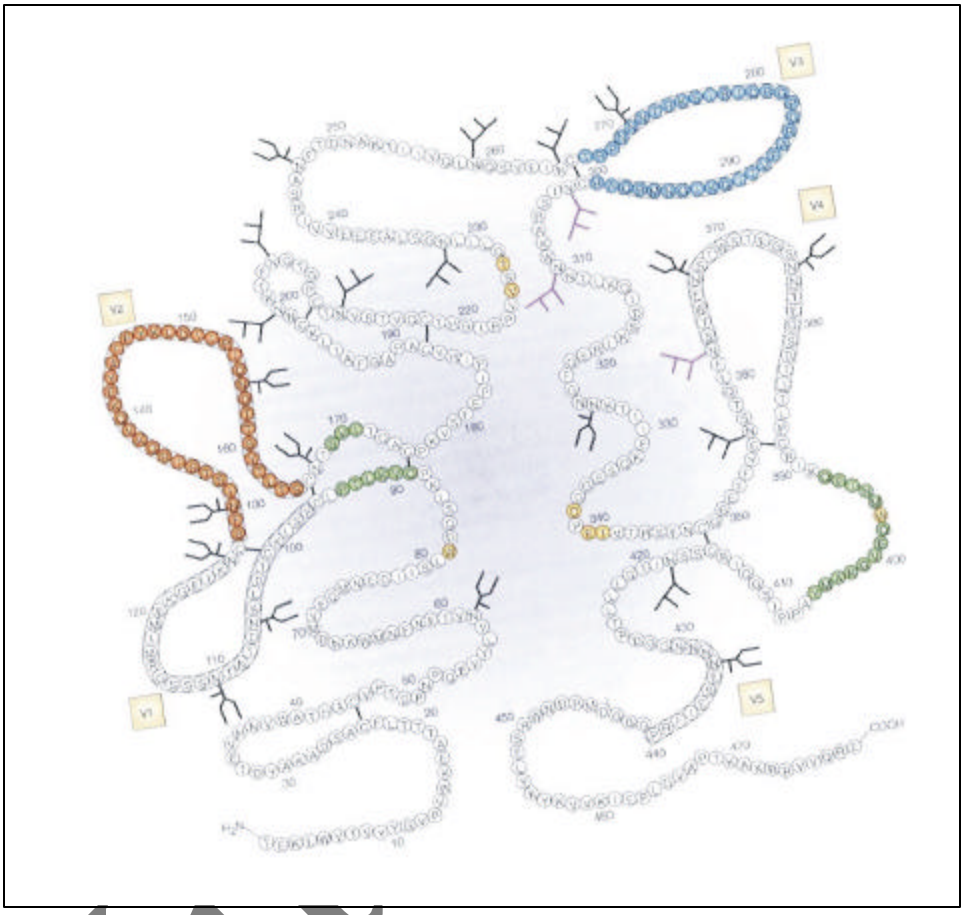


Figure 1.2: A schematic diagram of HIV-1 envelope. The variable loops are shown in boxes (V1-V5). Glycosylations sites containing mannose-type or hybrid-type oligosaccharide structures are indicated by branched structures and glycosylation sites containing complex-type oligosaccharides structures are indicated by the U-shaped branches. Epitopes able to induce neutralizing antibodies are highlighted in color.
Taken from (Zolla-Pazner, 2004)

1.4.2 Coreceptor usage

The discovery of the chemokine receptors CCR5 and CXCR4 as HIV-1 entry coreceptors allowed for a more precise understanding of biological phenotypes (Doms and Peiper,

1997). The MT-2 cell line, which traditionally is used to classify viruses as SI or NSI, express high levels of CXCR4, but do not express CCR5. Thus the ability to grow in MT-2 cells is a function of the ability of SI isolates to use CXCR4. HIV-1 strains that use CXCR4 are designated X4 and those that use CCR5 are called R5 viruses (Berger *et al*., 1998). Viral strains often show preferential use of a coreceptor, although some dual tropic strains have the ability to use both CCR5 and CXCR4 equivalently and are termed R5X4 variants (Berger *et al*., 1998; Glushakova *et al*., 1999). CCR5 and CXCR4 are used by most, if not all HIV-1 strains regardless of genetic subtype (Bjorndal *et al*., 1997; Vodicka *et al*., 1997). CCR5 is expressed on activated lymphocytes, macrophages, dendritic cells and brain cells while CXCR4 is expressed on resting T cells and monocytes (He *et al*., 1997; Lusso, 2000). Viruses isolated from recently infected individuals are almost exclusively CCR5-using indicating that R5 viruses are selectively transmitted. It is thought that macrophages and dendritic cells, which are abundant in mucosal tissues, transport the virions to the lymphoid tissues and are responsible for dissemination of the virus throughout the body.

1.4.3 Structure of CCR5 and CXCR4

The chemokine receptors CCR5 and CXCR4 are seven-transmembrane molecules of the G-protein coupled receptor family (Berger, 1997) (Figure 1.3 and 1.4). They share approximately 32% amino acid identity, although this is reduced to 20% when only the amino acids on the extracellular surface are compared (Doms and Peiper, 1997). Both CCR5 and CXCR4 contain 352 amino acids and have three extracellular loops on the surface of the cell. The extracellular domain contains four cysteine residues (indicated in

red) (Figures 1.3 and 1.4), which form disulfide bonds between the amino terminus and the third extracellular loop and between the first and second extracellular loops (Berson *et al*., 1996). It is likely that mutations in one extracellular domain may indirectly affect the function of neighboring regions and that residues in all four extracellular regions can influence coreceptor usage (Horuk *et al*., 1994). Alanine scanning mutagenesis and the use of monoclonal antibodies has identified important regions in the coreceptor for gp120 binding (Blanpain *et al*., 2003; Dragic, 2001; Dragic *et al*., 1998; Rabut *et al*., 1998; Wu *et al*., 1997a). For both CCR5 and CXCR4, specific amino acids within the amino-terminal domain including negatively charged and tyrosine residues are essential for binding and entry of viral isolates. The second extracellular loop of CXCR4 has also been implicated in entry of X4 and R5X4 strains (Brelot *et al*., 1997; Kajumo *et al*., 2000; Lu *et al*., 1997). Despite the low amino acid homology between CCR5 and CXCR4, HIV–1 isolates have been identified that are able to use both coreceptors very efficiently (Doranz *et al*., 1996; Simmons *et al*., 1996). This might be explained by the fact that coreceptors have common structural features or that a single gp120 molecule engages different determinants for each coreceptor. HIV can evolve to use CXCR4 during disease progression suggesting that the ability of gp120 to interact with specific amino acids within the coreceptor may change over time (Doranz *et al*., 1997; Picard *et al*., 1997). The surface of CXCR4 is negatively charged whereas CCR5 has an almost neutral charge (Moore and Stevenson, 2000). This is an indication that the interaction between CCR5, CXCR4 and the HIV envelope might be charge related and the higher the charge of the gp120 the more likely a viral isolate is to use CXCR4 (Edinger *et al*., 1998).

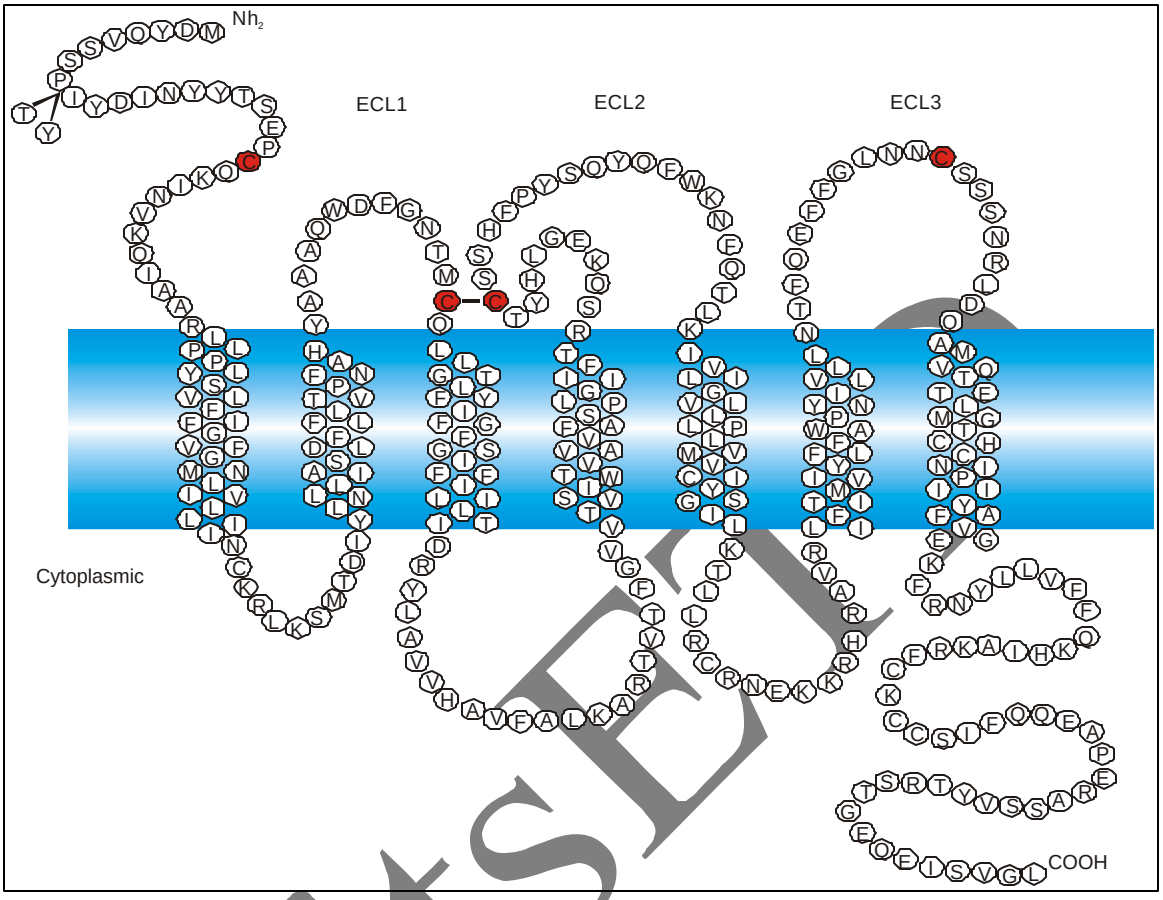


Figure 1.3: Two-dimensional structure of CCR5. The receptor transcends the membrane 7 times. The amino terminus is on the outside and the carboxy terminus on the inside of the cell. The three extracellular domains are indicated. The cysteine residues are shown in red.

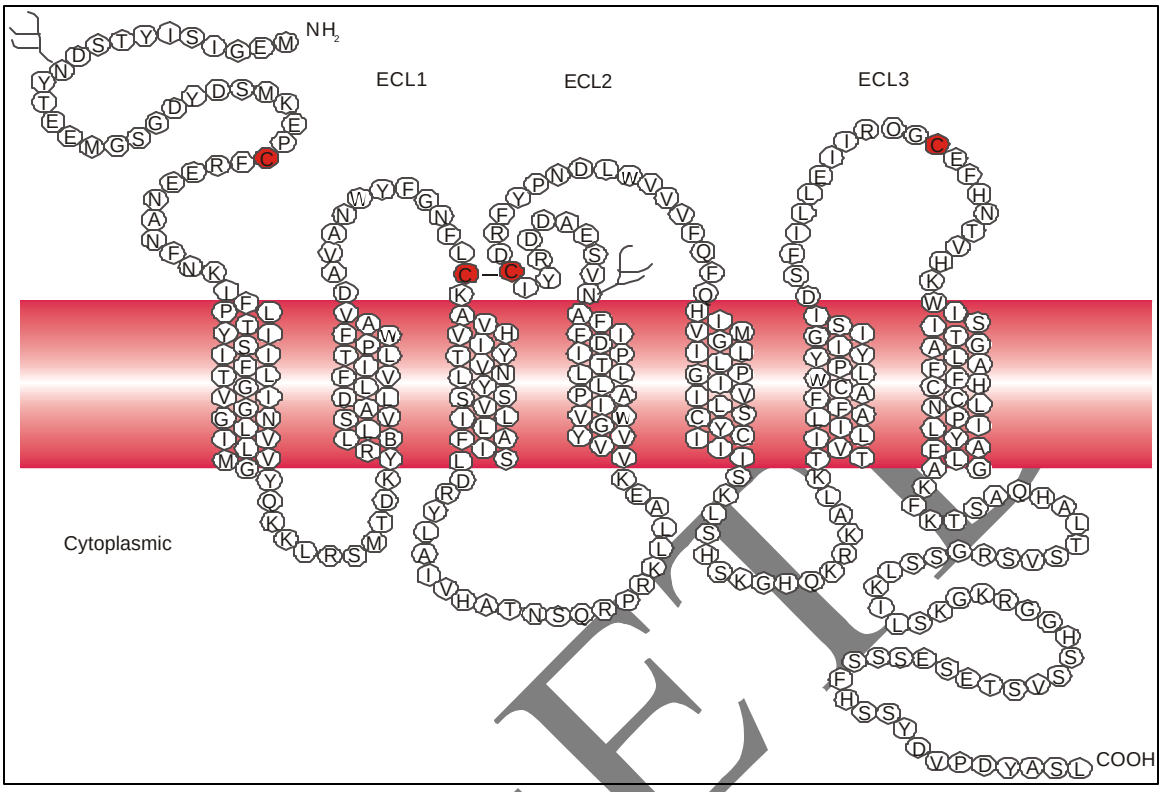


Figure 1.4: Two-dimensional structure of CXCR4. The receptor transcends the membrane 7 times. The amino terminus (NH_2) is on the outside and the carboxy terminus (COOH) on the inside of the cell. The three extracellular domains are indicated. The cysteine residues are shown in red. ECL refers to the extracellular loops 1-3.

1.4.4 Alternate coreceptors

In addition to CCR5 and CXCR4, some HIV-1 isolates are able to utilize other coreceptors, namely CCR1, CCR2b, CCR3, CCR8, CXCR6 (Bonzo/STRL33), Bob/GPR15, and GPR1 (Edinger *et al.*, 1998; Zhang *et al.*, 1998) (Table 1.1). The use of alternate coreceptors is rare and is normally associated with viral isolates from late-stage disease (Pohlmann, Krumbiegel, and Kirchhoff, 1999). In some cases this may provide a selective advantage, for example, the ability of some isolates from cerebrospinal fluid to use CCR3 may allow them to infect microglia, which express both CD4 and CCR3 (He *et*

al., 1997). HIV-1 isolates able to infect thymocytes and utilizing CCR8 was reported indicating use of alternate coreceptors on primary cells (Lee *et al*., 2000). Of all the alternate coreceptors proposed to be utilized by HIV-1, CCR3 and CCR8 appear to be the most commonly used (Table 1.1). Hoffman and co-workers have shown that the V1 and V2 loops play an important part in the utilization of minor coreceptors *in vitro* (Hoffman *et al*., 1998). However, the role of these minor coreceptors *in vivo* still remains uncertain (Zhang and Moore, 1999).

Use of known and unknown alternate coreceptors has been more commonly reported amongst HIV-2 isolates (Guillon *et al*., 1998; McKnight *et al*., 1998; Reeves *et al*., 1998; Reeves *et al*., 1997). Two HIV-2 isolates were able to use an unidentified coreceptor as they were able to productively infect PBMC but were unable to replicate in cell lines expressing CCR5, CXCR4, CCR1, CCR2, CCR3, CXCR6 and Bob/GPR15 (Azevedo-Pereira *et al*., 2003). Similar results were found by Willey *et al*., in that HIV-2, SIV and HIV isolates were able to infect CCR5 defective PBMC and brain microvascular endothelial cells (BMVEC's) which lack both CCR5 and CXCR4 (Willey *et al*., 2003). Most of these HIV-2 isolates tested had an R5X4 phenotype except one, which used CXCR4 exclusively (Willey *et al*., 2003). These viruses were inhibited by vMIP-1, a chemokine secreted by human herpes virus-8 (HHV-8), which is able to bind to a wide range of chemokine receptors including CCR8, GPR1 and CXCR6 thus indicating an unknown route of entry into cells relevant to *in vivo* infection (Willey *et al*., 2003).

Table 1.1: *In vitro* use of coreceptors by HIV-1.

Coreceptor	Ligand	HIV-1	Reference for coreceptor use
CCR1	MIP-1a, MPIF-1, MCP-3, RANTES	-*	Bron et al., 1997; McKnight et al., 1998
CCR2b	MCP-1, MCP-2, MCP-3	+	Doranz et al., 1996
CCR3	Eotaxin, eotaxin-2, MCP-3, MCP-4, RANTES	++	Choe et al., 1996; Doranz et al., 1996
CCR4	MDC, TARC, RANTES, MIP-1	-	McKnight et al., 1998
CCR5	**MIP-1a, MIP-1ß, RANTES, MCP-2**	**++++**	**Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996**
CCR8	I-309	++	Rucker et al., 1997
CCR9	TECK	+	Choe et al., 1998
CXCR4	**SDF-1**	**+++**	**Feng et al., 1996**
CX3CR1/V28	Fractalkine	+	Reeves et al., 1997
CXCR6/Bonzo	CXCL16	+	Alkhatib et al., 1997; Deng et al., 1997
GPR1	?	+	Farzan et al., 1997
Bob/GPR15	?	+	Farzan et al., 1997
Apj	Apelin	+	Choe et al., 1998; Edinger et al., 1998
Chem R23	?	+	Samson et al., 1998
RDC1	?	+	Shimizu et al., 2000

* Rarely or never used as a coreceptor (-); occasional use by a few isolates (+); used by 5-20% of isolates (++); frequent use by many isolates or by major subgroup (+++) (for example CXCR4 used by X4 and R5X4 isolates in bold); major coreceptor used by predominant virus *in vivo* (++++) (for example CCR5 usage by HIV-1 R5 isolates).

Taken from (Clapham and McKnight, 2002)

1.5 Resistance to HIV-1 infection

HIV-1 variants transmitted sexually are associated with CCR5 as the coreceptor of preference (Rana *et al*., 1997; Samson *et al*., 1996a). This coreceptor was first discovered by analyzing cells from people who engaged in high-risk sexual behavior, but who remained HIV-uninfected (Connor *et al*., 1996; Paxton *et al*., 1996; Samson *et al*., 1996a). Subsequently a 32-base pair deletion (Δ32) in the CCR5 gene in the region encoding the second extra-cellular loop was discovered which results in a frame shift and premature truncation of the protein (Liu *et al*., 1996; Samson *et al*., 1996b). This truncated polypeptide is expressed, but does not appear on the cell surface and lacks coreceptor activity (Liu *et al*., 1996; Rana *et al*., 1997; Samson *et al*., 1996a). Individuals

who are homozygous for Δ32 CCR5 are highly resistant, but not immune to HIV-1 infection as they can be infected by viruses that use CXCR4 (Biti *et al.*, 1997; O'Brien *et al.*, 1997; Theodorou *et al.*, 1997). Individuals who are heterozygous for Δ32 CCR5 progress to disease more slowly and have a much lower viral load, probably as a result of reduced expression of CCR5 (Dean *et al.*, 1996; Huang *et al.*, 1996; Michael *et al.*, 1997). The allele frequency of Δ32 CCR5 in the Caucasian population is approximately 10%, with 1% being homozygous and approximately 18% being heterozygous (Dean *et al.*, 1996; Samson *et al.*, 1996b). This mutation is very rare among African populations (Williamson *et al.*, 2000). The surprisingly high allelic frequency in the western European population suggests some selective pressure in the past, which gave individuals with this allele a survival advantage. The Δ32 CCR5 polymorphism was proposed to have a protective advantage against bubonic plague but no difference in protection or survival rate were noted when BALB/c and SCID mice were challenged with *Yersinia pestis* or *Y. pseudotuberculosis* (Mecsas *et al.*, 2004). This indicates that some other pathogen or natural selection is responsible for the occurrence of this mutation. While the Δ32 CCR5 polymorphism explains why some individuals remain uninfected by HIV, despite multiple exposures to the virus, only a minority of highly exposed persistent seronegative individuals are homozygous for the Δ32 CCR5, indicating that there are other naturally occurring mechanisms for HIV resistance.

1.6 Entry inhibitors

HIV-1 entry into a cell is a multi-step process and involves the envelope region of HIV-1 and two molecules on the cell surface (Wyatt and Sodroski, 1998). This entry step occurs

in three distinct phases, allowing multiple opportunities for intervention. Entry inhibitors can act at all three stages during this process: the attachment of gp120 to CD4, the interaction of the gp120-CD4 complex with a coreceptor and the gp41 mediated membrane fusion process. Agents that target these 3 steps are broadly referred to as entry inhibitors (Figure 1.5). Novel compounds targeting each one of these three stages are in development and one, T-20, has already been registered by the Food and Drug Administration and is in use as a therapeutic agent in combination with other antiretroviral agents.

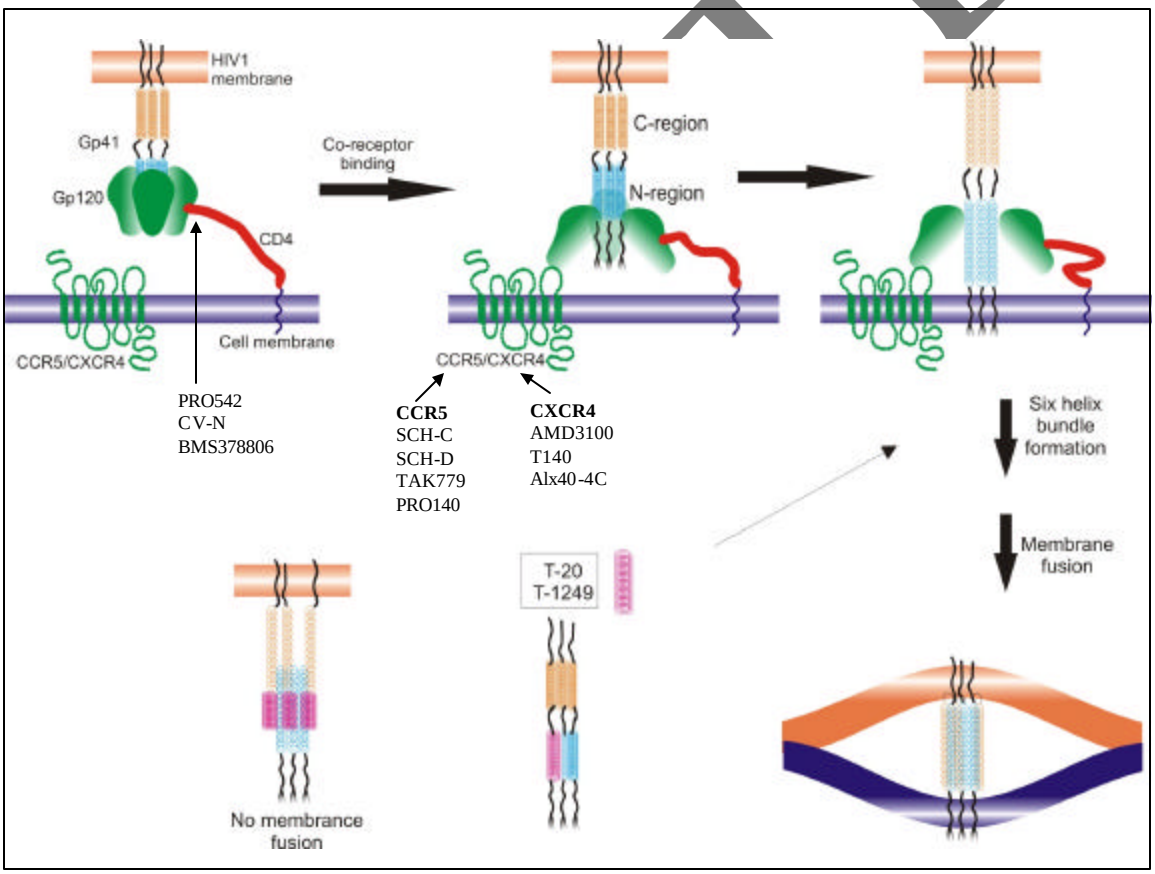


Figure 1.5: Three stages of HIV-1 entry and specific inhibition of each stage. The sequential interactions of HIV-1 gp120 with the host cell membrane are depicted. The first interaction is gp120-CD4 followed by coreceptor binding which then allows fusion of the host and virus cell membranes. Compounds able to prevent CD4 binding and coreceptor binding are indicated using arrows to show binding sites and which interactions are interrupted. The mechanism of action for T-20 and T1249 is shown.

1.6.1 Preventing CD4-gp120 interactions

1.6.1.1 PRO542

PRO542 is a tetravalent CD4-immunoglobin fusion protein that comprises the D1 and D2 domains of human CD4 genetically fused to the heavy and light constant regions of human IgG2. This agent is also known as CD4-IgG2 (Allaway *et al*., 1995). It consists of two heavy chains from a human-CD4-IgG2 heavy chain fusion protein and two chains of a CD4-human kappa light-chain fusion protein (Figure 1.6) (Allaway *et al*., 1995). PRO542 has four gp120 binding sites, which make it more stable and far more effective than soluble CD4 (Allaway *et al*., 1995). Phase-I clinical trials have shown that PRO542 is safe and has satisfactory pharmacological properties (Jacobson *et al*., 2000). The antiviral effect of PRO542 was very effective and a reduction of up to 2-$\log_{10}$ copies/ml was observed one to two weeks post administration (Jacobson *et al*., 2004; Jacobson *et al*., 2000). Similar results were seen for HIV-infected children who took part in a Phase-I clinical trial (Shearer *et al*., 2000). No free virus was detected up to 72 hours after treatment, which demonstrate long lasting effects of PRO542. PRO542 has the ability to selectively eliminate X4 and R5X4 viruses from treated patients rendering R5 viruses post-treatment, which is consistent with other antiretroviral agents when treating patients. (Jacobson *et al*., 2004). PRO542 may be particularly useful as a salvage therapy as the most notable effects were seen in patients with high viral loads and low CD4 counts (Jacobson *et al*., 2004).

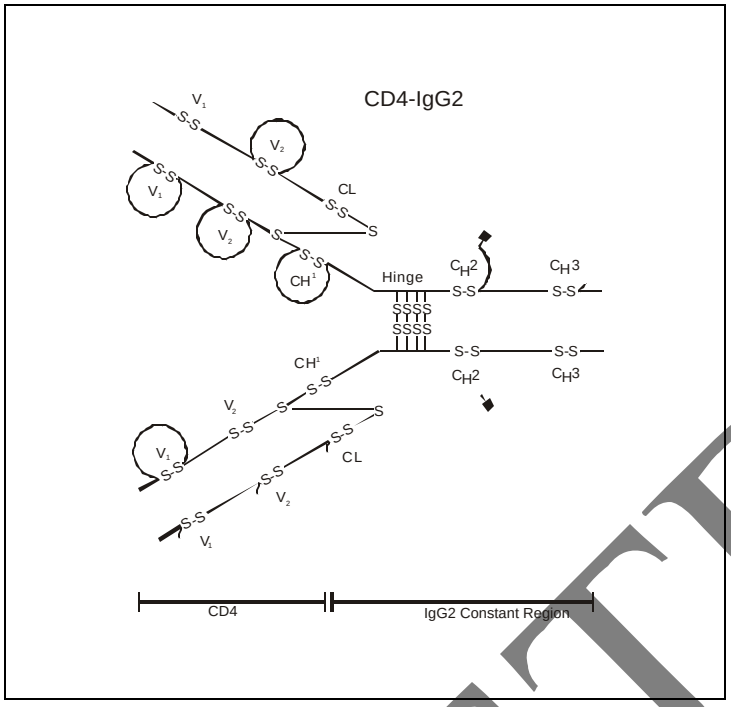


Figure 1.6: The structure of PRO542 (Taken from Allaway *et al*., 1995)

1.6.1.2 BMS-378806

BMS-378806 is a small molecule compound and has inhibitory activity by binding to or close to the CD4 binding site on gp120 (Guo *et al*., 2003; Lin *et al*., 2003). It has been shown to have high potency with an effective concentration of 40 nM against HIV-1 but is ineffective against HIV-2 and Simian Immunodeficiency Virus (SIV) (Lin *et al*., 2003). Its inhibitory activity is independent of coreceptor usage, which makes it a useful agent to treat HIV (Guo *et al*., 2003; Lin *et al*., 2003). It has significant pharmacological advantages such as low protein binding, minimal human sera effect on anti-HIV potency. It has good oral bioavailablity and low toxicity in animal studies (Lin *et al*., 2003). Although it was shown to be very potent against most HIV-1 subtype B strains resistance to this compound developed by amino acid substitutions in the CD4 binding region

within 20 days or more (Lin *et al*., 2003). Three amino acid differences were prominent with an M426L, M434I/V/T and M475I being able to render these isolates resistant to the compound (Guo *et al*., 2003; Lin *et al*., 2003). Mutations in other regions were also noted and had a complementary role in developing resistance (Lin *et al*., 2003). Analysis of sequences from non-B subtypes show a high frequency of naturally occurring resistance mutations, suggesting that it may be less useful in other subtypes (Moore *et al*., 2004).

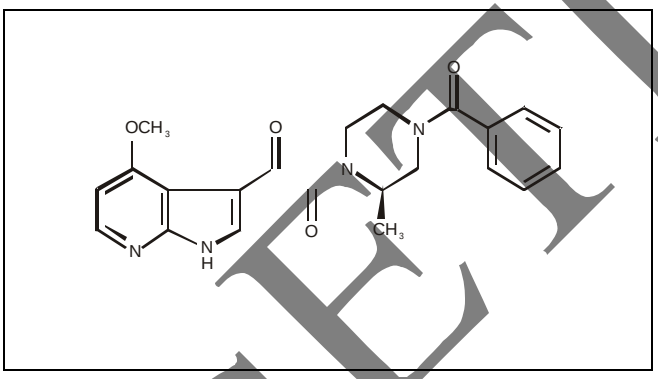


Figure 1.7: The structure of BMS-378806 (Taken from Guo *et al*., 2003)

1.6.1.3 Cyanovirin-N

Cyanovirin–N (CV-N) is a protein compound consisting of 101 amino acids (11-kDa) and was extracted from the cyanobacterium *Nostoc ellisporum* (Boyd *et al*., 1997). It has been produced in recombinant form in *Escherichia coli* and has identical properties to natural CV-N (Boyd *et al*., 1997). It is extremely stable and able to withstand denaturation by heat, denaturants, detergents, organic solvents and can withstand multiple freeze thaw cycles (Boyd *et al*., 1997). CV-N targets the virion attachment to the host cell and blocks the CD4-gp120 interaction. CV-N recognizes Man (alpha) 1→ 2Man-linked

mannose residues. CV-N thus also is able to block 2G12 binding to the viral envelope since this antibody recognizes an epitope that includes complex and hybrid carbohydrate residues. CV-N targets the same epitope as the 2G12 monoclonal antibody and prevents CD4 binding to HIV envelope possibly through steric hindrance, importantly the binding patterns of CV-N overlap but are not identical (Esser *et al*., 1999; Wyatt and Sodroski, 1998). CV-N has shown antiviral activity against a broad range of viruses such as HIV-1, HIV-2, SIV, human herpes virus 6 (HHV-6), Ebola and influenza virus (Barrientos *et al*., 2003; Boyd *et al*., 1997; O'Keefe *et al*., 2003). This compound might be especially useful as a therapeutic agent as well as a topical microbicide as a result of its broad activity (Dey *et al*., 2000; O'Keefe *et al*., 2003).

1.6.2 CCR5 Inhibitors

1.6.2.1 RANTES

RANTES (regulated upon activation, normal T cell expressed and secreted) is a ß-chemokine secreted by cytotoxic CD8 T-cells and binds to CCR5 and other chemokine receptors. It plays an important role during the inflammatory response and is responsible for leukocyte migration (Fernandez and Lolis, 2001; Wu *et al*., 1997a). It is the natural ligand for CCR5 and prevents HIV from entering the cell by binding to CCR5 (Cocchi *et al*., 1995; Trkola *et al*., 1996; Trkola *et al*., 2001). RANTES has been speculated to play a major role during disease progression in that high RANTES concentrations might inhibit R5 strains and thus prolong the asymptomatic phase or predict the switch to X4 variants (Saha *et al*., 1998). It was shown by Trkola and coworkers that very high concentration of RANTES (>500 ng/ml) might enhance HIV infection (Trkola *et al*.,

1999). A proposed mechanism might be that RANTES bind free virions and enhances virus cell interactions (Trkola *et al*., 1999). Escape of R5 variants from RANTES has been shown in rapid progressors but not in long-term survivors predicting a role for RANTES in controlling HIV-1 viral load as well as progression to disease (Koning *et al*., 2003).

RANTES has proven to be a useful agent to screen for other small molecule inhibitors of CCR5 as they compete for the same binding site on CCR5 (Strizki *et al*., 2001). Examples of compounds identified in this way include SCH-C and SCH-D, which are potent inhibitors of R5 isolates (Strizki *et al*., 2001) (see below).

A derivative of RANTES called AOP-RANTES was generated by adding an aldehyde-like group to the amino terminal and then adding amino-oxy-pentane (Simmons *et al*., 1997). Met-RANTES was developed by adding a methionine at the amino terminus and has potent antagonistic effects to RANTES (Simmons *et al*., 1997). Met-RANTES and AOP-RANTES have similar abilities at inhibiting R5 isolates compared to natural RANTES (Gordon *et al*., 1999; Simmons *et al*., 1997). In contrast, excess concentrations of AOP-RANTES can enhance HIV infectivity possibly via crosslinking with glycosaminoglycans (Gordon *et al*., 1999; Marozsan *et al*., 2001). Both Met-RANTES and AOP-RANTES are only effective against R5 isolates and are not effective against X4 isolates (Simmons *et al*., 1997).

1.6.2.2 TAK779

TAK779 prevents HIV-1 infection by binding a pocket formed by the trans-membrane helices 1, 2, 3 and 7 of CCR5 and does not induce down-modulation or signaling of the

receptor (Baba *et al*., 1999; Dragic *et al*., 2000). It has high specificity for CCR5 and CCR2 (Baba *et al*., 1999; Dragic *et al*., 2000). It has an IC_{50} ranging between 2-4 nM (Dragic *et al*., 2000). TAK779 is not being considered for clinical application but is a useful inhibitor of R5 isolates *in vitro* (Trkola *et al*., 2001).

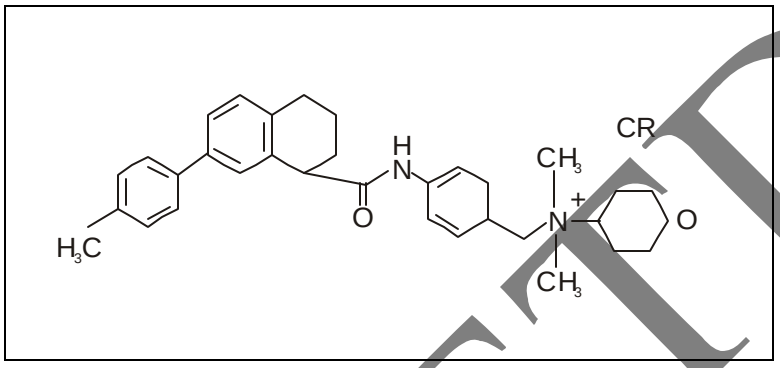


Figure 1.8: Structure of TAK779 (Taken from Baba *et al*., 1999)

1.6.2.3 PRO140

PRO140 is an anti-CCR5 monoclonal antibody and has high specificity for the cellular coreceptor CCR5 (Olson *et al*., 1999). It does not induce down-modulation or signaling of the receptor (Olson *et al*., 1999). It is very potent and prevents R5 strains from binding to the coreceptor and has no ability to block X4 isolates and has an IC_{90} of 2 µg/ml (Trkola *et al*., 2001). PRO140 binds amino acids in the amino terminus and second extracellular loop of CCR5 preventing both gp120 and chemokine binding (Olson *et al*., 1999). PRO140 is effective against most R5 HIV-1 strains including HIV-1 subtype C isolates (Cilliers *et al*., 2003; Trkola *et al*., 2001). No resistance mutants have thus far been shown for PRO140, which makes it very useful as a possible drug therapy (LaBranche *et al*., 2001). It is still in pre-clinical testing.

1.6.2.4 SCH-C (SCH-351125) and SCH-D

SCH-C is a small non-peptide molecule called oxime-piperidine and has a molecular weight of 557.5 KDa (Strizki *et al*., 2001). It is very specific for CCR5 with no cross reactivity to other chemokine receptors and prevents R5 viruses from binding to CCR5 (Strizki *et al*., 2001). It has a mean IC_{50} concentration ranging between 0.4 and 0.9 nM for primary R5 HIV-1 isolates (Strizki *et al*., 2001). SCH-C binds to a part of the second extracellular loop of CCR5 and binding is dependent on a cavity formed in helices 1, 2, 3, 5 and 7 (Tsamis *et al*., 2003). Binding of SCH-C to CCR5 disrupts the structure and thus prevents chemokine and HIV-1 interacting with the coreceptor (Tsamis *et al*., 2003). The residues that play a role in SCH-C binding to CCR5 were found to be L33 and Y37 in the first transmembrane region (TM1), D76 and W86 in TM2, F113 in TM3, I198 in TM5 and E283 in TM7 (Tsamis *et al*., 2003). It was found to be inactive against one HIV-1 subtype G isolate and it might be due to this virus' ability to use another coreceptor or use CCR5 in the presence of SCH-C (Strizki *et al*., 2001). It has good pharmacokinetic profile in rats and monkeys and has a serum half-life of 5-6 hours (Strizki *et al*., 2001). However human clinical trials have been less favorable due to cardiac irregularities (Reyes, 2001).

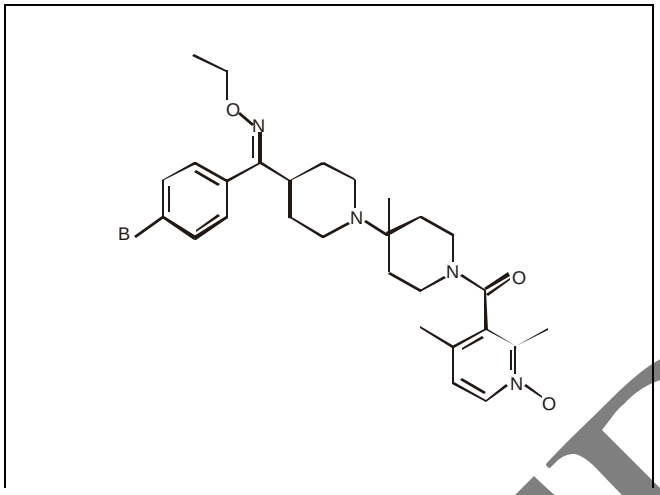


Figure 1.9: Chemical structure of SCH-C (Taken from Tremblay *et al*., 2002)

SCH-D is a follow-up compound of SCH-C. It has a different structure compared to SCH-C but the mechanism of action is more or less the same. It is reported to be more potent than SCH-C *in vitro* (Schurmann *et al*., 2004). SCH-D has a longer half-life, better absorption and higher bioavailability in rat and monkey studies (Schurmann *et al*., 2004). It has entered clinical trials and shown to be more effective at the higher dose range. It had a longer lag phase before viral rebound compared to patients in the lower dose trial group. This might be due to saturation of the CCR5 receptors giving prolonged inhibition of viral rebound (Schurmann *et al*., 2004).

1.6.3 CXCR4 Inhibitors

Inhibitors targeting CXCR4 are highly cationic compounds and specific for CXCR4 (Fujii, Nakashima, and Tamamura, 2003). Targeting CXCR4 as a potential therapy may

prove to be very difficult as CXCR4 is important for embryogenesis and both CXCR4 and its natural ligand stromal cell derived factor-1 (SDF-1) play important immunological roles (Fernandez and Lolis, 2001). Nevertheless a number of compounds have been developed.

1.6.3.1 SDF-1a

SDF-1 consists of 67 amino acids and is the natural ligand for CXCR4 (Bleul *et al*., 1996; Oberlin *et al.*, 1996). It is highly specific for CXCR4 as no other chemokine receptor has been shown to be able to interact with SDF-1 (Bleul *et al*., 1996; Oberlin *et al*., 1996). SDF-1 is highly efficient at attracting lymphocytes (Bleuel *et al* 1996). Maximal lymphocyte chemotaxis was induced at 1µg/ml of SDF-1 (Bleuel *et al*., 1996).

1.6.3.2 AMD3100

AMD3100 is a bicyclam and is a cationic compound that binds to the extracellular loops of CXCR4 (Labrosse *et al*., 1998; Schols *et al*., 1997) (Figure 1.9). It has an effective concentration in the nanomolar range and it prevents X4 and R5X4 isolates from binding to CXCR4 and has no activity against R5 viruses (Donzella *et al*., 1998; Labrosse *et al*., 1998; Schols *et al*., 1997). Resistance to AMD3100 takes a long time to develop *in vitro* and occurs after 50-60 serial passages, which is likely to give it a favorable resistance profile (Hendrix *et al*., 2000). It is not orally available and is administered intravenously or sub-cutaneously. It has an *in vivo* half-life of approximately 0.9 hours (Hendrix *et al*., 2000). AMD3100 was the first known chemokine receptor blocker to enter Phase I clinical trials (Hendrix *et al*., 2000). However AMD3100 caused an elevated white blood

cell count and due to this side effect no further clinical trials were undertaken (Hendrix *et al*., 2000).

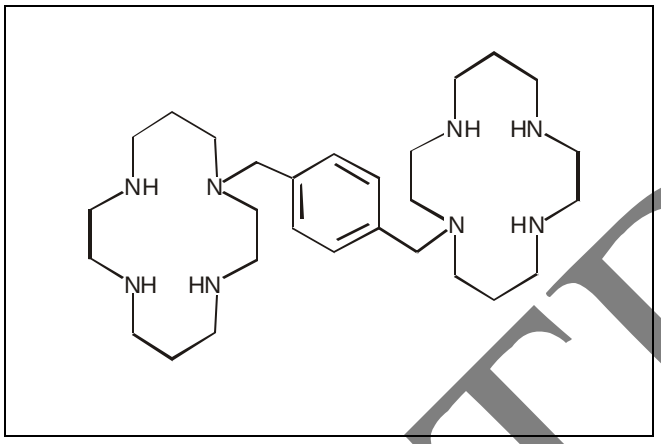


Figure 1.10: Structure of AMD3100. (Taken from Hendrix *et al*., 2000)

1.6.3.3 T-140 and T22

T22 is the precursor peptide compound of T-140 and is a synthetic polyphemusin analogue consisting of 18 amino acids and two disulfide bridges forming an anti-parallel ß-sheet structure (Figure 1.10) (Tamamura *et al*., 1996; Tamamura *et al*., 1998b). It has a half maximal effective concentration at 290 nM *in vitro* and has specificity for X4 viruses (Tamamura *et al*., 1996). T-140 is a smaller peptide and consists of 14 amino acid residues with a single disulfide bond (Tamamura *et al*., 1998a; Tamamura *et al*., 1998b). It has higher anti-HIV activity at a lower IC_{50} of 12 nM.

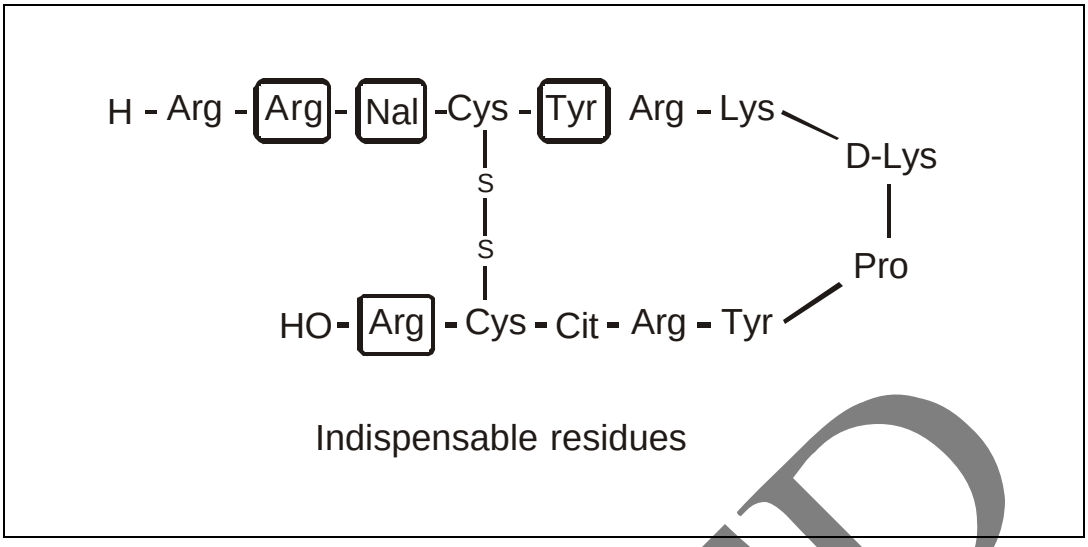


Figure 1.11: The peptide structure of T-140 taken from (Tamamura *et al*., 2003)

1.6.3.4 ALX40-4C

ALX40-4C is a peptide consisting of nine-D-Arginine residues (Doranz *et al*., 1997; Nakashima *et al*., 1992; O'Brien *et al*., 1996). It has specificity against X4 viruses but is ineffective against R5 viruses. It has an approximate IC_{50} of 3 nM for X4 strains (O'Brien *et al*., 1996). Due to displacement studies with both 12G5 (a monoclonal antibody to CXCR4) it is postulated that ALX40-4C binds to the first and second extracellular loops of CXCR4 (Doranz *et al*., 2001; Doranz *et al*., 1997). ALX40-4c proved to be safe and well tolerated in Phase-I/II clinical trials. It was tested on 40 patients, 12 of whom harbored X4 viruses (Doranz *et al*., 2001). Due to low efficiency of this compound to broadly inhibit HIV-1 clinical development of this compound was halted (Doranz *et al*., 2001). Even though this clinical trial did not result in the desired outcome it proved that X4 inhibitors could be safely administered without severe adverse side effects even though the effects on white blood cell counts were not investigated (Doranz *et al*., 2001).

1.6.4 Inhibiting gp41

The fusion process is another useful target for entry inhibitors. The gp41 consists of a fusion peptide and 2-terminal heptad repeat regions (HR-1 and HR-2), which are coiled coils and fold into each other to enable fusion to take place (Chan *et al*., 1997). These two heptad regions contain conserved areas that can serve as target areas for development of entry inhibitors.

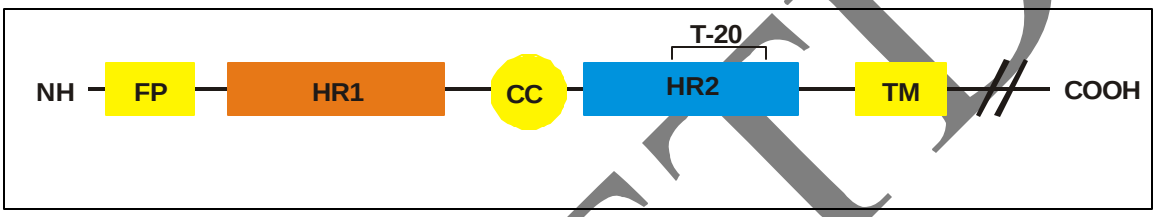


Figure 1.12: A schematic map of gp41 is shown from the amino terminus to the carboxy terminus. The fusion peptide (FP) is situated at the amino terminus and acts as a spike that inserts into the host cell membrane. The HR-1 region is the first heptad-repeat region and forms part of the coiled coil structure. The CC is the linker between HR-1 and HR-2 and acts as a hinge during fusion. The second heptad-repeat region (HR-2) is shown in blue and is situated near the transmembrane region (TM) of the virus**.** The region from which T-20 was derived is indicated (Taken from (Kilby and Eron, 2003).

1.6.4.1 T-20

T-20 is a 36 amino acid peptide derived from the HR-2 sequence of the HIV-1_{LAI} subtype B gp41 envelope glycoprotein (Wild *et al*., 1994) (Figure 1.11 and Figure 1.12). It interferes with the formation of the six-helical bundle by binding to the HR-1 region of gp41, thereby preventing fusion between the viral and host cell membranes (Kilby *et al*., 1998; Wild *et al*., 1994). T-20 is the first in a new class of anti-retroviral drugs termed entry inhibitors licensed for use in humans and is known by the name Enfuvirtide or Fuzeon. In Phase III human clinical trials this drug has been shown to be effective for patients in deep salvage therapy including those with viruses resistant to reverse

transcriptase and protease inhibitors (Lalezari *et al*., 2003b). Phase III clinical trials involving T-20 were referred to as T-20 versus Optimized Regimen Only (TORO). TORO I trials enrolled 491 patients from North America and Brazil and the TORO II trial enrolled 504 patients in Europe and Australia (Clotet *et al*., 2004; Lalezari *et al*., 2003a). Results from these clinical trials showed that patients who received T-20 as part of their combination regimen achieved a mean reduction in HIV levels of 1.43 $\log_{10}$ copies/ml compared to a mean of 0.65 $\log_{10}$ copies/ml in a control group at 24 week analysis (Clotet *et al*., 2004; Lalezari *et al*., 2003a). Serum antibodies to T-20 and preexisting antibodies to gp41 had no effect on T-20 efficacy (Walmsley *et al*., 2003). Adverse effects included reactions at the site of injection and other minor side effects, which could not be linked to T-20 (Clotet *et al*., 2004; Lalezari *et al*., 2003a). Bacterial pneumonia was more frequent among patients taking T-20 than in patients on the optimized regimen alone (Lalezari *et al*., 2003a). T-20 was effective for children at lower dosages but the same target plasma viral loads were achieved (Soy *et al*., 2003). Thus T-20 is an effective therapeutic agent with application for both adults and children with good tolerability combined with good pharmacokinetics profiles and efficacy. T-20 needs to be injected, as it is not orally available. It is very expensive to produce and was initially used as a salvage therapy but has become more freely available (www.fuzeon.com). The use of the drug is likely to be restricted in developing countries due to high cost.

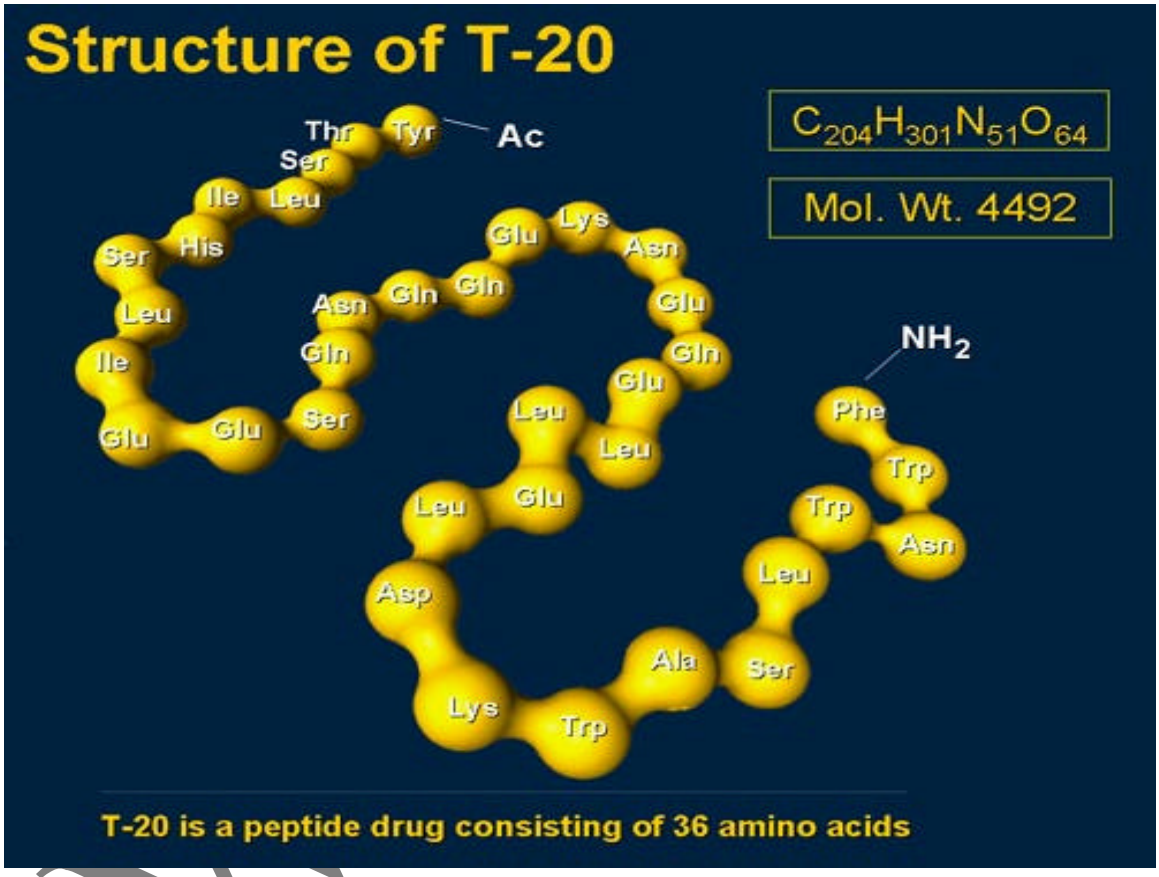
Coreceptor interactions have been reported to play a role in T-20 efficiency with CCR5-using isolates requiring higher concentrations of T-20 to inhibit replication compared to CXCR4-using viruses (Derdeyn *et al*., 2000; Derdeyn *et al*., 2001; Reeves *et al*., 2002). It

was found that expression levels of CCR5 on the host cell could influence the effectiveness of T-20 (Reeves *et al*., 2002). Reduced coreceptor affinity can also affect fusion levels and viral infectivity although there is no correlation between coreceptor affinity and entry inhibitor sensitivity (Reeves *et al*., 2004). A point mutation outside the ß-21 sheet did affect coreceptor and TAK779 affinity but had no impact on the fusion kinetics or sensitivity to T-20 (Reeves *et al*., 2004).

Resistance to T-20 is associated with changes in the HR-1 region, particularly the GIV motif at positions 36 and 38 in gp41 (Hanna *et al*., 2002; Kilby *et al*., 2002; Villahermosa *et al*., 2003; Wei *et al*., 2002). The changes occur mostly in G36D/S/V/A and V38M and G37V (Poveda *et al*., 2002; Rimsky, Shugars, and Matthews, 1998; Roman *et al*., 2003; Wei *et al*., 2002). A V38 mutation has an approximately 20-fold increase in resistance (Lu *et al*., 2004). The N42T and N42D mutants confer two and fourfold resistance and the N43S and N43K confer five-and six-fold resistance. Combinations of these resistance mutations confer much higher levels of resistance with N42T/N43K approximately 32-fold resistance and V38A/N42T approximately 149-fold higher resistance (Lu *et al*., 2004). An order of fitness was determined and shown to be WT>N42T>V38A>N42T/N43K~N42T/N43S>V38A/N42D~V38AN42T>V38E/N42S (Lu *et al*., 2004). These orders of fitness were determined on X4 envelopes, which may be different for R5 envelopes (Lu *et al*., 2004). Changes in the V3 loop as well as sensitivity to T-20 are determined in the V3 loop based on coreceptor usage (Derdeyn *et al*., 2000; Derdeyn *et al*., 2001; Reeves *et al*., 2002). Changes in the HR-2 region from

which T-20 was derived have also been reported to undergo changes to facilitate resistance (Poveda *et al*., 2002).

Figure 1.13: The structure of T-20 consists of 36 amino acids and targets the HR-1 region of gp41. (www.trimeris.com)



1.6.4.2 T-1249

T-1249 is the follow-up compound to T-20. It is a designer peptide consisting of 39 amino acids and was developed to be effective against T-20 resistant isolates and was derived from sequences of HIV-1, HIV-2 and SIV (Eron *et al*., 2004). The inclusion of HIV-2 and SIV sequences gives it a broader range of activity. It is currently in Phase I clinical trials and was used for patients failing phase II/III clinical trials with T-20 due to

resistance. Among these patients a drop of HIV plasma RNA of 1.1-log10 copies/ml was seen (Eron *et al*., 2004). T-1249 was well tolerated over a 14-day treatment period (Eron *et al*., 2004). Most patients had injection site reactions and these increased as the dose of T-1249 was increased. Cross serum antibody responses were noted for some patients receiving T-1249 and for some patients the response was accompanied with rash or fever (Eron *et al*., 2004). The response was specific for T-1249 and some patients had preexisting antibodies to T-1249 most probably because T-1249 share some homology with HIV-1 gp41 (Eron *et al*., 2004). Evidence of T-1249 resistance was seen in some patients with reduced sensitivity to T-1249 when the HR-1 region had V38E and Q40K mutations (Eron *et al*., 2004). Preliminary results appear to be promising for T-1249 to be used as a substitute or follow-up treatment for T-20 as a salvage regimen. The clinical development of T1249 is no longer being pursued (www.trimeris.com).

1.6.4.3 5-Helix

5-Helix is a recombinant C-peptide derived from the carboxy terminal of gp41 (Root, Kay, and Kim, 2001). It was designed to consist of five of the six helix bundles when the trimer of hairpins form and is connected by small peptide linkers. This peptide has one linker missing, which enables it to bind to the carboxy terminus of gp41 (Root, Kay, and Kim, 2001). It was derived from the region directly adjacent to the 2F5 epitope suggesting its action would be antagonistic with other C-peptides. Its efficacy is in the nanomolar range (Root, Kay, and Kim, 2001).

1.7 Combinations of entry inhibitors

A promising approach is to use a combination of entry inhibitors and existing anti-retrovirals. This approach will target different stages of viral entry and replication cycle and might give a better prognosis in delaying drug resistance (Tremblay *et al*., 2003). Combination of antiretroviral drugs has already been shown to be synergistic or antagonistic and these can only be established during clinical trials (Havlir *et al*., 2000; Merril *et al*., 1996; Tremblay *et al*., 2003). Pre-clinical studies showed a marked synergy in drug combination therapies between the CCR5 inhibitor SCH-C and reverse transcriptase inhibitors (zidovudine and lamivudine) and non-nucleoside reverse transcriptase inhibitors (efavirenz) and protease inhibitors (Indinavir). All these combinations with SCH-C were shown to be synergistic (Tremblay *et al*., 2002). SCH-C worked synergistically with T-20 and AOP-RANTES (Tremblay *et al*., 2002). Synergy between AMD3100 and T-20 was observed and is a useful combination to inhibit CXCR4 using viruses (Tremblay *et al*., 2002). Synergy was detected between 1-B-D-2.6-diaminopurinedioxolane (DAPD) a nucleoside reverse transcriptase inhibitor effective against both R5 and X4 isolates (Tremblay *et al*., 2003). T-20 and PRO542 proved to be a good combination as both CD4 binding and fusion was targeted to prevent virus entry (Nagashima *et al*., 2001). All these drug combinations targeting different stages of the virus life cycle provide good proof of concept that drug combinations will be effective in combating drug resistance and gives ample opportunity to switch drug combinations. Clinical trials are necessary to establish which combinations will be most favorable and to establish first line and follow-up drug regimens.

1.8 Monoclonal antibodies to CCR5 and CXCR4

Various monoclonal antibodies to CCR5 and CXCR4 have been identified. Of these 2D7 and PRO140 seem to be the most effective at inhibiting HIV entry via the CCR5 coreceptor. Both 2D7 and PRO140 are mouse monoclonal antibodies. However PRO140 was subsequently transformed into a humanized version (Olson *et al*., 1999; Wu *et al*., 1997b). Antibodies that bind to other regions especially the amino terminus of CCR5 have been developed but their efficacy at inhibiting HIV-1 replication was poor (Olson *et al*., 1999; Wu *et al*., 1997b). PRO140 was described earlier in section 1.6.2.3. Naturally occurring antibodies to CCR5 has been found in individuals who were exposed to HIV but remained uninfected (Lopalco *et al*., 2000). This suggests that the effectiveness of CCR5 antibodies may play a role in preventing HIV-1 replication.

1.8.1 2D7

2D7 was generated from a mouse and binds to the second extracellular loop of CCR5. It was shown to prevent the natural ligands RANTES, macrophage inflammatory protein-1a and 1ß from binding which make 2D7 a very potent CCR5 antagonist (Wu *et al*., 1997b). The monoclonal antibody has proven that the second extracellular loop of CCR5 plays an important role in binding gp120 (Wu *et al*., 1997b). Resistance to 2D7 has been shown for isolates able to replicate in the presence of 2D7 without the use of other coreceptors (Aarons *et al*., 2001).

1.8.2 12G5

12G5 targets CXCR4 and binds to the second extracellular loop of CXCR4 and has the ability to prevent X4 viruses from binding to this coreceptor (McKnight *et al*., 1998). Full inhibition of X4 isolates was obtained at between 10-20 µg/ml (Strizki *et al*., 1997). It was found that not all X4 viruses were inhibited by this monoclonal antibody suggesting that other regions of CXCR4 can be used to bind CXCR4 and enter cells (McKnight *et al*., 1998; Strizki *et al*., 1997). This monoclonal antibody is useful in screening for other agents able to bind to CXCR4.

1.9 Monoclonal antibodies targeting HIV envelope

Monoclonal antibodies (Mabs) targeting the virus have been identified to play an important role in neutralization of HIV-1 and have assisted in identifying epitopes sensitive to antibodies. Several monoclonal antibodies have been described and some of their binding epitopes are known. These Mabs are IgG1b12 (b12), 2G12, 2F5 and 4E10 (Burton and Montefiori, 1997; Burton *et al*., 1994; Moulard *et al*., 2002; Trkola *et al*., 1995).

1.9.1 IgG1b12

One of the most effective cross-clade neutralizing antibodies is IgG1b12. It has a conformational epitope overlapping the CD4 binding site and thus prevents CD4 gp120 interactions (Burton and Montefiori, 1997; Burton *et al*., 1994; Roben *et al*., 1994). It has an IC_{50} of between 3 and 7 ng/ml for laboratory adapted HIV strains and was higher for

primary isolates at less or equal to 1 µg/ml (Burton and Barbas, 1994). Trials with macaques proved that IgG1b12 could prevent HIV-1 infection at 25 mg/ml making it a possible therapeutic agent (Parren *et al*., 2001). Its efficacy has been demonstrated in an animal model where cocktails of monoclonal antibodies, which included IgG1b12, were able to protect macaques from infection (Ferrantelli *et al*., 2003). It is effective against most but not all HIV-1 subtype C strains, which accounts for most infections worldwide (Binley *et al*., 2004; Bures *et al*., 2002).

1.9.2 2G12

The monoclonal 2G12 binds to gp120 and has broad cross-clade neutralization and its mannose rich glycosylation sites at positions N295, N332, N339, N386 and N392 play a major role in binding 2G12 (Moulard *et al*., 2002). 2G12 binding is ineffective if changes in these regions occur (Sanders *et al*., 2002; Scanlan *et al*., 2002). 2G12 has been proven not to be able to neutralize most HIV-1 subtype C probably due to the absence of the glycosylation site at position 295 (Bures *et al*., 2002; Binley *et al*., 2004).

1.9.3 2F5

2F5 is an antibody targeting a linear epitope ELDKWA on the C-terminal region of gp41 (Purtscher *et al*., 1994; Muster *et al*., 1994; Zwick *et al*., 2005). It is very potent against viruses harboring this epitope especially subtype B with an IC_{50} ranging between 0.7 µg/ml and 9.7 µg/ml (Purtscher *et al*., 1994). Most HIV-1 subtype C does not have the ELDKWA epitope and have natural polymorphisms in this region thus rendering 2F5 largely ineffective against this subtype (Bures *et al*., 2002; Binley *et al*., 2004).

1.9.4 4E10

4E10 is an IgG1 monoclonal antibody targeting the gp41 region near the 2F5 epitope (Stiegler *et al*., 2001). 4E10 recognizes the sequence NWFDIT and binding to this epitope is dose related (Stiegler *et al*., 2001; Zwick *et al*., 2005). The NWFDIT epitope is adjacent to the ELDKWA epitope and is very conserved for most subtypes (Stiegler *et al*., 2001; Zwick *et al*., 2001). The IC_{50} of 4E10 ranged from 0.3 to 12.5 µg/ml and was effective against a variety of HIV-1 subtypes and was also effective against subtype C viruses (Stiegler *et al*., 2001; Xu *et al*., 2001; Binley *et al*., 2004).

1.9.5 sCD4-17b

A novel approach to inhibit HIV-1 was to link soluble CD4 (sCD4) with 17b, which is an antibody able to bind to a region after gp120 bound to CD4 (CD4 induced epitope) and it is able to prevent coreceptor binding. The sCD4 was linked to 17b via a polypeptide linker (Dey, Del Castillo, and Berger, 2003). sCD4-17b was found to be a very potent inhibitor of HIV-1 and was reactive against a broad range of subtypes. It was effective at an IC_{50} of 3.2 nM and more than 99% neutralization was obtained at 32 nM (Dey, Del Castillo, and Berger, 2003). sCD4-17b was more potent than other monoclonal antibodies such as 2G12, 2F5 and IgG1b12 and it was effective against both R5 and X4 isolates (Dey, Del Castillo, and Berger, 2003).

1.10 Combinations of monoclonal antibodies

Monoclonal antibodies with an effective cross-clade neutralizing ability can be used as a possible therapeutic intervention. Combinations of monoclonal antibodies can be used against subtype A, B, C and D if one antibody fails to neutralize, another monoclonal antibody might do so. Combinations of monoclonal antibodies can work synergistically in lowering the concentration needed to neutralize virus (Ferrantelli *et al*., 2004; Kitabwalla *et al*., 2003; Xu *et al*., 2001). The effectiveness of combinations of monoclonal antibodies has been demonstrated in a macaque model where neonate macaques were protected from SHIV89.6P infection by a cocktail of monoclonal antibodies while control macaques were infected (Veazey *et al*., 2003b). This suggests that monoclonal antibodies might be useful in preventing mother-to-child transmission via breastfeeding (Veazey *et al*., 2003b). Further immunogens able to induce such antibodies may be useful in the prevention of infection by vaccination. Thus neutralizing antibodies can either be used as a prophylactic or can have therapeutic applications.

1.11 Entry inhibitors as microbicides

An effective microbicide is urgently needed to help stem the spread of HIV. Entry inhibitors present an attractive option as they can prevent HIV from entering the cell. The first microbicide in human clinical trials was Nonoxynol-9. It was unable to prevent HIV and may have increased infection (Van Damme *et al*., 2002). Other potential agents are PRO2000 and CV-N (Dey *et al*., 2000; Mayer *et al*., 2003; O'Keefe *et al*., 2003). PRO2000 is a synthetic naphthalene sulfonate polymer and is contained in a gel

formulation with a pH of 4.5 (Mayer *et al*., 2003). PRO2000 efficacy has been proven to be at 100 µg/ml or less and is effective at blocking HIV-1 infection (Mayer *et al*., 2003). PRO2000 binds to gp120 and prevents CD4 binding as well as V3 loop interactions with the coreceptor (Mayer *et al*., 2003). As with CV-N it is also effective against other viruses such as herpes viruses and sexually transmitted pathogens such as *Chlamydia trachomatis* and *Neisseria gonorrhea* (Mayer *et al*., 2003). Phase I trials demonstrated that PRO2000 was safe and well tolerated by women and men (Mayer *et al*., 2003; Tabet *et al*., 2003). Only minor side effects were reported which makes PRO2000 a potential candidate to prevent sexual transmission of HIV-1 (Mayer *et al*., 2003).

A study using monoclonal antibodies in macaques has demonstrated the efficacy of a microbicide containing monoclonal antibodies targeting the virus (Veazey *et al*., 2003b). Monoclonal antibodies are very expensive and would not be a financially viable option for use in a microbicide but demonstrate proof of concept that agents targeting the virus will be effective (Veazey *et al*., 2003b). Microbicides using non-nucleoside reverse transcriptase inhibitors such as TMC120 and entry inhibitors may also be very effective as they target both the virus as well as the host cell receptors and further studies needs to be done to prove their usefulness in preventing sexual transmission of HIV-1 (Di Fabio *et al*., 2003; Veazey *et al*., 2003a).

1.12 OBJECTIVES

1.12.1 Primary objective

To determine the sensitivity of HIV-1 subtype C viruses to inhibitors of viral entry.

1.12.2 Secondary objectives

1. To determine the effectiveness of coreceptor inhibitors against HIV-1 subtype C isolates from adult AIDS patients with different coreceptor usage patterns.

2. To determine coreceptor usage of isolates from an acutely infected cohort and to establish if isolates from two individuals who showed a coreceptor switch become less sensitive to entry inhibitors over time.

3. To determine alternate coreceptor use on primary cells of two unusual HIV-1 isolates using chemokines and entry inhibitors with defined receptor-binding profiles.

4. To assess the effectiveness of T-20 against HIV-1 subtype C isolates and to compare the sensitivity of HIV-1 subtype C isolates to subtype B isolates.

5. To determine T-20 resistance patterns of HIV-1 subtype C and to compare these to HIV-1 subtype B.

CHAPTER 2

SENSITIVITY OF SUBTYPE C ISOLATES FROM AIDS PATIENTS TO CCR5 AND CXCR4 INHIBITORS IN PBMC

2.1 INTRODUCTION

Transmission of HIV-1 is almost always associated with viruses that utilize CCR5 (R5 viruses) which predominate during the acute and asymptomatic phases of infection (Michael *et al.*, 1997; Rana *et al.*, 1997). Disease progression is often associated with the emergence of viruses that have acquired the ability to use CXCR4 (X4 viruses), instead of, or as well as, CCR5 (R5X4 viruses) (Connor *et al*., 1997; Scarlatti *et al*., 1997). These patterns of coreceptor usage correspond to the phenotypes previously defined by the MT-2 assay; here, syncytium-inducing (SI) viruses use CXCR4 while non-syncytium-inducing (NSI) viruses use CCR5 (Bjorndal *et al.*, 1997). Some isolates from AIDS patients can also use other chemokine receptors as coreceptors in receptor-transfected cell-lines, including CCR1, CCR2b, CCR3, B0b/GPR15 and CXCR6 (Bonzo/STRL33) (Deng *et al*., 1996; Edinger *et al*., 1998; Pohlmann, Krumbiegel, and Kirchhoff, 1999; Sharron *et al*., 2000; Xiao *et al*., 1998; Zhang *et al*., 1998). Rarely, however, are such alternative coreceptors used in primary cells (Sharron *et al*., 2000; Zhang and Moore, 1999).

HIV-1 cellular tropism and coreceptor specificity is largely determined by the sequence of the third hypervariable loop (V3) of the viral gp120 glycoprotein, and distinct changes in this region have been associated with the NSI/SI phenotype among subtype B viruses (Chan *et al*., 1999; De Jong *et al*., 1992; Ivanoff *et al*., 1992; Shimizu *et al*., 1999). Compared to other subtypes, the V3 regions of the subtype C viruses studied to date tend to be highly conserved and have a low overall positive charge, which is consistent with

the NSI phenotypes and restricted coreceptor usage of subtype C viruses (Gaschen, Korber, and Foley, 1999).

The reported low frequency of SI HIV-1 subtype C isolates might be that in most cases isolates were studied, from asymptomatic patients or from poorly defined disease stages (Abebe *et al*., 1999; Bjorndal *et al*., 1999; Cecilia *et al*., 2000; Morris *et al*., 2001; Ping *et al*., 1999; Tien *et al*., 1999). We have therefore isolated viruses from patients with subtype C infection and advanced AIDS, to determine whether SI viruses are identified more frequently under these conditions than previously reported. We have also explored whether subtype C isolates able to use either or both CCR5 and CXCR4 are sensitive to coreceptor-specific entry inhibitors.

2.2 Materials and Methods

2.2.1 Isolation of NSI and SI HIV-1 subtype C viruses

Patients admitted to the Sizwe Infectious Diseases Hospital in Johannesburg, South Africa between August and December 1999 with HIV-1-related opportunistic infections and CD4 T cell counts less than 100 cells/µl were selected for this study. Ethical clearance was obtained from the University of the Witwatersrand Committee for Research on Human Subjects (Medical) and informed consent was obtained from all patients prior to drawing 5 ml of blood in EDTA (Appendix E). Plasma viral levels were measured using the Versant HIV-1 RNA 3.0 assay (bDNA assay from Bayer Nucleic Acid Diagnostics), previously shown to amplify HIV-1 RNA from all HIV-1 group M genetic subtypes (Elbeik *et al*., 2002; Swanson *et al*., 2001). The median plasma viral load for this cohort was high at 321,070 HIV-1 RNA copies/ml consistent with advanced HIV-1 disease. Twenty-two patients had tuberculosis (TB), 4 had cryptococcal meningitis (CM), 2 had *Pneumocystis carinii* pneumonia (PCP) and one had mycobacterium other than tuberculosis (MOTT). HIV-1 was isolated from 22 patients when their peripheral blood mononuclear cells (PBMC) were co-cultured with phytohemagglutinin (PHA)-activated donor PBMC as previously described (Morris *et al*., 2001). Isolates from the remaining 7 patients were obtained by culturing 100 µl of plasma overnight with PHA-activated PBMC. Culture supernatants were tested weekly using a commercial p24 antigen kit (Beckman Coulter Ltd., Buckinghamshire, UK) and expanded when high levels of p24 antigen were present. Cultures were replenished with fresh interleukin –2 (IL-2) medium every 3-4 days and with PHA-stimulated donor PBMC every 7 days for up to 3 weeks. Virus-containing supernatants were spun to remove cellular debris and

frozen at –70°C until use. Some isolates were re-expanded using PBMC stimulated with both PHA and surface-immobilized anti-CD3 Mab plus IL-2 as previously described (Trkola *et al*., 1999), to generate high titer stocks for further experiments. These isolates had limited expansion in PBMC *in vitro* and are likely to reflect the viral swarm present *in vivo*, although we cannot exclude that some selection, albeit limited, occurred during *in vitro* passage.

2.2.2 Determination of biological phenotype

The MT-2 cell line was used to determine virus phenotype. MT-2 cells were grown in RPMI 1640 media containing 10% fetal calf serum (FCS) and seeded at $0.5x10^6$ cells/ml in 12 well plates. The MT-2 cells were infected with 500µl of virus and monitored daily for the appearance of syncytia (Morris *et al*., 2001).

2.2.3 Coreceptor usage in transfected cell lines

Viral isolates were tested for their abilities to replicate in U87.CD4 cells transfected with CCR5, CXCR4, CCR1, CCR2b or CCR3. Cells were plated in 12-well plates at 10^5 cells/well in 2 ml of selection medium (Dulbecco's modified eagle's medium (DMEM) containing 10% FCS, antibiotics plus 500 µg/ml G418 (Boehringer Mannheim GmbH) and 1 µg/ml puromycin (ICN Biomedicals Inc., Ohio, USA). The following day, 1,000 $TCID_{50}$ (50% tissue culture infectious doses) of virus was added. After incubation overnight, the cultures were washed three times with DMEM plus 10% FCS to remove unbound virus, then monitored for syncytium formation and p24 antigen production on days 4, 8 and 12. The in-house p24 antigen ELISA has a detection limit of 250 pg/ml

(Trkola *et al*., 2001). Isolates that induced syncytia formation and generated increasing concentrations of p24 antigen were considered to be replication positive.

GHOST.3 cell lines expressing Bob/GPR15 or CXCR6 were used to determine alternate coreceptor use. The assays were performed essentially as described for the U87.CD4 cell assays except that cells were grown in the presence of 100 µg/ml hygromycin (Sigma-Aldrich, South Africa) in addition to the other two antibiotics mentioned above. Because GHOST.3 cell lines express endogenous CXCR4 it was necessary to add 1.3 µM of AMD3100 to prevent entry via this coreceptor (Zhang *et al*., 2000; Zhang and Moore, 1999). Viral replication in the presence of AMD3100 therefore indicated use of the transfected coreceptor (either Bob/GPR15 or CXCR6).

2.2.4 Viral replication in wild type and Δ32/Δ32-CCR5 PBMC

PBMC from a CCR5 Δ32/Δ32 donor and CCR5 +/+ control donors were isolated and stimulated using PHA and surface-immobilized anti-CD3 Mab, as described above. On the following day, the cultures were washed 3 times and incubated in IL-2 containing medium. Supernatants were assayed for p24 antigen levels using a commercial assay with a kinetic readout (Dupont/NEN Life Sciences, Boston, MA) on days 4, 8 and 11.

2.2.5 Inhibitor assays in PBMC

Entry inhibitor assays were performed using CD8+ T cell depleted PBMC utilizing RosetteSep CD8 antibody cocktail (StemCell Technologies, Vancouver, Canada) and stimulated with phytohemagglutinin (PHA). CD8 depletion was confirmed using flow

cytometry (Becton Dickinson, San Jose, CA). The PBMC were incubated with the specific inhibitor for 1 hour before adding 1,000 $TCID_{50}$ (50% tissue culture infectious dose per ml) of virus. Assays were performed on 96 well plates using RPMI 1640, 10% fetal calf serum and IL-2. Inhibitors tested included the anti-CCR5 Mab PRO140 (Progenics Pharmaceuticals, New York), chemokines RANTES (PeproTech Inc., Rocky Hill, N.J) and the small molecule CXCR4 antagonist, AMD3100. Viral replication was measured by p24 antigen (ag) production. The reduction in p24 ag levels was calculated relative to cultures without inhibitors and the percentage inhibition at a high and low concentration of each inhibitor is reported. Results shown are from duplicate cultures and each experiment was repeated twice as previously described (Trkola *et al*., 2001).

2.2.6 Sequencing of V3 region

The V3 region was amplified using primers BF (5'-TAACACAAGCCTGTCCAAAGG-3') and BR (5'-AATTCTAGGTCCCCTCCTGA-3'). RNA was reverse transcribed with AMV Reverse Transcriptase (Roche Molecular Biochemicals) and amplified with Super-Therm Polymerase (Southern Cross Biotechnologies); the PCR product was cleaned with a High Pure PCR Product Purification kit (Roche Applied Science). Sequencing was performed in both directions with the BF and BR primers.

2.3 RESULTS

2.3.1 Characteristics of AIDS patients with NSI or SI isolates

Twenty-nine isolates were generated from 13 men and 16 women with a median age of 34 years and a median CD4 count of 40 cells/µl. Among the 29 isolates, 5 (17%) were able to grow in MT-2 cells, leading to syncytia formation (SI isolates) (Table 2.1). The subset of patients with SI viruses had significantly reduced CD4 T cell counts, which is similar to observations made on patients infected with subtype B strains (Richman and Bozzette, 1994). Viral loads among the patients with SI viruses were not particularly high, compared to levels in the cohort as a whole, perhaps because too few memory CD4 T cells remain to support high-level viral replication. However, viral load measurements were available from only 3 patients with SI viruses, so no conclusions can be drawn from the statistical perspective. SI viruses were isolated from 3 of 22 (14%) TB patients and 2 of 4 (50%) cryptococcal meningitis (CM) patients (Table 2.1). However, overall, there were no significant differences in the age, gender or opportunistic infections of patients with SI isolates compared to those that harbored NSI isolates. A cohort containing larger numbers of patients with SI isolates will be needed to fully address such issues.

2.3.2 Sub-typing of HIV-1 isolates

The HIV-1 isolates were subtyped in the C2-V5 region of *env* gene by heteroduplex mobility assay (HMA) and all shown to belong to HIV-1 subtype C, the predominant subtype in South Africa (Bredell *et al*., 1998; Van Harmelen *et al*., 1999). Thirteen isolates were also subtyped in gag by HMA and shown to belong to subtype C (data not shown) suggesting that these isolates were unlikely to be inter-subtype recombinants,

although this cannot be excluded. Two viruses, CM9 and SW7, have been confirmed as being subtype C throughout its genome (Papathanasopolous *et al*., 2002).

2.3.3 Coreceptor use of HIV-1 subtype C isolates from AIDS patients

There was an absolute correlation between the NSI and SI phenotype and the ability of an isolate to use CCR5 or CXCR4, respectively (Table 2.2). Thus, all 24 NSI isolates were able to replicate in CCR5-expressing cells, but not in the CXCR4-expressing cells (hence they are conventionally designated as R5 viruses). The 5 SI isolates all replicated in CXCR4-expressing cells. Among them, 3 could replicate in CCR5-expressing cells (designated as R5X4 viruses) while 2 did not (designated as X4 viruses). Although PCP1 was classified as an R5 virus it did show low-level usage of CXCR4 that was below the cut-off for the p24 antigen assay (data not shown). The ability of this virus to use CXCR6 (see below) very efficiently may account for these low levels of replication as U87.CD4 cells express this receptor, and also GPR1, endogenously (Farzan *et al*., 1997; Unutmaz *et al*., 2000). CM9 could use CXCR6, Bob/GPR15 and CCR3 (Table 2.2). In each case, entry via the alternate coreceptor was at a comparable level of efficiency but less than CCR5 or CXCR4 (Table 2.2).

Table 2.1: Characteristics of 29 South African AIDS patients with NSI or SI isolates

	Total	NSI isolates	SI isolates
Number (% of total)	30	25 (83%)	5 (17%)
Female (% of total)	16	13 (81%)	3 (19%)
Male (% of total)	14	12 (86%)	2 (14%)
Median age	34	34	29
Median viral load (copies/ml)	26	776100*	68410*
Median CD4 count (cells/ul)	42	48**	10**
Diagnosis			
Tuberculosis (TB) (% of NSI/SI)	22	19 (76%)	3 (60%)
Cryptococcal meningitis (% of NSI/SI)	5	3 (12%)	2 (40%)
Pneumocystic Carinii pneumonia (PCP) (% of NSI/SI)	2	2 (8%)	0
MOTT/Mac (% of NSI/SI)	1	1 (4%)	0

* significantly different (p=0.0134) by Mann Whitney test, data from only 3 of 5 ppatients available

** significantly different (p=0.0033) by Mann Whitney test

2.3.4 Sensitivity of HIV-1 subtype C isolates to coreceptor inhibitors in PBMC

To confirm the use of CCR5 or CXCR4 in PBMC, viral isolates were cultured in the presence of CCR5-specific (RANTES and PRO140) or CXCR4-specific (AMD3100) entry inhibitors. A subset of 13 subtype C viruses able to use different coreceptors was selected for analysis, in comparison with a panel of 7 isolates from subtypes A, B and E (from National Institute for Health, Reference and Reagents Repository). The replication of 7 of the 8 R5 subtype C viruses was effectively inhibited (>90%) by the highest concentrations of RANTES and PRO140 that were tested (Table 2.3). One such isolate (CM1) was marginally less sensitive to RANTES (87% inhibition) and PRO140 (78% inhibition). In contrast, the CCR5 inhibitors were ineffective against R5X4 and X4 viruses, suggesting that these viruses can all use CXCR4 to enter PBMC efficiently. AMD3100 effectively inhibited all the R5X4 and X4 viruses, although two isolates (SW30 and SW12) were slightly less sensitive (85% and 86% inhibition, respectively) than the others. The ability of AMD3100 to restrict the replication of all 3 R5X4 viruses suggests that they use CXCR4 in preference to CCR5 to enter PBMC *in vitro* as

previously described (Yi *et al*., 1999). As expected, AMD3100 was ineffective against all the R5 isolates, because it is known not to interfere with CCR5-mediated entry (Donzella *et al*., 1998; Schols *et al*., 1997). The ability of an isolate to use alternate coreceptors in transfected cell lines did not influence whether it was sensitive to any of the entry inhibitors in PBMC cultures. Thus, both PCP1 and CM9 were fully sensitive to RANTES, PRO140 and AMD3100 up to day 6, despite being able to use alternate co-receptors (Table 2.3). Either expression levels of the alternate co-receptors on PBMC are lower than on the transfected cell lines, or these coreceptors simply do not function as HIV-1 entry receptors on primary cells (Sharron *et al*., 2000) (Refer to Chapter 4).

The CCR5 inhibitors were also effective against the R5 isolates from subtypes A, B and E. One R5X4 subtype B isolate (92US077) was effectively inhibited by AMD3100 but not by RANTES and PRO140, suggesting that it too uses CXCR4 preferentially to enter PBMC. Overall, there was a good, and subtype-independent, correlation between the classification of an isolate as R5 or X4 by use of the co-receptor-transfected cell lines, and its sensitivity to CCR5- or CXCR4-specific entry inhibitors in PBMC (Trkola *et al*., 2001; Trkola *et al*., 1998). However, the isolates classified as R5X4, because they could use both CCR5 and CXCR4 in co-receptor-transfected cells (Table 2.3), were sensitive only to the CXCR4 inhibitor AMD3100 in PBMC, and were little affected by the CCR5 antagonists PRO140 and RANTES (Table 2.3). Hence CXCR4 is the co-receptor of choice for these R5X4 isolates in primary T-cells, with only a minority of the viruses present entering the cells via CCR5.

Table 2.2: Coreceptor usage by HIV-1 subtype C isolates from AIDS patients

Isolate ID	Source	CD4 count	Viral load	Subtype *env*	MT-2 assay	U87.CD4 cells expressing*		Minor coreceptors	Biotype
						CXCR4	CCR5		
PCP1	PBMC	2	ND	C	NSI	-	++++	CXCR6+++	R5
CM1	PBMC	43	146,514	C	NSI	-	++		R5
CM7	PBMC	79	14,089	C	NSI	-	++++		R5
SW2	Plasma	84	157,150	C	NSI	-	++++		R5
SW3	Plasma	53	261,880	C	NSI	-	++++		R5
SW4	Plasma	76	1,496,620	C	NSI	-	++++		R5
SW5	PBMC	40	1,374,235	C	NSI	-	++++		R5
SW8	Plasma	67	1,198,880	C	NSI	-	+		R5
SW9	Plasma	65	301,605	C	NSI	-	++++		R5
SW14	PBMC	32	>5,000,000	C	NSI	-	+++		R5
SW16	PBMC	9	151,630	C	NSI	-	+		R5
SW22	PBMC	67	2,749,340	C	NSI	-	++		R5
SW23	PBMC	10	696,000	C	NSI	-	++++		R5
SW26	PBMC	25	ND	C	NSI	-	++++		R5
SW28	Plasma	43	3,814,130	C	NSI	-	++++		R5
SW29	PBMC	12	1,190,180	C	NSI	-	++++		R5
SW34	PBMC	40	127,000	C	NSI	-	++++		R5
SW35	PBMC	37	1,652,870	C	NSI	-	++++		R5
SW36	PBMC	55	201,340	C	NSI	-	++++		R5
SW37	PBMC	121	34,210	C	NSI	-	+		R5
SW38	PBMC	37	1,777,930	C	NSI	-	++++		R5
SW39	PBMC	94	321,070	C	NSI	-	++++		R5
SW40	PBMC	67	856,180	C	NSI	-	++++		R5
SW41	PBMC	60	3,162,240	C	NSI	-	+++		R5
CM9	PBMC	24	ND	C	SI	++++	++++	CCR3+++ CXCR6+++ BOB+++	R5X4
SW7	PBMC	10	ND	C	SI	++++	-		X4
SW12	Plasma	27	68,410	C	SI	++++	-		X4
SW20	PBMC	2	43,595	C	SI	++	++++		R5X4
SW30	PBMC	2	73,860	C	SI	++++	++++		R5X4

Data shown of day 12

*scoring system (In house endpoint p24 assay)

- = <250 pg/ml p24 antigen, + = 250 pg-1 ng/ml p24 antigen ++ = 1-2 ng/ml p24 antigen +++ = 2-4 ng/ml p24 antigen ++++ = >4 ng/ml p24 antigen

Table 2.3 Inhibition of HIV-1 subtype C viruses by CCR5 and CXCR4 inhibitors

Virus	Subtype	Phenotype	Day	CCR5 inhibitors*				CXCXR4 inhibitor*	
				RANTES at:		PRO140 at:		AMD3100 at:	
				19 nM	4 nM	167 nM	33 nM	500 nM	100 nM
PCP1	C	R5 CXCR6	6	**97**	*63*	**98**	*88*	*34*	*18*
CM1	C	R5	6	*87*	*49*	*78*	*59*	*0*	*8*
CM7	C	R5	8	**94**	*89*	**97**	**100**	*3*	*19*
SW5	C	R5	4	**100**	*75*	**100**	**100**	*19*	*6*
SW23	C	R5	8	**100**	*89*	**98**	**94**	*0*	*0*
SW26	C	R5	8	**100**	**91**	**93**	*87*	*8*	*0*
SW29	C	R5	8	**98**	*74*	**98**	**91**	*13*	*0*
SW38	C	R5	8	**100**	*34*	**100**	**96**	*0*	*0*
CM9	C	R5X4 CXCR6, Bob, R3	8	*36*	*36*	*34*	*13*	**97**	*58*
SW7	C	X4	4	*0*	*24*	*0*	*5*	**100**	*87*
SW12	C	X4	10	*0*	*0*	*0*	*0*	*86*	*31*
SW20	C	R5X4	8	*0*	*0*	*79*	*0*	**100**	*0*
SW30	C	R5X4	8	*24*	*9*	*8*	*39*	*85*	*3*
DJ258	A	R5	8	**100**	*82*	**100**	**98**	*46*	*0*
92RW026	A	R5	8	**98**	*69*	**100**	**100**	*63*	*23*
JR-FL	B	R5	10	**99**	*71*	**100**	**98**	*0*	*36*
92US714	B	R5	4	**95**	*60*	**100**	**100**	*0*	*0*
92US077	B	R5X4	6	*17*	*48*	*48*	*41*	**99**	*76*
2044	B	X4	6	*20*	*23*	*30*	*22*	*39*	*21*
CM235	E	R5	10	**95**	*62*	**100**	**97**	*22*	*2*
92TH001	E	R5	10	**100**	*88*	**99**	**98**	*60*	*21*

* Inhibition >90% is bolded and inhibition <90% is in italics

2.3.5 Replication of NSI and SI viruses in wild type and Δ32/Δ32-CCR5 PBMC

The subtype C isolates used above were also tested for their ability to replicate in PBMC deficient of CCR5 (i.e., cells from an HIV-1-negative individual was confirmed by PCR to have the CCR5 Δ32/Δ32 genotype). None of the R5 isolates were able to replicate in the CCR5 Δ32/Δ32 PBMC but all replicated well in the CCR5 +/+ PBMC, confirming their dependence on CCR5 to infect cells (Table 2.4). The R5X4 and X4 isolates were able to replicate in both the CCR5 Δ32/Δ32 and the CCR5 +/+ PBMC, although p24 production from the CCR5 Δ32/Δ32 cells was relatively low. In particular, the replication of isolate SW20 was sporadic in the CCR5 Δ32/Δ32 PBMC, with only some of the replicate wells producing p24 antigen. This contrasts with the consistent replication of the same virus in the CCR5 +/+ PBMC.

2.3.6 CXCR4-using subtype C viruses show alterations in V3 loop

The V3 loop is involved in determining coreceptor usage (Hoffman *et al*., 2002a). Certain genetic changes in this region are associated with CXCR4 use, in the context of viruses from subtypes B and D (De Wolf *et al*., 1994). To determine whether CXCR4-using subtype C isolates also have distinctive V3 loop sequences, RNA was isolated and their V3 regions sequenced. Phylogenetic analysis of the nucleotide sequences of all 16 V3 loop sequences showed that all clustered with HIV-1 subtype C (data not shown), confirming the HMA data. The V3 loops of all the R5 viruses were 35 amino acids in length and contained the GPGQ motif at the crown that is characteristic of HIV-1 subtype C viruses (Figure 2.1). A neutral serine residue was always present at position 11 and, in most cases, an amino acid with a negatively charged side chain (either D or E) at position

25. The net charge of the V3 loop was +3 to +5. In contrast, the V3 loops of the R5X4 and X4 viruses varied markedly from the consensus sequence, containing substitutions, deletions and insertions. A common, and unusual, feature was a two-residue insertion between positions 13 and 14, most frequently isoleucine and glycine, which increased the length of the loop to 37 amino acids.

Table 2.4: HIV-1 subtype C replication in PBMC from Δ32/Δ32 and wt/wt donors

	p24 antigen level for genotype [a]	
Isolates	D**32**/D**32**	**wt/wt**[b]
R5		
PCP1	-	++++
CM1	-	++++
CM7	-	++++
SW5	-	+++
SW23	-	++++
SW26	-	++++
SW29	-	++++
SW38	-	++++
R5X4		
CM9	+	++
SW20	+/-	+
SW30	+	++++
X4		
SW7	+	+++
SW12	++	+++

[a] p24 antigen level on day 8 (kinetic p24 assay). -, <250 pg of p24 antigen/ml; +, 250 pg to 10 ng of p24 antigen/ml; ++, 10-30 ng of p24 antigen/ml; +++,30-60 ng of p24 antigen/ml; ++++, >60 ng/ml p24 antigen/ml.

[b] wt, wild type.

A tetrapeptide motif containing positively charged arginine residues was also frequently present in the V3 loops of the R5X4 and X4 isolates, resulting in a marked increase in the overall positive charge of the loops to +6 to +9. Furthermore, all the R5X4 and X4 isolates had either a neutral or positively charged amino acid substitution or a deletion at

position 25. There were no sequence motifs that distinguished between R5X4 and X4 viruses.

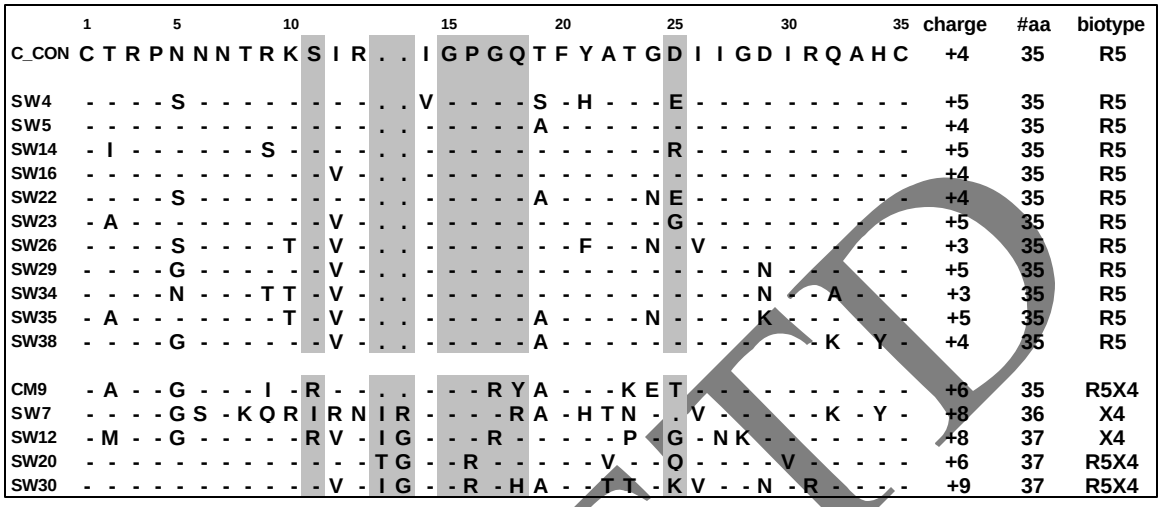


Figure 2.1: Predicted V3 amino acid sequence of 16 South African HIV-1 subtype C isolates (11 R5 and 5 R5X4 or X4) compared to a HIV-1 subtype C consensus sequence. The overall positive charge, number of amino acids and biotype of each isolate is shown on the right. Dashes (-) indicate concurrence and a period (.) indicates a deletion or lack of an insertion. Changed amino acids are indicated. Positions 11 and 25 (associated with changes in biological phenotype), the tip of the V3 loops and region with insertions are highlighted in grey. Amino acid abbreviations are shown in Appendix B. GenBank accession numbers are listed in Appendix D (Sequencing was performed by Jabulani Nhlapo and amino acid sequence analysis by myself)

2.4 Discussion

Here we describe HIV-1 subtype C isolates from patients with late-stage AIDS that grow in MT-2 cells (SI viruses) and use CXCR4 for entry into cells. These isolates were fully sensitive to AMD3100, a CXCR4-specific entry inhibitor and were able to grow in PBMC devoid of CCR5. Most of these SI viruses had significant genetic changes in the V3 loop compared to NSI viruses isolated from the same cohort. This is a similar observation to those reported for viruses from subtypes B and D (De Wolf *et al*., 1994; Hoffman *et al*., 2002b). Hence the HIV-1 subtype C envelope glycoproteins can accommodate the amino acid changes needed to use CXCR4 as a coreceptor. Probably, the gp120 proteins from all subtypes interact with CXCR4 in a broadly similar manner.

Our observations stand in contrast to previous reports, including our own, that subtype C infections rarely involved viruses with the SI phenotype (Abebe *et al*., 1999; Bjorndal *et al*., 1999; Cecilia *et al*., 2000; Morris *et al*., 2001; Ping *et al*., 1999; Tien *et al*., 1999). These earlier descriptions of subtype C isolates mostly involved cohorts of patients in the relatively early stages of HIV-1 infection, identified relatively soon after subtype C viruses started to spread rapidly in the geographic area under study. Now, we have deliberately selected for study patients with advanced AIDS since they were more likely to harbour SI viruses. The isolation of CXCR4-using viruses from some of our late-stage AIDS patients suggests that subtype C viruses may undergo a phenotypic switch during disease progression, as occurs during subtype B infection (Connor *et al*., 1997; Richman and Bozzette, 1994; Scarlatti *et al*., 1997). However because our cohort was observational, we cannot know whether any of the patients with SI viruses had NSI

viruses at an earlier stage. Prolonged, longitudinal follow-up of individuals with acute subtype C-infection will be needed to address this issue (See Chapter 3). Although viral stocks were grown in PBMC *in vitro* it is likely that the dominant population is representative of the *in vivo* swarm as V3 sequence analysis of cultured isolates and from uncultured plasma samples from 5 of these patients showed them to be identical (data not shown). Thus phenotypes identified in vitro are likely to reflect the dominant phenotype in the patients.

Although only 5 of our 29 (17%) AIDS patients were infected with SI viruses, this represents a higher frequency than was observed in previous studies on subtype C infection (Abebe *et al*., 1999; Bjorndal *et al*., 1999; Cecilia *et al*., 2000; Morris *et al*., 2001; Ping *et al*., 1999; Tien *et al*., 1999). In subtype B cohorts, from 50-90% of patients with late-stage AIDS have been reported to harbour SI isolates (Richman and Bozzette, 1994; Scarlatti *et al*., 1997). Whether the frequency of subtype C X4 viruses will increase over time or will remain relatively low compared to subtype B infections remains to be determined. HIV-1 subtype C might be compared to subtype A/E in Thailand where SI viruses were identified later during the epidemic with ever increasing frequency (De Wolf *et al*., 1994; Menu *et al*., 1999; Sutthent *et al*., 2002; Sutthent *et al*., 2001; Utaipat *et al*., 2002). Further studies on subtype C infection may reveal the same trend. In any case, the assumptions that subtype C viruses use CCR5 almost exclusively are unlikely to be valid with more reports of SI viruses being detected (Cilliers *et al*., 2003; Janse Van Rensburg *et al*., 2002; Johnston *et al*., 2003; Pollakis *et al*., 2004).

Most of the patients in our study had TB, which is the most common opportunistic infection in AIDS patients in South Africa. CCR5 expression levels are affected by environmental factors, such as TB, among African patients, which could favor a relatively high frequency of R5 viruses throughout the course of disease (Clerici *et al*., 2000; Fraziano *et al*., 1999). The transmission of R5 viruses may be particularly efficient in areas such as South Africa where there is a high incidence of early HIV-1 infection (Gouws *et al*., 2002), which is associated with high viral loads and R5 viruses. Furthermore, any individuals who harbour SI viruses early in infection may not survive long enough in an African health-care setting to become enrolled in a clinical cohort. Hence this factor might lead to under-representation of SI isolates in a cohort such as ours. Patients with cryptococcal meningitis were particularly likely to have CXCR4-using viruses, which is probably related to the association between this opportunistic infection and advanced HIV-1 disease.

We previously showed that subtype C viruses were as sensitive as subtype B viruses to the CCR5 inhibitors, PRO140, RANTES and TAK-779 (Trkola *et al*., 2002). Here, we have extended our observations to a more diverse group of viruses and we have also tested the CXCR4 inhibitor, AMD3100. Together, these studies support the clinical evaluation of coreceptor-specific entry inhibitors in South Africa; the antiviral responses are likely to be similar to those that have been observed in studies on subtype B infections in North America and Europe (Hendrix *et al*., 2002; Reynes, 2002). Subtype C accounts for the vast majority of global HIV-1 infections that are spread by heterosexual and perinatal transmission in developing countries. The ability of coreceptor inhibitors to

prevent infection makes them particularly attractive as interventions in high incidence settings. Novel approaches could include the use of entry inhibitors as microbicides to prevent sexual transmission or as supplements to be added to breast-milk to block perinatal infection. Such interventions would however need to be effective, cheap and simple to administer. Our data suggest that therapeutic or preventative approaches based on coreceptor entry inhibitors could be useful in developing countries where subtype C circulates and where such interventions are most desperately needed.

CHAPTER 3

LONGITUDINAL ANALYSIS OF TWO HIV-1 SUBTYPE C ISOLATES UNDERGOING A CORECEPTOR SWITCH

3.1 Introduction

The use of CCR5 by HIV-1 isolates is normally associated with early transmission and the asymptomatic phase of HIV-1 infection, while the use of CXCR4 occurs mostly later during the disease stage. The occurrence of a switch from a CCR5-using (R5) to CXCR4-using (X4) or CXCR4/CCR5 (R5X4) leads to rapid depletion of CD4 cells and accelerated disease progression (Karlsson *et al*., 1994; Koot *et al*., 1993). Coreceptor switching has been shown for subtype B (Scarlatti *et al*., 1997). In subtype C, the occurrence of SI isolates have been demonstrated but there have been no longitudinal studies to show a switch in coreceptor use over time (Janse Van Rensburg *et al*., 2002, Cilliers *et al*., 2003; Johnston *et al*., 2003; Pollakis *et al*., 2004). Coreceptor switching has been shown to be associated with changes in sensitivity to coreceptor inhibitors or their natural ligands (Scarlatti *et al*., 1997). A switch in coreceptor use will therefore render CCR5 coreceptor inhibitors ineffective (Scarlatti *et al*., 1997).

The ability to use the CXCR4 coreceptor is associated with genetic changes in the V3 region often including an increase in the overall positive charge (Hoffman *et al*., 2002a). Changes at specific positions 11 and 25 with positively charged amino acids (i.e arginine (R), lysine (K) and histidine (H)) determines an X4 or R5/X4 phenotype (Milich, Margolin, and Swanstrom, 1993). The length of V1 and V2 regions as well as insertions and deletions in V3 has also been implicated in contributing to the X4 or R5X4 phenotype (Jansson *et al*., 2001). The higher charge of the V3 loop is more likely to interact with a negatively charged CXCR4 (Hoffmann *et al*., 1999).

The aim of this study was to examine coreceptor usage of HIV-1 subtype C isolates from an acutely infected cohort. Two individuals were analyzed further as they were shown to have undergone a coreceptor switch. The next aim was to establish if these isolates became less sensitive to CCR5 coreceptor inhibitors over time and to determine genetic changes that allowed the isolates to switch coreceptor use.

3.2 Materials and Methods

3.2.1 HIV viral isolates

Ten commercial sex workers in Kwa-Zulu Natal participating in a Phase III clinical trial of a vaginal microbicide (N9) were followed longitudinally (Table 3.1). The women were HIV antibody negative at enrollment and were tested monthly for HIV-1/2 antibodies. When the women tested antibody positive they were enrolled and samples were collected longitudinally for virus isolation. HIV-1 was isolated from PBMC by co-culturing with normal donor PBMC. Viral loads were determined using Chiron bDNA assay (Chiron, San Francisco, CA). CD4 counts were determined using a FACS count (Becton Dickinson, San Jose, CA) (as described in Chapter 2). Two women who underwent a coreceptor switch were studied intensively. Du151 was followed for 20 months and six samples were collected including one from pre-seroconversion. Four samples over 33 months were collected from Du179.

3.2.2 Coreceptor assays

Viruses were tested for their ability to replicate in U87.CD4 cells transfected with either CCR5 or CXCR4 cells as previously described in Chapter 2. Phenotypic assays were performed on MT-2 cell lines as described in Chapter 2.

3.2.3 Inhibition assays

The viral isolates were tested for their sensitivity to entry inhibitors to determine preferential coreceptor use. These assays were performed on CD8-depleted PBMC with

p24 antigen readout as described for the coreceptor inhibitors as well as for the fusion inhibitor T-20 as described in Chapter 2 and Chapter 4.

3.2.4 V3 sequencing

V3 sequencing was performed as described in Chapter 2 and Chapter 6.

3.3 Results

3.3.1 Patient characteristics

Viral isolates from ten patients with acute HIV infection were characterized (Table 3.1). All the isolates were subtype C and all except Du179 used CCR5 (Table 3.1). Six women were followed longitudinally: Du123, Du151, Du156, Du179, Du368 and Du422. Of these six, two isolates (Du151 and Du179) had either an SI phenotype or underwent a phenotypic switch and were investigated more fully. The remaining four individuals retained the R5 phenotype for the duration of the study (data not shown).

Du151 was estimated to be infected around September 1998 and the first isolate was obtained approximately 1 month and 3 days post infection when no serum antibodies were detectable (pre-seroconversion). No CD4 count was available for this sample but the viral load was very high consistent with acute infection (Table 3.2). Follow-up samples were obtained 2.1, 8.8, 12, 18.6 and 19.9 months post infection. During this time the CD4 counts dropped from 367 to 66 cells/µl. Viral levels remained high throughout the study (>500 000 copies RNA/ml). This individual developed AIDS and died within 2 years and was termed a rapid progressor. (Table 3.2).

It was estimated that Du179 was infected from the first of August 1997 and the first isolate was obtained 16.1 months. In this study the first isolate studied was obtained 19.8 months post infection. Follow-up samples were obtained 21.9, 31.2 and 33.8 months post infection and viral isolates were generated at these time points. The CD4 count at 19.8

months post infection was 435 cells/µl, but declined. The viral load remained low during the study period (Table 3.3).

Table 3.1 Viral isolates from a cohort of acutely infected patients

Isolate ID	Estimated months after infection	CD4 cell count (cells/ml)	VL RNA copies/ml	Viral genotype (gag/pol/env)	MT-2 Phenotype	Biotype
Du123	3.5	841	19,331	C/-/C	NSI	R5
Du151-Nov 98	2.1	367	>500,000	C/C/C	NSI	R5
Du156	<1	404	22,122	C/C/C	NSI	R5
Du172	3.2	793	1,916	C/C/C	NSI	R5
Du174	14.1	634	9,454	C/C/C	NSI	R5
Du179-Nov 98	16.1	394	1,359	C/C/C	SI	R5X4
Du204	6.5	633	8,734	C/C/C	NSI	R5
Du281	4.7	594	24,689	C/C/C	NSI	R5
Du368	8.3	670	13,993	C/-/C	NSI	R5
Du422	2	409	17,118	C/C/C	NSI	R5

- indicates no result

(Adapted from Williamson *et al*., 2003)

Table 3.2: Longitudinal isolates of Du151 with clinical and phenotypic data

Isolate ID	Sample date	Estimated months after infection	CD4 cell count (cells/ml)	VL RNA copies/ml	MT-2 Phenotype	Biotype
Du151-Oct 98	26-Oct-98	1.1	N/A	579,480	NSI	R5
Du151-Nov 98	24-Nov-98	2.1	367	569,300	NSI	R5
Du151-Jun 99	15-Jun-99	8.8	239	756,660	NSI	R5
Du151-Sep 99	23-Sep-99	12.0	N/A	321,040	NSI	R5
Du151-Mar 00	30-Mar-00	18.6	143	>500,000	SI	R5X4
Du151-May 00	11-May-00	19.9	66	1,163,800	SI	R5X4

N/A not available

Table 3.3: Longitudinal isolates of Du179 with clinical and phenotypic data

Isolate ID	Sample date	Estimated months after infection	CD4 cell count (cells/ml)	VL RNA copies/ml	MT-2 Phenotype	Biotype
Du179-Mar 99	18-Mar-99	19.8	435	4,895	SI	R5X4
Du179-May 99	20-May-99	21.9	N/A	N/A	SI	R5X4
Du179-Feb 00	24-Feb-00	31.2	259	3,131	SI	R5X4
Du179-May 00	13-May-00	33.8	231	2,228	SI	X4

N/A not available

3.3.2 Coreceptor usage

Du151 and Du179 isolates were grown in U87.CD4.CCR5 and U87.CD4.CXCR4 to determine coreceptor usage. The first four isolates of Du151 were able to replicate efficiently in U87.CD4.CCR5 cells but were unable to replicate in cells expressing CXCR4. The last two isolates, Du151-Mar 00 and Du151-May 00 were able to replicate in cells expressing both CCR5 and CXCR4. This demonstrates that the isolates from the last two time points were fully dual tropic. These data correlated with the MT-2 data used to determine the phenotype of the isolate.

Du179 was able to replicate in both CCR5 and CXCR4 cells indicating that the virus isolate for the first three time points were dual tropic. The isolate from the last time point was only able to replicate in CXCR4 expressing cells demonstrating that it was an X4 isolate.

3.3.3 Sensitivity of Du151 and Du179 to entry inhibitors

Du151 and Du179 isolates were tested against two CCR5 (RANTES and PRO140) and two CXCR4 coreceptor inhibitors (AMD3100 and T-140) and the fusion inhibitor T-20 (Figures 3.1 and 3.2). The first four isolates of Du151 were effectively inhibited 100% at

the highest concentration tested, indicating that CCR5 is the major coreceptor used by these isolates. The last two isolates of Du151 were not inhibited by the CCR5 inhibitors, indicating that they were using another coreceptor to infect PBMC. The two CXCR4 inhibitors AMD3100 and T-140 were unable to inhibit any of the R5 isolates and were ineffective against Du151-Mar 00 an R5X4 isolate. This might suggest that this isolate was able to use both CCR5 and CXCR4 with the same efficiency. However the later isolate (Du 151-May 00) preferentially uses CXCR4 (Table 3.4). This data indicates that Du151 is initially an R5 isolate but evolves from using CCR5 to a dual tropic isolate and to using CXCR4 preferentially on PBMC.

Du179-Mar 99 was not inhibited by either RANTES or PRO140 indicating that it uses CXCR4 to infect PBMC. The CXCR4 inhibitors AMD3100 and T-140 were unable to inhibit the first two isolates, which may indicate that these isolates were fully dual tropic and able to use CCR5 when CXCR4 is blocked. AMD3100 was able to partially inhibit the last two isolates. T-140 only partially inhibited the first three isolates but was effective at inhibiting the last isolate Du179-May 00 (Table 3.4). This indicates that the last two isolates were more prone to using CXCR4 even though it is not fully inhibited by the CXCR4 inhibitors.

Table 3.4: Inhibition of Du151 and Du179 longitudinal samples.

Isolate number	Biotype	% Inhibition				
		RANTES	PRO140	AMD3100	T-140	T-20
		150 mg/ml	25 mg/ml	500 nM	2 mg/ml	1 mg/ml
Du151-Oct 98	R5	**100**	**100**	9	3	**100**
Du151-Nov 98	R5	**100**	**100**	*0*	*6*	**100**
Du151-Jun 99	R5	**99**	**98**	*15*	*51*	**100**
Du151-Sep 99	R5	**100**	**100**	*1*	*12*	**100**
Du151-Mar 00	R5X4	*41*	*11*	*3*	*0*	**100**
Du151-May 00	R5X4	*31*	*0*	**91**	**100**	**91**
Du179-Mar 99	R5X4	*29*	*39*	*24*	*13*	**99**
Du179-May 99	R5X4	*23*	*19*	*32*	*24*	**97**
Du179-Feb 00	R5X4	*4*	*5*	*77*	*46*	**93**
Du179-May 00	X4	*0*	*0*	*71*	**95**	76

% Inhibition higher than 90% is bolded

3.3.4 Inhibition with T-20

No naturally occurring resistance mutations for T-20 were noted in the HR-1 sequence of either Du151 or Du179 (data not shown). Both Du151 and Du179 were sensitive to T-20 (Figures 3.1 and 3.2). All Du151 isolates were inhibited 100% at 1 µg/ml except the last isolate, which was slightly less sensitive at 91% (Table 3.2). Du179 was inhibited more than 90% at 1 µg/ml T-20 except the last isolate, which was only inhibited 76% at 1 µg/ml. Thus it appears if there was a tendency for the later isolates to become slightly less sensitive to T-20 (Figure 3.2).

3.3.5 Analysis of V3 sequences

The six Du151 and four Du179 isolates were sequenced in the V3 region, aligned and compared. The V3 region of Du151 had 35 amino acids for all 6 isolates but the overall charge decreased from 4.5 to 2.5 (Figure 3.3). The last two isolates Du151-Mar 00 and

Du151-May 00 had an increased charge of 8.5 and were accompanied by a large number of amino acid substitutions in the V3 region (Figure 3.3). These two isolates both had an R5X4 biotype. Changes in the crown of V3 changed for the R5 isolates from GPGQ to GPGL for the two R5X4 isolates. A positive charge was noted at position 25, which is associated with coreceptor switching.

Du179 had fewer changes in the V3 region with most changes occurring for the latter two isolates (Du179-Feb 00 and Du179-May 00). A change in charge from 4.5 to 6 for the latter isolate in the V3 region was noted which coincided with a change from dual tropism (R5X4) to mono-tropism (X4 isolate) in culture as well as on PBMC assays. The V3 region consisted of only 34 amino acids and the last isolate Du179-May 00 had only 32 amino acids. The switch from dual tropism to monotropism (X4) was accompanied by a deletion next to position 25, which is normally associated with charge changes. This is in contrast to Du151 and other X4 isolates (Chapter 2) which either retain 35 amino acids or have insertions to be able to undergo a phenotypic switch. This data indicate that charge related changes in the V3 region are required for Du179 to be able to switch from a dual tropic isolate to a monotropic (X4) isolate.

3.3.6 Changes in other regions of gp160

Insertions were noted in the V5 region of Du151-Mar 00 and Du151-May 00. A change in the fusion peptide was seen when Du151 switched phenotype from LLGFLG to FLGFLG (Mia Coetzer, personal communication). No changes were seen in other regions for Du179.

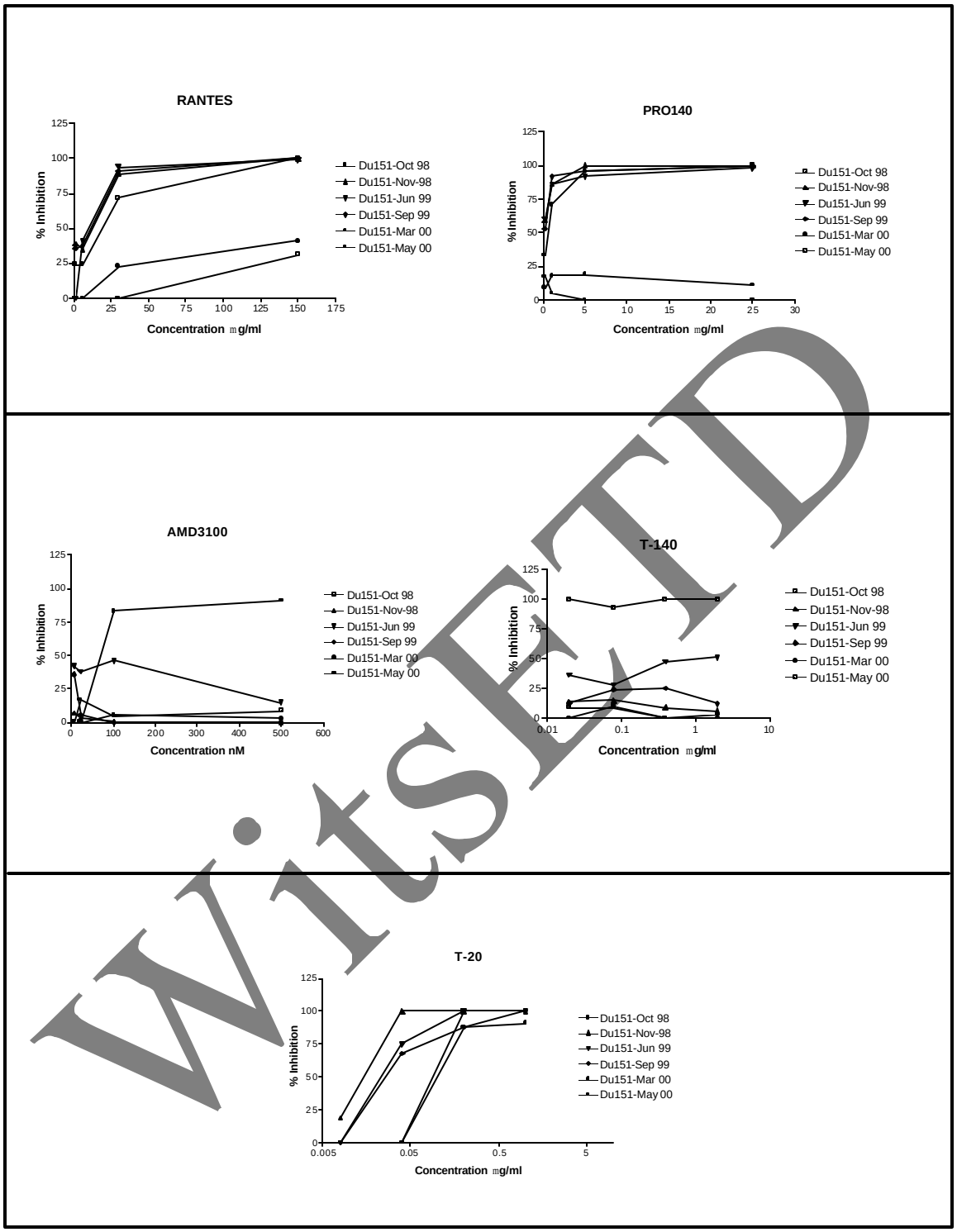


Figure 3.1: Inhibition of Du151 by coreceptor and fusion inhibitors. The longitudinal isolates become less sensitive to the CCR5 inhibitors as a result of the coreceptor switch.

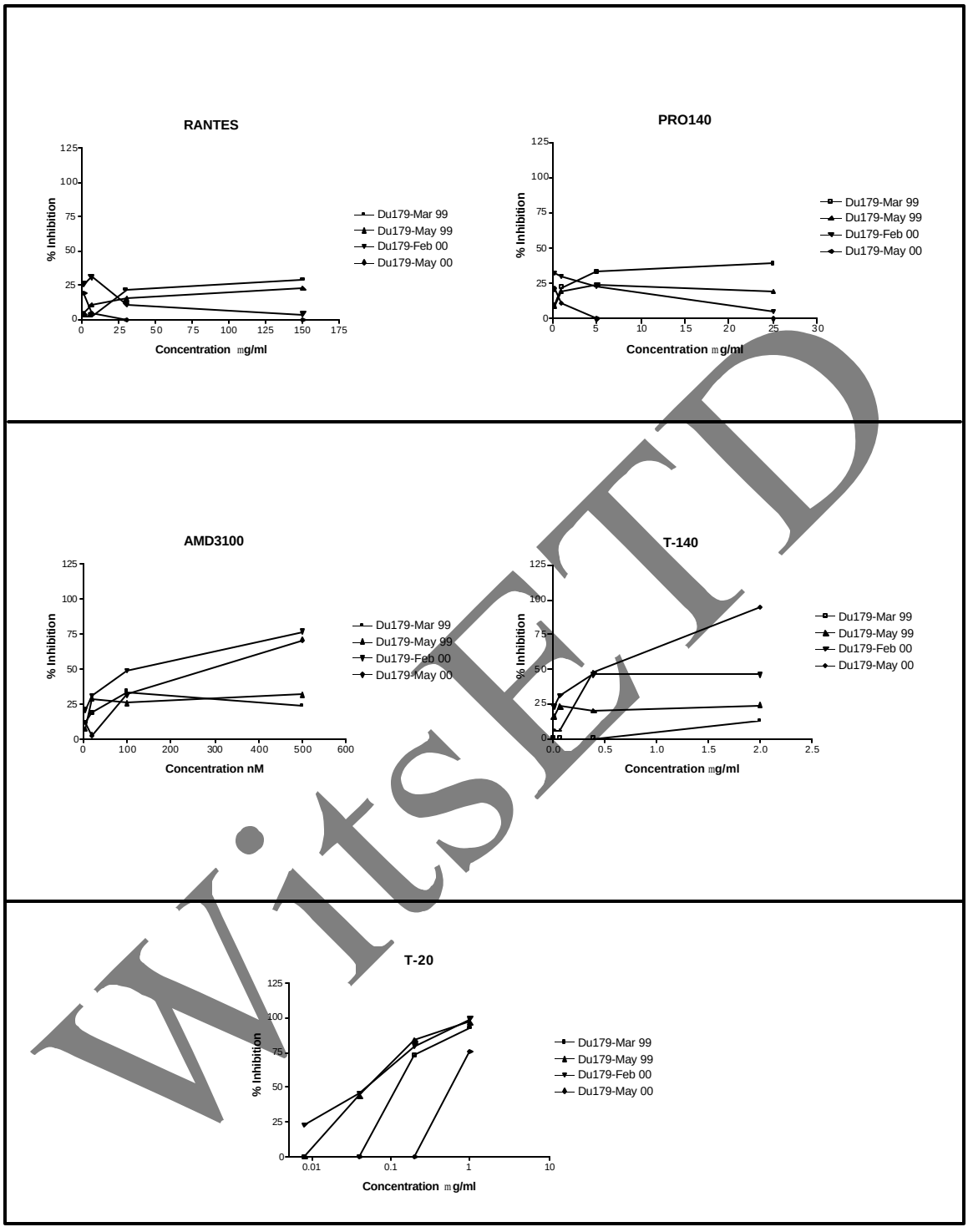
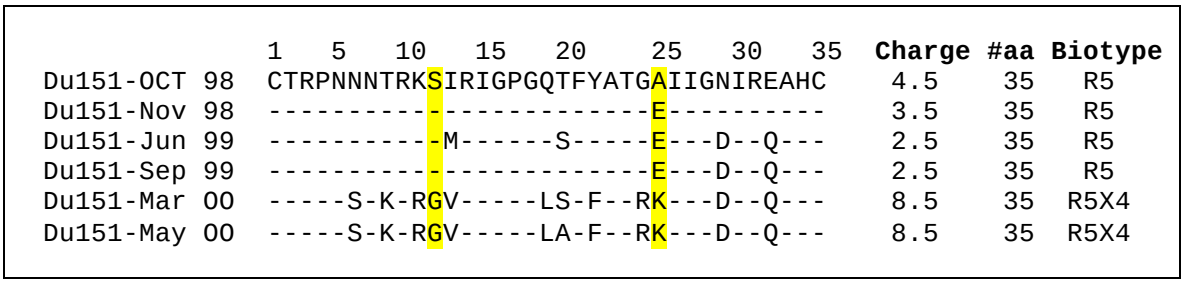
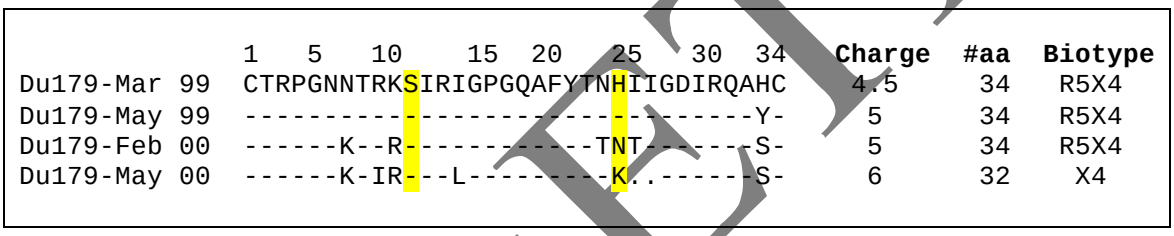


Figure 3.2: Inhibition of Du179 by two CCR5 inhibitors and two CXCR4 inhibitors as well as a fusion inhibitor.



	1 5 10 15 20 25 30 35	Charge	#aa	Biotype
Du151-OCT 98	CTRPNNNTRKSIRIGPGQTFYATGAIIGNIREAHC	4.5	35	R5
Du151-Nov 98	----------E----------	3.5	35	R5
Du151-Jun 99	----------M------S-----E---D--Q---	2.5	35	R5
Du151-Sep 99	----------E---D--Q---	2.5	35	R5
Du151-Mar 00	-----S-K-RGV-----LS-F--RK---D--Q---	8.5	35	R5X4
Du151-May 00	-----S-K-RGV-----LA-F--RK---D--Q---	8.5	35	R5X4

Figure 3.3: V3 amino acid sequences of Du151 longitudinal isolates. Positions 11 and 25, which are associated with a coreceptor switch (highlighted in yellow). The number of amino acids (#aa) is indicated and the biotype indicates coreceptor usage. (Amino acid abbreviations are shown in Appendix B) (Sequences courtesy of Mia Coetzer)



	1 5 10 15 20 25 30 34	Charge	#aa	Biotype
Du179-Mar 99	CTRPGNNTRKSIRIGPGQAFYTNHIIGDIRQAHC	4.5	34	R5X4
Du179-May 99	----------Y-	5	34	R5X4
Du179-Feb 00	------K--R----------TNT------S-	5	34	R5X4
Du179-May 00	------K-IR---L---------K..------S-	6	32	X4

Figure 3.4: V3 amino acid sequences of Du179 longitudinal isolates. Positions 11 and 25 are highlighted in yellow (according to consensus sequence), which are associated with a coreceptor switch. The number of amino acids (#aa) is indicated and the biotype indicates coreceptor usage. (Sequences courtesy of Mia Coetzer)

3.4 Discussion

Ten HIV-1 subtype C isolates from an acutely infected patient cohort were selected for this study. Longitudinal follow-up samples were obtained from 6 patients of whom two (Du151 and Du179) had undergone a coreceptor switch or already had an SI phenotype. The other four isolates continued using the CCR5 coreceptor. The occurrence of an SI phenotype for HIV-1 subtype C is rare with only a few reported cases (Cilliers *et al*., 2003; Janse Van Rensburg *et al*., 2002; Johnston *et al*., 2003; Pollakis *et al*., 2004). The frequency of CXCR4 using isolates was low (2/10) 20% similar to what we found in our previous study described in Chapter 2. Longitudinal follow-up of 2 patients undergoing a coreceptor switch gave some clues as to the necessary changes required to engage CXCR4.

The first isolate for Du151 was obtained pre-seroconversion and six isolates were generated over a period of 20 months. The coreceptor switch for Du151 was accompanied by changes in the V3 region with a charge change from 4.5 to 2.5, which increased, to 8.5. Changes were also seen in the V1 and V5 loops and in the gp41 region. Du151 was shown to be dually infected with two HIV-1 subtype C strains and this may be accountable for the coreceptor switch due to recombination (Grobler *et al*., 2004). Coreceptor inhibition data indicated that the change was gradual from an R5 isolate to a fully dual tropic isolate and then preferential use of CXCR4 on PBMC. Even though the isolate became less sensitive to CCR5 inhibitors, CXCR4 inhibitors were effective at inhibiting the last isolate of Du151. The gradual change in coreceptor usage is reflected

in the steady decline in CD4 count which was associated with the emergence of X4 isolates.

The first isolate obtained from Du179 was 19.8 months post infection and already possessed the ability to use both CCR5 and CXCR4 in cell lines. It too was shown to be dually infected (Grobler *et al*., 2004). Coreceptor inhibition data suggested that this isolate was fully dual tropic from onset and was resistant to both CCR5 and CXCR4 coreceptor inhibitors for the initial three isolates. Even though V3 loop charges resembled R5 isolates it appeared as if they were able to use both CCR5 and CXCR4 efficiently on PBMC. The later isolate became sensitive to the CXCR4 entry inhibitor indicating that it was evolving to use CXCR4 preferentially in PBMC. This switch in coreceptor use was accompanied by an increase in charge as well as a two amino acid deletion, which rendered the V3 loop only 32 amino acids in length.

This is the first study using longitudinal samples to show a coreceptor switch in subtype C. The switch in coreceptor use will benefit the virus in that it provides new targets and possible escape from the immune system. This switch in coreceptor use will have implications for CCR5 coreceptor inhibitors, as they will become ineffective against these isolates. Dual infection has been shown to be responsible for rapid progression to AIDS in some patients (Gottlieb *et al*., 2004). Here we describe two individuals dually infected with different strains of HIV-1 subtype C. The one individual progressed rapidly to AIDS and the other did not, which may indicate differences in immune systems to control the virus.

Targeting different regions of the entry process could be used to prevent HIV-1 from replicating even if the virus undergoes a coreceptor switch. Most HIV-1 subtype C isolates use CCR5 and the occurrence of X4 isolates are very rare. Here we showed two isolates that did switch coreceptor usage. More longitudinal follow-up studies are required to determine if this phenomenon occur at a higher rate as the epidemic progresses.

CHAPTER 4

USE OF ALTERNATE CORECEPTORS ON PRIMARY CELLS BY TWO HIV-1 ISOLATES

4.1 Introduction.

The two most important coreceptors for HIV-1 entry are CCR5 and CXCR4, and all HIV-1 isolates tested to date use one or both (Doms, 2000). A few rare isolates can also use other coreceptors present on coreceptor-transfected cell-lines, including CCR1, CCR2b, CCR3, Bob/GPR15, CXCR6 (STRL33) and CCR8 (Cilliers *et al*., 2003; Deng *et al*., 1997; Edinger *et al*., 1998; Pohlmann, Krumbiegel, and Kirchhoff, 1999; Xiao *et al*., 1998). Some of these receptors such as CXCR6 and CCR8 have been shown to function as HIV entry coreceptors on primary cells *in vitro*. (Lee *et al*., 2000; Sharron *et al*., 2000; Zhang *et al*., 2000; Zhang *et al*., 2001). Further evidence that some isolates can use alternate coreceptors comes from studies where both major coreceptors are either absent or blocked. In this regard, the use of PBMC with a 32-base pair deletion in CCR5 (Δ32/Δ32 CCR5) in the presence of a CXCR4 inhibitor has proven particularly useful (Azevedo-Pereira *et al*., 2003; Willey *et al*., 2003; Zhang *et al*., 2000). Identification of alternative HIV-1 coreceptors is hampered by the lack of suitable ligands although in some instances chemokines have been used to provide suggestive evidence (Willey *et al*., 2003). Collectively these data demonstrate that some rare isolates can exploit alternate coreceptors on primary cells *in vitro*, although whether such receptors are used by HIV-1 *in vivo* remains unclear (Sharron *et al*., 2000; Willey *et al*., 2003; Zhang and Moore, 1999; Zhang *et al*., 2001).

The ability of some viral isolates to use alternate coreceptors could impact on the efficacy of entry inhibitors and possibly also HIV pathogenesis. Hence it is relevant to identify and characterize HIV isolates able to use alternate coreceptors on primary cells.

Viral macrophage inflammatory protein-1 (vMIP-1), a promiscuous chemokine expressed by Human Herpes Virus 8, has been shown to bind to a range of coreceptors, including CCR8, GPR1, CXCR6 and to a lesser extent Bob/GPR15 (Dairaghi *et al*., 1999; Willey *et al*., 2003). This chemokine has been shown to prevent HIV-1 infection of some isolates on primary cells when the major coreceptors are absent or blocked. This suggests that vMIP-1 might be useful in identifying alternate coreceptors that could be used by HIV-1 isolates (Willey *et al*., 2003). The aim of this study was to further investigate how two unusual HIV-1 isolates can use alternate coreceptors on primary cells, by using chemokines with defined receptor binding profiles.

4.2 Materials and Methods

4.2.1 HIV-1 viral isolates:

Two previously described isolates, CM4 (99ZACM4) and CM9 (99ZACM9) from a cohort of AIDS patients, were selected for further analysis (Bredell *et al*., 2002; Cilliers *et al*., 2003; Papathanasopoulos *et al*., 2002; Trkola *et al*., 2001). CM4 was isolated in 1999 from a thirty-four year old male in Johannesburg, South Africa who had advanced HIV infection and cryptococcal meningitis. This patient had a viral load of 163,755 HIV-1 RNA copies/ml (by bDNA assay) and CD4 count of 47 cells/µl. CM9 was described in Chapter 2 and was isolated in 1999 from a thirty- year old female also with cryptococcal meningitis and with a CD4 count of 24 cells/µl. No viral load was available for this patient. Viral isolates were generated from both patients as described in Chapter 2. CM4-P1 was the first isolation reported in a previous publication (Trkola *et al*., 2001), which was later re-expanded using PHA-stimulated PBMC and IL-2 to generate high titre stocks for these experiments (CM4-P2). The gp160 encoding regions of both CM4-P1 and CM4-P2 were sequenced and shown to be more than 99% identical (Figure 4.1a). CM4 was reported initially as an *env* subtype C by HMA (Bredell *et al*., 2002), but subsequent sequence analysis indicated that this virus was a complex recombinant of at least five different subtypes with multiple breakpoints in the *env* region (Papathanasopoulos *et al*., 2002). CM9 was subtype C throughout the genome (Papathanasopoulos *et al*., 2002).

4.2.2 Coreceptor assays on cell lines

Both viruses were tested for their ability to replicate in U87.CD4 cells transfected with either CCR5 or CXCR4 cells as previously described in Chapter 2. In addition, U87.CD4 cell lines expressing CCR1 and GHOST.3 cell lines expressing Bob/GPR15, CCR1,

CCR3 and CXCR6 were used to measure alternate coreceptor usage. These assays were performed as described in Chapter 2.

4.2.3 Δ32/Δ32 CCR5 PBMC cell preparation

Blood was drawn from individuals identified with the Δ32/Δ32 CCR5 mutation and PBMC isolated and stimulated with PHA and IL-2 as previously described in Chapter 2 and (Willey *et al*., 2003). AMD3100 (1 μM) was used to inhibit entry via CXCR4.

CM4 titrations experiments were performed using Δ32/Δ32 CCR5 PBMC stimulated with PHA and used at 1 $X10^5$ cells/well. CM4 was titred from 1000, 800, 600, 400, 200, 100, 50 and 10 TCID50 and added to the cells. The experiments were performed in 96 well round bottom plates and cells were washed 24 hours after infection. The levels of p24 antigen production were measured as described in Chapter 2. The same protocol was followed for the normal donor PBMC assays.

4.2.4 Entry inhibitor assays

Entry inhibitor assays were performed using PHA-stimulated PBMC from wt/wt (wild-type/wild-type) CCR5 and Δ32/Δ32 CCR5 donor cells. Cells were incubated with the specific inhibitor for 1 hour before adding 1,000 $TCID_{50}$ (50% tissue culture infectious dose per ml of virus). Assays were performed on 96 well plates using RPMI 1640, 10% fetal calf serum and IL-2 as previously described in Chapter 2 (Willey *et al*., 2003). Inhibitors tested included the anti-CCR5 Mab PRO140 (Progenics Pharmaceuticals, New York), chemokines RANTES (PeproTech Inc., Rocky Hill, N.J), I309, eotaxin, vMIP-I (all from R&D Systems, Inc) and AMD3100 (AnorMED Inc., Langley, Canada). Viral

replication was measured by p24 antigen production or reverse transcriptase (RT) activity using an RT-enzyme-linked immunosorbent assay (CavidiTech, Uppsala, Sweden). T-20 (American Peptide Company and Hoffmann-La Roche, Palo Alto, Ca) experiments were performed as described in Chapter 5.

4.2.5 RNA extraction and amplification of the gp160 region

HIV-1 viral RNA was extracted from 200 µl of culture supernatant from different cell lines using the MagNA Pure LC Total Nucleic Acid Isolation Kit and automated MagNA Pure LC Instrument (Roche Diagnostics GmbH, Mannheim, Germany). A reverse transcription step was performed and the cDNA was used to amplify the gp160 region with primers as previously described (Gao *et al*., 1996). PCR products were purified using the High Pure PCR Product Purification kit (Roche Diagnostics GmbH, Mannheim, Germany). Sequencing reactions were performed in a MicroAmp 96 well optical reaction plate using the ABI PRISM Big dye Terminator v3.0 Cycle Sequencing kit and ABI PRISM 3100 automated sequencer. Sequence analysis was performed using the Sequencher version 4.0 software (Gene Codes Corporation). The Clustal X program was used for alignments.

4.3 Results

4.3.1 Coreceptor usage of CM4 and CM9

Two HIV-1 isolates from patients with cryptococcal meningitis, a severe AIDS defining condition, were selected for this study. CM9 is an HIV-1 subtype C isolate able to use CCR5, CXCR4, CCR3, CXCR6 and Bob/GPR15 to replicate in transfected cell lines as described in Chapter 2 (Table 4.1). While the efficiency with which CM9 used the three minor coreceptors was considerably lower than for the two major coreceptors (i.e. CCR5 and CXCR4), their usage was highly reproducible. CM4, a complex recombinant virus with subtype C-like envelope was able to use both CCR5 and Bob/GPR15 with equal efficiency on transfected cell lines (Table 4.1). This isolate was unable to use CXCR4, as judged by its inability to replicate in U87.CD4.CXCR4 or MT-2 cells. It was also unable to use CCR3, CXCR6 or CCR1. CCR1 was included as a negative control for both CM4 and CM9 (Table 4.1).

Both CM4 and CM9 were able to replicate in Δ32/Δ32 CCR5 PBMC efficiently although CM4 replicated to higher levels (84 ng/ml versus 10 ng/ml p24 in one representative experiment) (see also Figures 4.2 and 4.3). The growth seen for CM9 in Δ32/Δ32 CCR5 PBMC is most likely related to the ability of this isolate to use CXCR4 (Figure 4.2). Since CM4 is unable to use CXCR4, growth in Δ32/Δ32 CCR5 PBMC must be due to an alternate coreceptor. Whether CM4 is using Bob/GPR15 to enter PBMC cannot be assessed at present, as no inhibitor or natural ligand to block this coreceptor is available.

Table 4.1: Growth of CM4 and CM9 on coreceptor transfected cell lines

Isolate	Coreceptor usage (Day12 p24 ng/ml)						
	U87.CD4			GHOST.3			
	CCR5	CXCR4	CCR1	Bob/GPR15	CCR1	CCR3	CXCR6
CM4	70	<1	<1	65	<1	<1	<1
CM9	69	48	<1	3	<1	3	8.5

4.3.2 Use of CCR5 and CXCR4 inhibitors to determine coreceptor use

To further define coreceptor specificity of CM9 and CM4, specific inhibitors were used. The CCR5 inhibitors included PRO140 and RANTES. AMD3100 was used to block CXCR4. The assays were performed on wt/wt CCR5 PHA-stimulated PBMC with a p24 antigen read-out on day 6. CM4 was not inhibited by either PRO140 or RANTES at the same concentrations that were active against other HIV-1 isolates (Table 4.2) (Chapter 2), (Trkola *et al*., 2001). This isolate was also insensitive to AMD3100, consistent with its inability to use CXCR4. Hence, neither CCR5 nor CXCR4 inhibitors were effective against CM4. In contrast, CM9 was sensitive to AMD3100 (97% inhibition on day 6, Table 4.2). However, like CM4, CM9 was insensitive to the CCR5 inhibitors. Thus the major coreceptor of preference for this pleiotropic isolate is CXCR4 (Chapter 2).

We next tested the ability of CM4 and CM9 to be inhibited by the fusion inhibitor T-20, which is not coreceptor-specific. Both isolates were fully sensitive to T-20 and were inhibited by >99% at 1 µg/ml (Table 4.2). This demonstrates that use of an alternate coreceptor does not radically alter the envelope-mediated fusion process.

Table 4.2: Inhibition data on normal donor (wt/wt) CCR5 PBMC

Isolate	% Inhibition			
	PRO140	**RANTES**	**AMD3100**	**T-20**
	167 nM	19 nM	500 nM	1 µg/ml
CM4	33	24	4	**99**
CM9	36	34	**97**	**100**

4.3.3 Env sequence analysis of CM4 and CM9 grown in different cell lines.

To determine if minor quasispecies were accounting for multiple coreceptor use, CM4 and CM9 were grown in different cell lines. The *env* genes were then sequenced and compared to those of the original infecting strain. CM4-P2 was grown in normal donor PBMC, Δ32/Δ32 CCR5 PBMC, and GHOST.3 cells expressing Bob/GPR15. The amino acid sequences of the expanded viruses were then compared to the original infecting stock (CM4-P1). All gp160 sequences from CM4-P2 grown in different cells were identical and had only 1 amino acid change in V3 region (T300N) compared to the earlier stock (CM4-P1) (Figure 4.1a). Hence, the major viral population of CM4 uses both CCR5 and Bob/GPR15 as coreceptors on transfected cell lines as well as on Δ32/Δ32 CCR5 PBMC, suggesting that the same envelope glycoprotein configuration can use both CCR5 and Bob/GPR15. CM4 had a low overall charge of +3 in the V3 loop, which is more consistent with usage of CCR5 than of CXCR4 (Hoffman *et al*., 2002a).

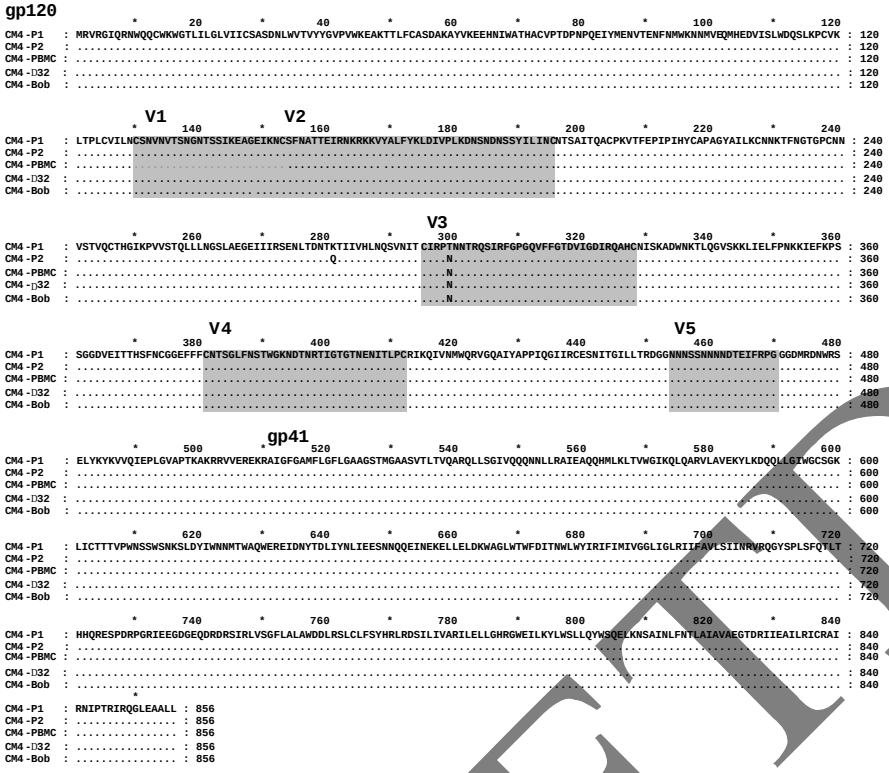


Figure 4.1a: Full-length envelope amino acid sequence alignment of CM4 grown in different cell lines and PBMC. The original culture supernatant is labelled CM4-P1. The regrown strain is labelled CM4-P2. CM4-P2 grown in PBMC (CM4-PBMC), Δ32/Δ32 CCR5 PBMC (CM4-Δ32) and GHOST.3.Bob cell line (CM4-Bob) is shown. The variable loops are highlighted in grey and amino acid changes shown. Dots indicate identical amino acids. (Sequences provided by Dr Maria Papathanasopoulos and Trudy Patience)

A similar analysis was done using CM9 grown in cell lines expressing CCR5, CXCR4, CCR3, CXCR6 and Bob/GPR15, with the resulting sequences then compared to the original isolate grown in wt/wt PBMC. Distinct amino acid sequences changes in gp160 were noted between the selected populations, particularly in the V1/V2 and V3 regions of gp120, and in the cytoplasmic tail of gp41 (Figure 4.1b). Viruses that grew in the CXCR6/Bonzo and CCR3 cell lines were virtually identical in sequence, and most similar to the viruses that grew in the CCR5 transfected cells. The sequence of the virus

grown in CXCR4-expressing cells was identical to the original PBMC sequence and most similar to the virus that grew out of the Bob/GPR15 cells. Three changes were noted in the V3 crown region between these two populations. The viruses expanded on the CXCR4 or Bob/GPR15 cells had a GPRY motif while those grown in CCR5, CXCR6/Bonzo or CCR3 expressing cells had a GPGY sequence. There was no difference in the overall charge (+6) or number of amino acids in the V3 region of the different selected populations, so no prediction about coreceptor usage could be made from the genotype. An insertion in the cytoplasmic tail of the gp41 region was noted for almost all viruses grown in coreceptor-transfected cells except for those grown in CXCR4 transfected cells (Figure 4.1b). This suggests that the original CM9 isolate comprised at least 2 distinct populations, one that uses CCR5, CXCR6 and CCR3 and a second that uses CXCR4 and Bob/GPR15. This mixed population may account for the usage of at least 2 different coreceptors by this isolate.

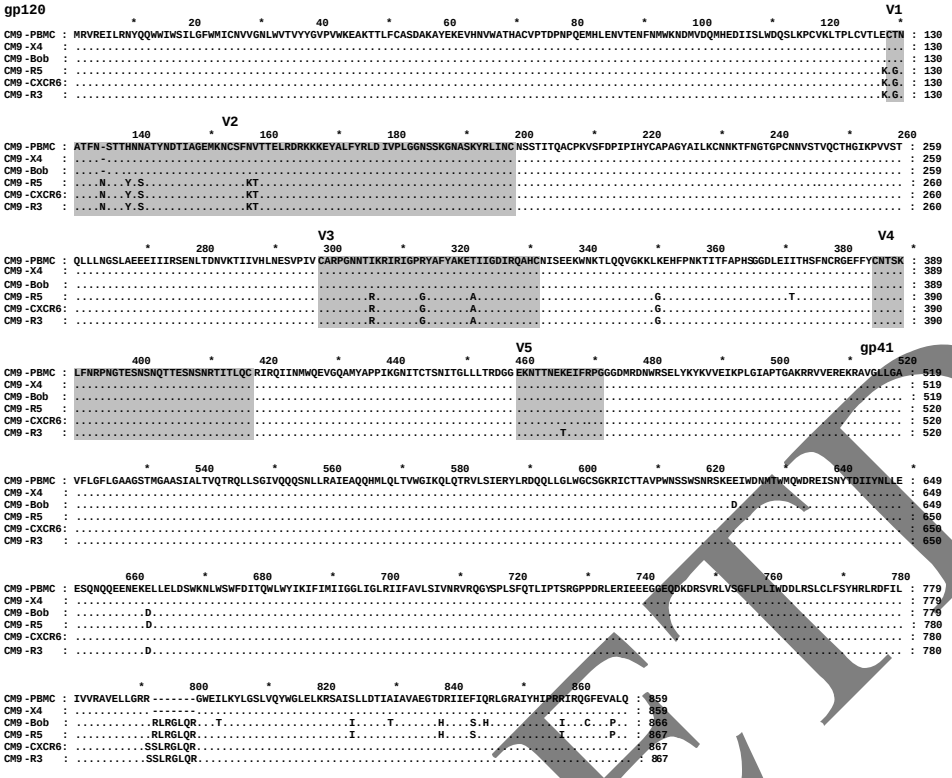


Figure 4.1b: Full length envelope amino acid sequence of CM9 grown in different cell lines and PBMC (CM9-PBMC). CM9 was grown in U87.CD4.CXCR4 (CM9-X4), GHOST.3 transfected with Bob/GPR15 (CM9-Bob), U87.CD4.CCR5 (CM9-R5) and GHOST.3.CXCR6 (CM9-CXCR6) and CCR3 (CM9-R3) respectively. The variable loops are highlighted in grey and amino acid changes shown. Dots indicate identical amino acids and dashes indicate amino acid insertions. (Sequences provided by Dr Maria Papthanasopoulos and Trudy Patience)

4.3.4 Inhibition assays on Δ32/Δ32 CCR5 PBMC

We next explored the ability of chemokines that act as natural ligands for alternate coreceptors to inhibit CM4 and CM9 replication in Δ32/Δ32 CCR5 PBMC. The chemokine vMIP-1 is a promiscuous chemokine secreted by Human Herpes Virus 8 that binds to CCR8, GPR1, Bob/GPR15 and CXCR6 (Dairaghi *et al*., 1999; Simmons *et al*., 2000; Willey *et al*., 2003). Eotaxin is the ligand for CCR3, and I309 is specific for CCR8. CM9 was grown in Δ32/Δ32 CCR5 PBMC in the presence of vMIP-1, I309 and eotaxin

either singly or in combination with AMD3100. It was previously shown that CM9 could be inhibited by AMD3100 for up to 6 days on normal donor PBMC, consistent with its efficient use of CXCR4 for entry (Chapter 2). However, in this study we found that CM9 was eventually able to replicate in the presence of AMD3100 on Δ32/Δ32 CCR5 PBMC when the assay was extended beyond 6 days (Figure 4.2A). When AMD3100 was combined with eotaxin, the natural ligand for CCR3, there was enhanced viral replication. vMIP-1 and I309 had no effect on CM9 replication when tested individually (Figure 4.2A) but when they were combined with AMD3100 inhibition was complete (Figure 4.2B). Since both I309 and vMIP-1 can bind to CCR8, CM9 may be using CCR8 to infect Δ32/Δ32 CCR5 PBMC in the presence of AMD3100.

CM4 was tested in Δ32/Δ32 CCR5 PBMC in the absence and presence of AMD3100, vMIP-1, I309 and CXCL16 (Figure 4.3). CXCL16 is the natural ligand for CXCR6. None of these inhibitors had any effect on CM4 replication except vMIP-1, which caused a delay. Hence, CM4 is using a coreceptor other than CCR5, CXCR4, CCR8 or CXCR6 to productively infect PBMC. Its identity is still unclear, but it may be a vMIP-1 receptor.

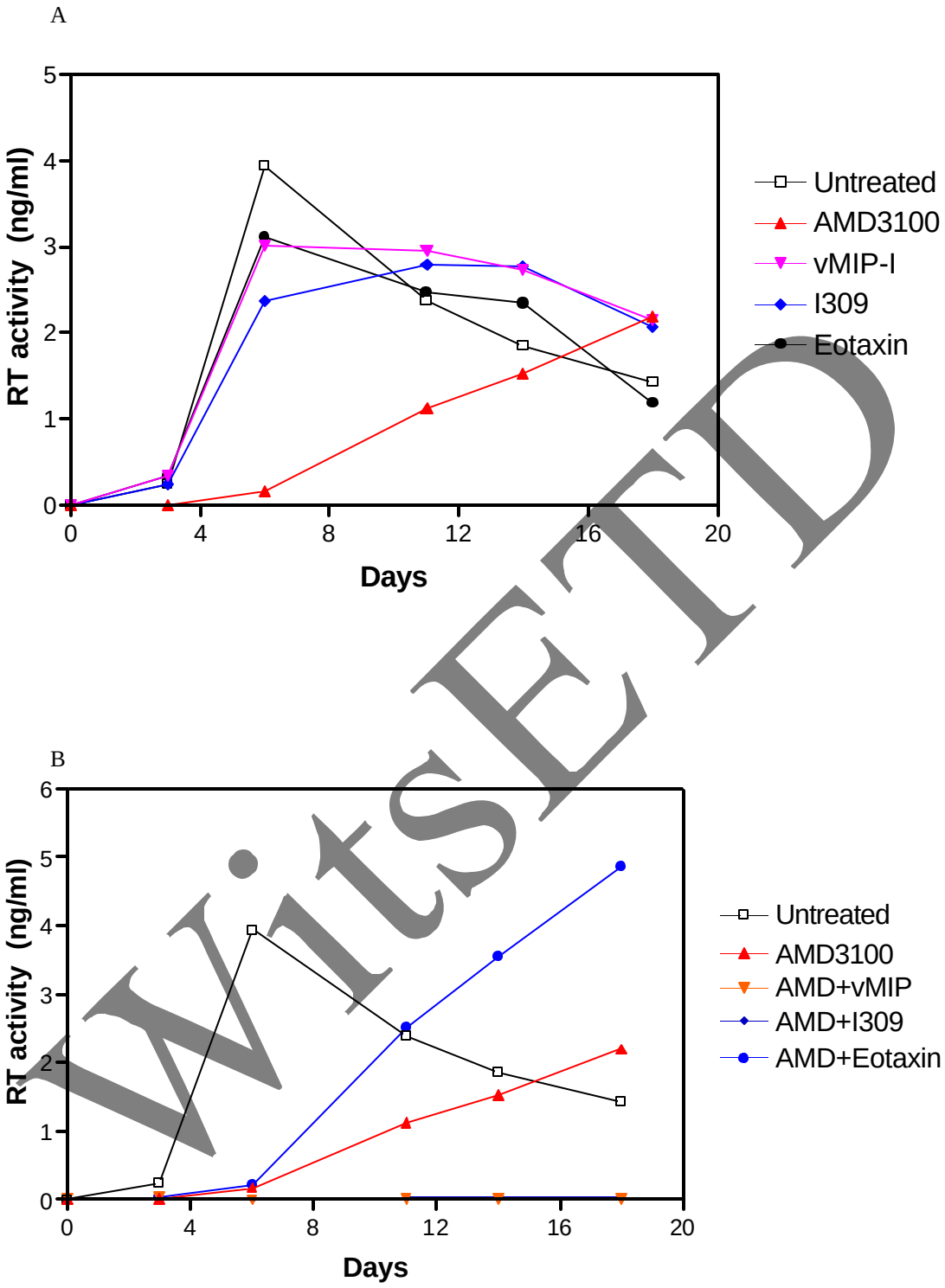


Figure 4.2: Inhibition of CM9 in Δ32/Δ32 CCR5 PBMC. (A) Growth curve of CM9 in the presence of AMD3100, vMIP-1, I309, eotaxin or without inhibitors. (B) CM9 in the presence of AMD3100 alone or in combinations with vMIP-1, I309 or eotaxin or no inhibitors. (Samantha Willey and Mathew Sullivan in Dr Paul Clapham's laboratory performed these experiments)

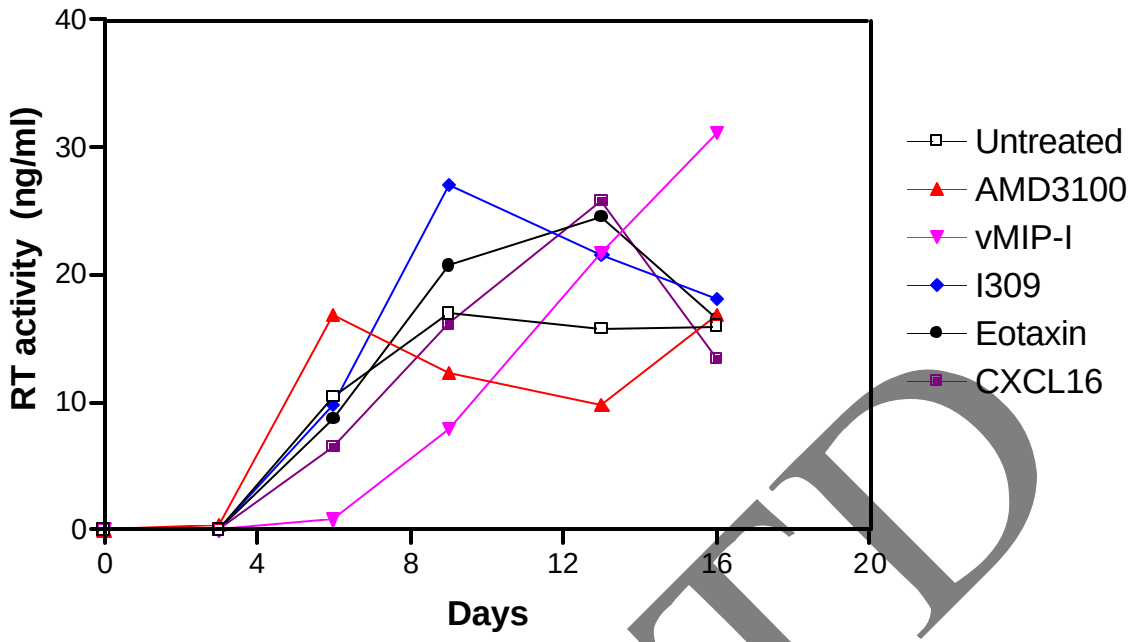


Figure 4.3: Growth curve of CM4 in Δ32/Δ32 CCR5 PBMC in the presence of AMD3100, vMIP1, I309, CXCL16 or without inhibitors. (Samantha Willey and Mathew Sullivan in Dr Paul Clapham's laboratory performed these experiments).

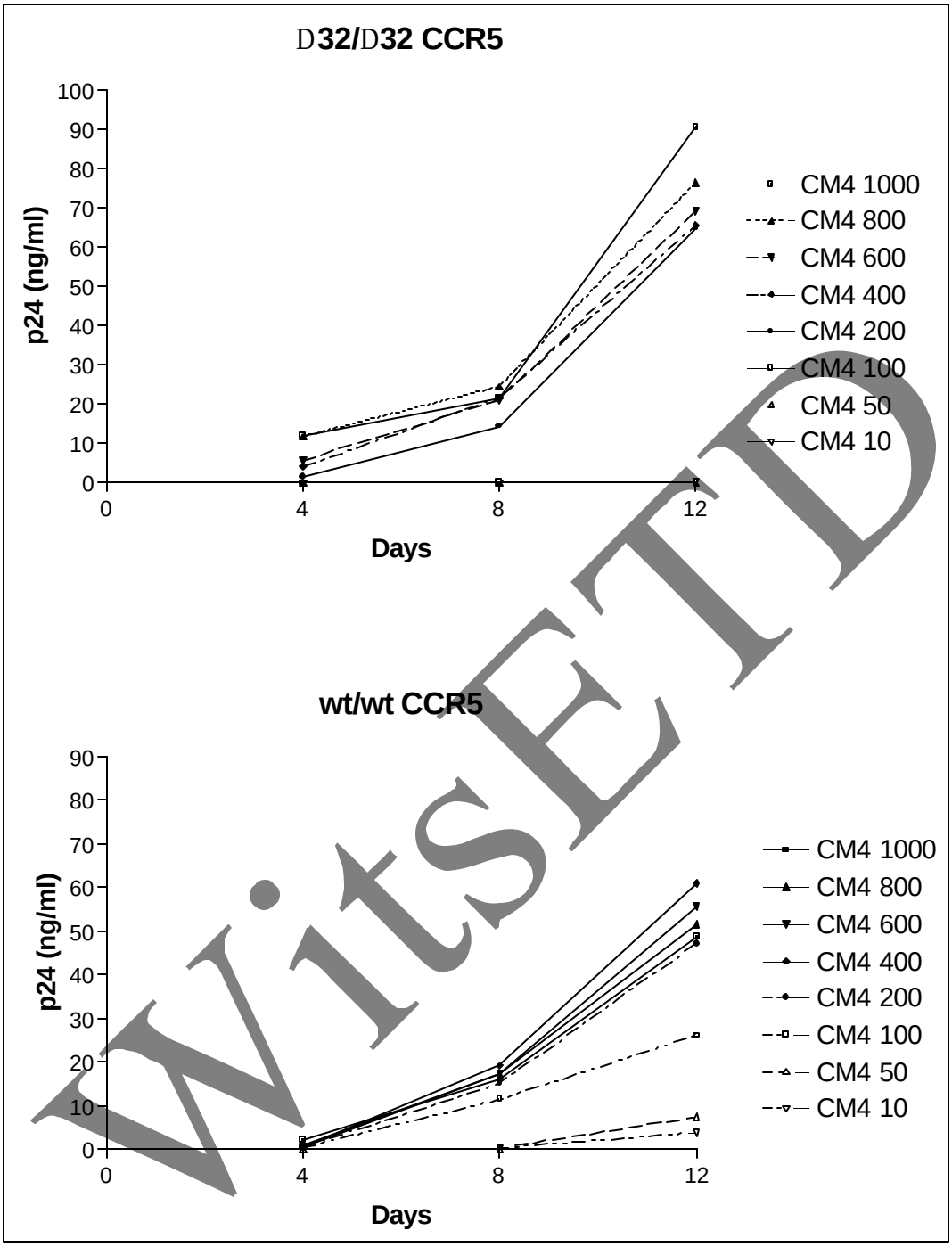


Figure 4.4: Titration of CM4-P2 performed on Δ32/Δ32 CCR5 PBMC and normal donor PBMC. CM4 was diluted starting at 1000 $TCID_{50}$ down to 10 $TCID_{50}$ and the levels of p24 were measured using an in-house p24 assay. The top figure was performed in Δ32/Δ32 CCR5 PBMC and the bottom experiment was performed on normal donor PBMC (wt/wt).

4.3.5 Titration of CM4 on Δ32/Δ32 CCR5 and normal donor PBMC

Titration experiments were performed in order to establish how well CM4 infects PBMC with or without CCR5 and to determine the levels of infection in the absence of CCR5. CM4-P2 was diluted using different $TCID_{50}$ concentrations starting at 1000, 800, 600, 400, 200, 100, 50 and 10 and was grown in Δ32/Δ32 CCR5 PBMC and normal donor PBMC. The ability of the virus to grow in the PBMC was measured using an in-house p24 antigen assay and were measured on days 4, 8 and 12. CM4 was able to replicate very efficiently in the Δ32/Δ32 CCR5 PBMC up to 200 $TCID_{50}$ and was unable to infect PBMC at a lower concentration (Figure 4.4). CM4-P2 was able to infect normal donor PBMC at 10 $TCID_{50}$ albeit at low levels. This suggests that CM4-P2 requires 200 $TCID_{50}$ or higher to be able to use an alternate coreceptor. This may explain why CM4-P1, which had a very low titre, was unable to infect PBMC deficient of CCR5.

4.4 Discussion

The identification of HIV-1 isolates able to use coreceptors other than CCR5 and CXCR4 on primary cells is rare (Lee *et al*., 2000; Willey *et al*., 2003; Zhang *et al*., 2000). While the significance of alternate coreceptor usage is uncertain, the possibility that HIV-1 can expand its cellular host range through the use of alternate coreceptors remains a theoretical possibility. Here we report on how two isolates, CM4 and CM9, can use alternate coreceptors to infect primary cells. Inhibition data suggests that CCR8 and an as yet unknown coreceptor can mediate HIV-1 entry for these two isolates respectively. Such isolates would be expected to be refractory to inhibition by agents directed at CCR5 and CXCR4.

CM4, an R5 isolate able to utilize Bob/GPR15, on transfected cell lines was isolated from a patient with advanced HIV disease. Its insensitivity to both CCR5 and CXCR4 inhibitors suggested that it could use an alternate coreceptor to infect PBMC. This receptor is apparently also present on Δ32/Δ32 CCR5 PBMC and can support high levels of replication. Whether CM4 is able to use Bob/GPR15 on primary cells remains to be determined. Bob/GPR15 is an orphan G-protein coupled receptor whose mRNA is widely expressed in the colon, spleen, PHA stimulated PBMC, purified T-cells and to a lesser extent in unstimulated PBMC (Deng *et al*., 1997). Until a natural ligand to Bob/GPR15 is identified it will remain difficult to prove that CM4 is using this receptor to enter primary cells. In a previous study a different batch of CM4 (CM4-P1) was inhibited by RANTES, PRO140 and TAK779 suggesting that entry was CCR5-dependent (Trkola *et al*., 2001). For the experiments reported here, CM4 had been expanded in PHA-stimulated PBMC to

generate a high titre stock (CM4-P2). The entry of this virus preparation was found to be independent of CCR5, as reflected by its ability to grow in Δ32/Δ32 CCR5 PBMC. Experiments directly comparing the properties of the original (CM4-P1) and expanded viral stock (CM4-P2) confirmed these findings (data not shown). The envelope sequences of CM4-P1 and CM4-P2 showed they were more than 99% identical (Figure 4.1a). It is unlikely that the single amino acid change T300N between CM4-P1 and CM-4P2 is responsible for the altered coreceptor phenotype. It is more likely that the ability of CM4-P2 to grow in Δ32/Δ32 CCR5 PBMC is related to the viral titre, with the ability to use alternate coreceptors only being revealed when the viral titre is high (Figure 4.3). Nevertheless we cannot formally rule out that the ability of CM4 to replicate in Δ32/Δ32 CCR5 PBMC, presumably by alternate coreceptor-use, was acquired or selected for during *in vitro* passage.

CM9 was also isolated from a patient with advanced HIV infection and it too is a pleiotropic isolate that has a preference for CXCR4-usage (Chapter 2). Other studies have shown that CXCR4 is the coreceptor of preference for most dual- or pleiotropic isolates (Yi *et al*., 1999). CM9 was previously shown to use alternate coreceptors, CCR3, CXCR6 and Bob/GPR15 albeit less efficiently than CXCR4 (Cilliers *et al*., 2003). In this study we show that in addition to using these five coreceptors, CM9 can also use CCR8. This was confirmed using NP2.CD4.CCR8 transfected cells, which supported CM9 replication (data not shown). However, the use of CCR8 appears to be less efficient than CXCR4. Thus during the first 6 days, replication in PBMC was fully inhibited by AMD3100, suggesting a dominant role of CXCR4. However, replication at later time points was

inhibited by both vMIP-1 and I309, a CCR8-specific inhibitor, in the presence of AMD3100. Thus it appears that growth in the presence of AMD3100 after 6 days is due to the ability of this isolate to use CCR8. This later use of CCR8 may be related to expression levels of this receptor on PBMC, which may vary in culture, or the utilization of CCR8 by different populations in the CM9 stock, or less efficient usage of CCR8 compared to CXCR4. CCR8 is expressed at high level in the thymus and has the ability to facilitate HIV-1 entry into thymocytes (Lee *et al*., 2000). This receptor has been reported to be used by a handful of simian immunodeficiency virus (SIV), HIV-1 and HIV-2 isolates and is one of the more commonly used alternate coreceptors (Horuk *et al*., 1998; Rucker *et al*., 1997). However, most of the above studies were performed only on cell lines, raising questions as to the significance of the findings for understanding replication in primary cells. Our finding that CM9 uses CCR8 on primary cells suggests that CCR8 may indeed prove to be a significant HIV-1 coreceptor for some isolates, a factor that may need to be taken into consideration in clinical studies of CCR5 and CXCR4 inhibitors. Chemokines that target CCR8, such as I309 may need to be considered for drug development.

Analysis of envelope sequences from viruses grown in PBMC and cell lines revealed that the same viral population of CM4 was able to use CCR5, Bob/GPR15 and a coreceptor on CCR5 deficient PBMC. In contrast, distinct viral populations may account for some of the promiscuous coreceptor activity of CM9. Two distinct viral populations could be discerned in gp160 from CM9. The population able to use CCR5, CXCR6 or CCR3 had three amino acid changes in the V3 region and 4 amino acid changes in V2 compared to

the population able to utilize CXCR4 or Bob/GPR15. However the population able to utilize Bob/GPR15 also had an insertion in the gp41 similar to that found in the CCR5, CXCR6 and CCR3 using virus. This suggests that the Bob/GPR15 utilizing envelope is distinct from all the other populations; it even may be a recombinant between different populations. Further studies on this virus may provide clues as to the genetic determinants of both major and alternate coreceptor usage. However the CCR8-using virus is likely to be a subpopulation different than the one that uses CXCR4, as the usage of the latter coreceptor was effectively inhibited up to day 6 in a PBMC culture. Hence, the sequence analysis performed on CM9 grown in the different cell lines may not used to conclusively indicate which viral population accounts for use of the CCR8 receptor on Δ32/Δ32 CCR5 PBMC.

Whether it is significant that both the CM4 and the CM9 isolates were derived from patients with cryptococcal meningitis still remains unclear. Cryptococcal meningitis is a severe AIDS condition that arises after the immune system has deteriorated and it is associated with high levels of viral replication in blood and cerebrospinal fluid (Morris *et al*., 1998). It is possible that when CD4 target cells are limited HIV-1 isolates exploit other receptors to maintain high levels of replication. Alternatively, cryptococcal meningitis could up-regulate other chemokine receptors or allow access of HIV-1 to other cell populations, such as in the central nervous system that may promote use of alternate coreceptors. This data may also point to the need for the use of a combination of entry inhibitors that target different stages in the entry cycle, such as the CD4-binding site or the fusion process. T-20 was able to efficiently inhibit both CM4 and CM9 strains

suggesting that it is not influenced by alternate coreceptor usage. Thus T-20 may be a useful adjunct to coreceptor inhibitors (Tremblay *et al*., 2000, Tremblay *et al*., 2002). Collectively these data suggest that alternate coreceptors need to be considered when developing entry inhibitors and that such isolates pose a potential threat to the clinical use of inhibitors targeting only CCR5 and CXCR4.

CHAPTER 5

SENSITIVITY OF HIV-1 SUBTYPE C ISOLATES TO THE ENTRY INHIBITOR T-20

5.1 Introduction

T-20 is the first in a new class of anti-retroviral drugs termed entry inhibitors licensed for use in humans and is known by the name T-20 or Fuzeon. In Phase III human clinical trials this drug has been shown to be effective for patients in deep salvage therapy including those with viruses resistant to reverse transcriptase and protease inhibitors (Lalezari *et al*., 2003b). Coreceptor interactions have also been reported to play a role in T-20 efficiency with CCR5-using isolates requiring higher concentrations of T-20 to inhibit replication compared to CXCR4-using viruses. (Derdeyn *et al*., 2000; Derdeyn *et al*., 2001; Reeves *et al*., 2002). Resistance to T-20 is associated with changes in the HR-1 region, particularly the GIV motif (Hanna *et al*., 2002; Kilby *et al*., 2002; Villahermosa *et al*., 2003; Wei *et al*., 2002). This motif is highly conserved among HIV-1 isolates, which has led to the assumption that all subtypes are naturally susceptible to T-20 (Hanna *et al*., 2002; Villahermosa *et al*., 2003; Xu *et al*., 2002). However, there have been almost no comprehensive phenotypic assessments of the sensitivities of non-B subtypes to T-20 to validate this assumption.

Subtype C viruses show significant genetic differences from subtype B in the HR-2 region used to design T-20, with 12 of the 36 amino acids in the consensus C sequence being different (Hanna *et al*., 2002). In addition subtype C viruses show differences in coreceptor preference with reported but infrequent use of CXCR4 (Cilliers *et al*., 2003; Janse Van Rensburg *et al*., 2002; Johnston *et al*., 2003). We therefore aimed to determine whether T-20 would be effective *in vitro* against subtype C viruses able to use both

CCR5 and/or CXCR4 as well as viruses from patients with resistance to other anti-retroviral drugs.

5.2 Materials and methods

5.2.1 Viral isolates

The primary isolates used in the study were derived from South African patients infected with HIV-1 subtype C viruses. This included 4 isolates from acutely infected sex workers (Williamson *et al*., 2003), 11 isolates from adult patients with AIDS (Chapter 2) and 3 isolates from pediatric AIDS patients (Appendix A). In addition viruses were isolated from 5 patients failing anti-retroviral therapies. These were DR10 (currently or previously treated with d4T, 3TC, EFV, AZT, ddI); DR19 (AZT, ddI, EFV); DR28 (AZT, 3TC, EFV, ddI, d4T); DR62 (3TC, d4T) and DR100 (d4T, 3TC, RTV). Clinical data for these isolates are described in Appendix A. Sequence analysis revealed the presence of drug resistance mutations in the *pol* gene (see list of mutations at bottom of Table 5.2). Primary virus stocks were generated as described in Chapter 2. Of these 23 subtype C isolates, 15 used the CCR5 coreceptor, 3 used CXCR4 and 5 used both CCR5 and CXCR4 (Chapter 2 and Chapter 3). Six HIV-1 subtype B R5 isolates from acutely infected South African patients were included for comparison.

5.2.2 Nucleic acid amplification and sequencing

Full-length gp160 was amplified from viral RNA in a nested RT-PCR using the first round outer primers *env* A and *env* N and second round inner primers *env* B and *env* M as described previously (Gao *et al*., 1996). Primers specific for the gp41 region were used to sequence the PCR products in both directions. The sequencing reactions were electrophoresed and analyzed on an ABI3100 sequencer (Applied Biosystems, CA).

Sequence data was edited and assembled and neighbor-joining trees were constructed from the nucleotide sequences as described previously (Papathanasopoulos *et al*., 2002).

5.2.3 T-20 phenotypic sensitivity assays

Experiments were performed using peripheral blood mononuclear cells (PBMC) with a p24 antigen read-out. Five-fold dilutions of T-20 (American Peptide Company, Inc, Sunnyvale, California) were pre-incubated with each virus (1,000 $TCID_{50}$) in 100 µl RPMI 1640 plus 10% FCS and IL-2 for 1 hr. Following this, 10^5 CD8-depleted activated PBMC (Chapter 2) in 100 µl was added to give a final concentration of 1, 0.2, 0.04 and 0.008 µg/ml T-20. Levels of p24 antigen in supernatants were measured on days 4, 6, 8 and 10 by chemiluminescence ELISA as described (Trkola *et al*., 2001) (Chapter 2). Experiments were done in duplicate and repeated twice with different batches of PBMC. Data are shown as the percentage inhibition by T-20 relative to viral cultures without T-20 (virus control). The mean 50% inhibitory concentration (IC_{50}) and IC_{90} values were calculated for each virus.

5.3 Results

We investigated the amino acid similarity between T-20 and the corresponding region in the gp41 sequences of the isolates used in this study. Phylogenetic analysis of the gp41 gene confirmed the subtype designation of all subtype C and B isolates used in this study (Figure 5.1). Analysis of the predicted amino acid sequences of the T-20 region in HR-2 showed that between 10 and 16 of the 36 amino acids were different (Table 5.1). These changes were present for almost all subtype C isolates at certain positions in the HR-2 region. The median number of changes for the South African subtype C viruses was 12 (33%) which is the same as the consensus subtype C. Collectively more than half the loci (19 of 36) could tolerate variation. However 17 loci were 100% conserved suggesting functionally relevant domains in this region. The 6 South African subtype B sequences showed the lowest level of variation, with only 2 to 6 amino acid differences (6-17% difference) as expected, given that T-20 is derived from a subtype B isolate. These data are shown graphically in Figure 5.2

This high level of genetic diversity prompted us to investigate the efficacy of T-20 to inhibit HIV-1 subtype C virus replication *in vitro*. Table 5.2 shows that all 23 subtype C isolates were inhibited >90% at 1 µg/ml T-20. The IC_{50} values ranged from 0.008-0.324 µg/ml with 7 isolates having IC_{50} values at the lowest concentration tested (0.008 µg/ml). Of these, 4 used CXCR4 and 3 used CCR5. There were, however, no significant differences in IC_{50} or IC_{90} values between the CCR5 and CXCR4-using viruses (Figure 5.3 A and B). There were no apparent differences in the sensitivity of viruses from adult

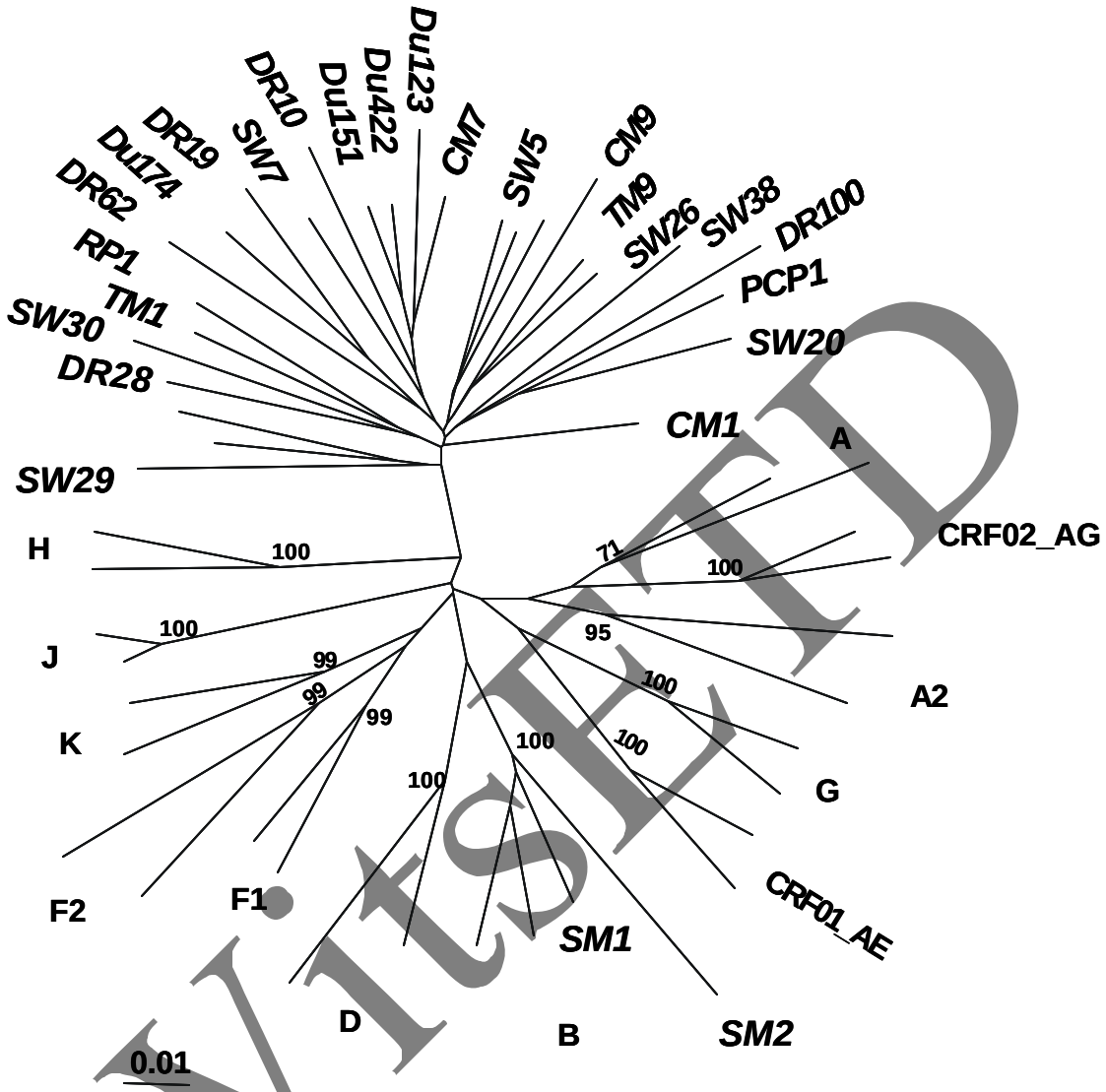


Figure 5.1: Phylogenetic analysis of gp41 sequences of isolates used in this study. All 23 subtype C isolates clustered significantly with C references and the two subtype B isolates clustered with the subtype B references.

Table 5.1: Amino acid sequences in the HR-2 region of gp41 subtype C isolates aligned to T-20

T-20 sequence	Y	T	S	L	I	H	S	L	I	E	E	S	Q	N	Q	Q	E	K	N	E	Q	E	L	L	E	L	D	K	W	A	S	L	W	N	W	F	# aa changes
Consensus C sequence	.	.	G	T	.	Y	R	.	L	.	D	.	.	.	.	.	.	.	.	.	K	D	.	.	A	.	.	S	.	N	N	.	.	.	.	.	12 (33%)
Subtype C-acute																																					
Du123a	.	.	G	T	.	Y	K	.	L	.	.	.	.	.	.	.	.	.	.	.	K	D	.	.	A	.	.	S	.	K	N	.	.	S	.	.	12 (33%)
Du151a	.	.	G	T	.	Y	R	.	L	.	D	.	.	.	.	.	.	.	.	.	K	D	.	.	A	.	.	S	.	K	N	.	.	.	.	.	12 (33%)
Du174	F	.	D	I	.	Y	N	.	L	.	V	.	.	.	.	.	.	E	.	.	K	D	.	.	A	.	N	.	.	Q	N	.	.	G	.	.	15 (42%)
Du422a	.	.	N	T	.	F	R	.	L	.	D	.	.	.	.	.	.	.	.	.	K	D	.	.	A	.	.	S	.	K	N	.	.	.	.	.	12 (33%)
Subtype C-AIDS																																					
PCP1	.	.	G	E	.	Y	R	.	L	.	.	.	.	.	.	.	.	.	.	.	K	D	.	.	A	.	.	S	.	N	N	.	.	.	.	.	11 (31%)
SW5	.	.	N	T	.	Y	R	.	L	.	.	.	T	.	.	.	N	.	.	.	K	D	.	.	A	.	.	S	.	K	N	.	.	.	.	.	13 (36%)
SW26	.	S	N	T	.	Y	K	.	L	.	D	.	.	I	.	.	.	Q	.	.	K	D	.	.	A	.	.	S	.	K	N	.	.	S	.	.	16 (44%)
SW29	.	.	N	I	.	Y	R	.	L	.	D	.	.	.	.	.	.	N	.	.	K	D	.	.	A	.	.	.	.	N	N	.	.	.	.	.	12 (33%)
SW38	.	.	D	T	.	Y	R	.	L	.	.	.	.	T	.	.	.	.	.	.	K	D	.	.	A	.	.	.	.	N	N	.	.	.	.	.	11 (31%)
CM1	.	.	D	T	.	Y	R	.	L	.	A	.	.	.	.	.	.	.	.	.	.	D	.	.	A	.	.	.	.	N	N	.	.	.	.	.	10 (28%)
CM7	.	.	N	T	.	Y	R	.	L	.	E	.	.	T	.	.	.	.	.	.	K	D	.	.	A	.	.	S	.	K	N	.	.	.	.	.	13 (36%)
CM9	.	.	D	I	.	Y	N	.	L	.	.	.	.	.	.	.	.	E	.	.	K	.	.	.	.	.	.	S	.	K	N	.	.	S	.	.	11 (31%)
SW20	.	.	N	T	.	Y	R	.	L	.	D	.	.	.	.	.	.	.	.	.	K	D	.	.	A	.	.	.	.	N	N	.	.	.	.	.	11 (31%)
SW30	.	.	E	T	.	Y	R	.	L	.	.	.	.	.	.	.	.	R	.	.	.	D	.	.	A	.	.	S	.	N	N	.	.	S	.	.	12 (33%)
SW7	.	.	D	T	.	Y	R	.	L	.	V	.	.	.	.	.	.	.	.	.	K	D	.	.	A	.	.	.	.	N	.	.	.	S	.	.	12 (33%)
Subtype C-pediatric AIDS																																					
RP1	.	.	E	T	.	Y	R	.	L	.	D	.	.	.	.	.	.	Q	.	.	K	D	.	.	A	.	.	R	.	N	N	.	.	S	.	.	14 (39%)
TM1b	.	.	N	T	.	Y	R	.	L	.	D	.	.	S	.	.	.	E	.	.	K	D	.	.	A	.	.	S	.	N	S	.	.	.	.	.	14 (39%)
TM9	.	.	N	T	.	Y	R	.	L	.	.	.	.	.	.	.	.	D	.	.	K	D	.	.	A	.	.	S	.	K	N	.	.	.	.	.	12 (33%)
Subtype C-drug resistant																																					
DR10	.	.	D	T	.	Y	R	.	L	.	.	.	.	T	.	.	.	.	.	.	K	D	.	.	.	.	.	.	.	D	.	.	.	.	.	.	10 (28%)
DR19	.	.	G	.	.	Y	N	.	L	.	V	.	.	.	.	.	.	Q	.	.	K	D	.	.	A	.	N	N	.	Q	.	.	.	.	.	.	13 (36%)
DR28	.	.	D	E	.	Y	R	M	L	.	D	.	.	I	.	.	.	.	.	.	K	D	.	.	A	.	.	R	.	N	N	.	.	.	.	.	14 (39%)
DR62	.	.	G	I	.	Y	Q	.	L	.	.	.	.	I	.	.	.	Q	.	.	K	D	.	.	A	.	.	.	.	Q	N	.	.	S	.	.	13 (36%)
DR100	.	.	D	I	.	Y	T	.	L	.	D	.	.	S	.	.	.	.	.	.	.	D	.	.	A	.	.	.	.	N	.	.	.	.	.	.	10 (28%)
Subtype B-acute																																					
SM1	.	.	G	.	.	Y	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3 (8%)
SM2	.	.	G	V	.	Y	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	T	.	6 (17%)
DS3	.	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2 (6%)
DS8	.	.	K	E	.	Y	K	.	.	I	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	6 (17%)
DS9	.	.	G	.	.	Y	T	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4 (11%)
DS12	.	.	G	I	.	Y	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4 (11%)

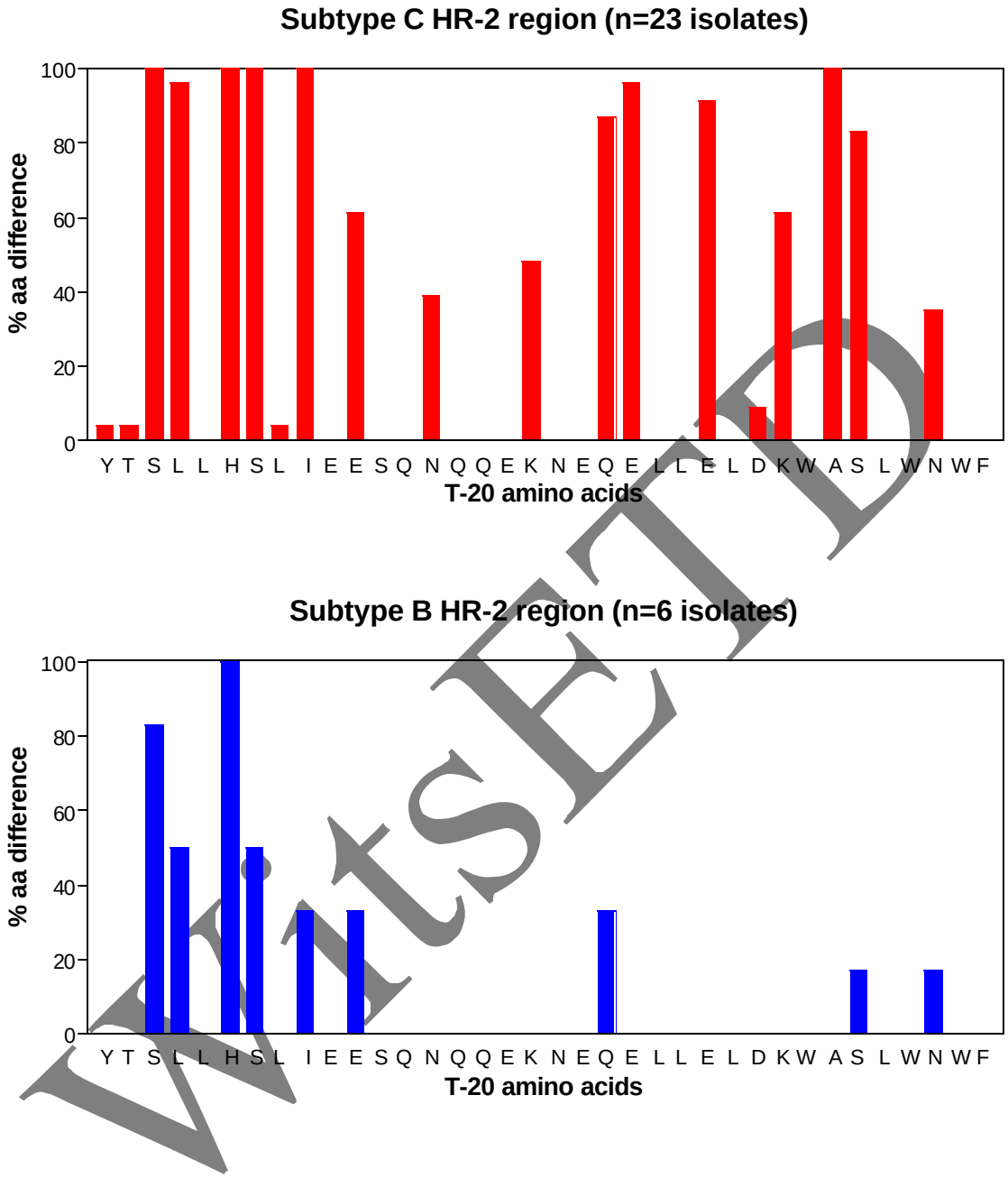


Figure 5.2: Variation in the HR-2 region of HIV-1 subtype C and B compared to T-20. The mean percentage amino acid changes at a certain position containing the total number of isolates with a change at that position. This was compared to the T-20 peptide in the HR-2 region comparing subtype C with subtype B isolates.

Table 5.2: Inhibition of HIV-1 subtype C viruses by T-20 and analysis of baseline resistance patterns

Virus	Biotype	Day	T-20 at*:		T-20 (mg/ml)		Region associated with T-20 resistance																		
							30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
			1 mg/ml	200 ng/ml	IC_{90}	IC_{50}	A	R	Q	L	L	S	**G**	I	**V**	Q	Q	Q	S	N	L	L	R
Subtype C-acute																							
Du123a	R5	8	**100**	***98***	0.088	0.010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Du151a	R5	8	**100**	**100**	0.078	0.014	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Du174	R5	6	**100**	**100**	0.073	0.014	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Du422a	R5	8	**100**	**93**	0.207	0.035	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Subtype C-AIDS																							
PCP1	R5, CXCR6	6	**93**	*87*	0.294	0.017	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SW5	R5	8	**100**	**100**	0.132	0.009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SW26	R5	6	**98**	**98**	0.073	0.008	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SW29	R5	6	**99**	**90**	0.312	0.046	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.
SW38	R5	6	**100**	**100**	0.173	0.063	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K
CM1	R5	6	**98**	**94**	0.191	0.090	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CM7	R5	6	**92**	**90**	0.249	0.123	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.
CM9	R5X4, CXCR6, Bob, R3	6	**100**	**93**	0.260	0.059	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SW20	R5X4	8	**96**	**96**	0.093	0.008	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SW30	R5X4	6	**100**	**94**	0.093	0.008	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SW7	X4	4	**98**	**92**	0.468	0.031	.	.	.	.	.	.	.	**V**	.	.	.	.	.	.	.	.	.
Subtype C-pediatric AIDS																							
RP1	R5X4	6	**90**	*53*	0.910	0.276	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TM1b	R5X4	6	**100**	**100**	0.073	0.008	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TM9	X4	8	**99**	**95**	0.008	0.008	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Subtype C-drug resistant**																							
DR10	R5	10	**100**	*89*	0.378	0.094	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.
DR19	R5	10	**99**	**99**	0.027	0.008	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DR28	X4	6	**100**	*89*	0.399	0.070	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DR62	R5	10	**100**	**100**	0.022	0.008	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DR100	R5	10	**99**	*57*	0.559	0.324	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.
Subtype B-acute																							
SM1	R5	6	**93**	*62*	0.966	0.079	.	.	L	.	.	.	.	.	.	.	.	.	N	.	.	.	.
SM2	R5	6	**93**	*69*	0.952	0.150	.	.	L	.	.	.	.	.	.	.	.	.	N	.	.	.	.
DS3	R5	6	**95**	*70*	0.766	0.156	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	M
DS8	R5X4	6	**100**	**100**	0.148	0.026	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.
DS9	R5	6	**98**	*62*	0.481	0.252	.	.	L	.	.	.	.	.	.	.	.	.	N	.	.	.	.
DS12	R5X4	6	*84*	*31*	>1	0.430	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.

* Inhibition >90% is bolded and inhibition <90% is in italics

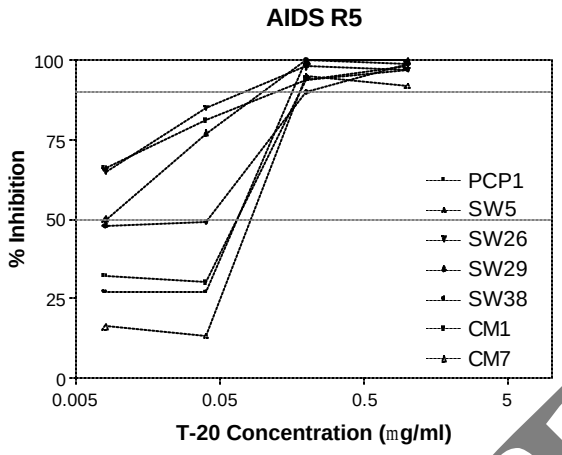
**Isolates have the following resistance mutations in reverse transcriptase: DR10 (M41L, D67N, T69D, M184I, Y188L, G190A, L210W, T215Y); DR19 (D67N, T69_SS, V106M, G190A, L210W, T215Y); DR28 (K103N, M184V, T215Y, M230L); DR62 (D67N, K70R, M184V, K219Q); DR100 (M184V, G333E). Protease mutations found in DR100 (M46I, I54V, V82A)

and pediatric patients collected at different stages of disease progression (Figure 5.3A and B). Inhibition was not affected by the presence of drug resistance mutations in the *pol* gene, as all 5 isolates from patients failing anti-retroviral therapies were fully sensitive to T-20 (Figure 5.3C).

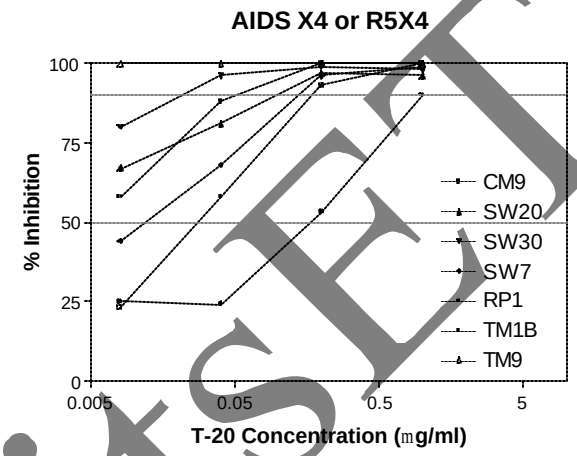
The mean IC_{90} for subtype C was 0.2243 µg/ml compared to 0.7188 µg/ml for subtype B. The IC_{50} for subtype C was 0.0578 µg/ml and 0.1822 µg/ml (Figure 5.4). The differences in IC_{90} and IC_{50} values between subtype C and B isolates were analyzed and found to be not significant according to the Wilcoxon signed rank test (Figure 5.4), although there were fewer subtype B isolates compared to subtype C isolates. The 2 viruses that used alternate coreceptors (PCP1 and CM9) were also effectively inhibited by T-20. The 6 subtype B isolates had the highest IC_{90} values ranging from (0.148 to >1 µg/ml) compared to the subtype C isolates (range 0.008-0.910 µg/ml). Thus despite the 33% difference in the consensus sequences of HR-2 between subtypes B and C, T-20 appears to be highly effective against both subtypes.

Genetic changes spanning amino acids 36-45 of HR-1 have been shown to confer resistance to T-20 (Kilby *et al*., 2002; Poveda *et al*., 2002; Rimsky, Shugars, and Matthews, 1998; Wei *et al*., 2002). The most important mutations are G36D/S and V38M/A (bolded in Table 5.2) but changes at position 37, 39, 40, 42 and 43 have also been found in patients failing T-20 therapy (Kilby *et al*., 2002; Poveda *et al*., 2002). Analysis of this region among the isolates used in this study showed no evidence for

(A)



(B)



(C)

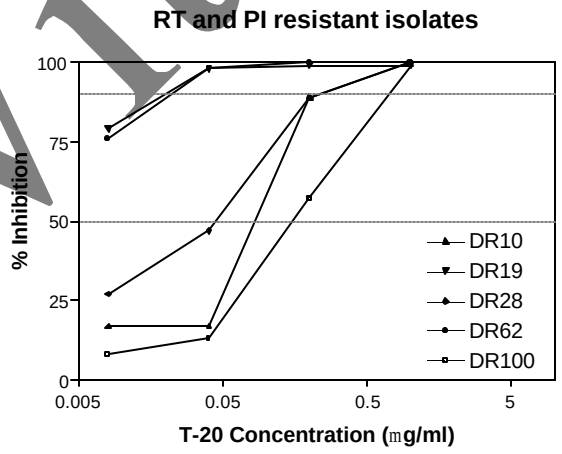


Figure 5.3: Sensitivity of HIV-1 subtype C isolates to T-20. The graphs show percentage inhibition plotted against the concentration of T-20 in µg/ml. The IC_{90} and IC_{50}'s are shown with a dotted line. (A) AIDS R5 viruses are compared to (B) AIDS X4 and R5X4 isolates. Isolates resistant to reverse transcriptase and protease inhibitors are shown to be fully sensitive to T-20 (C).

mutations associated with T-20 resistance except for 1 isolate (SW7) that had the I37V mutation (Table 5.2). This mutation was previously seen among patients failing T-20 (Roman *et al*., 2003; Wei *et al*., 2002) but in the case of SW7 this is likely to be a naturally occurring polymorphism as this isolate was fully sensitive to T-20. Other polymorphisms in this region also had little to no impact.

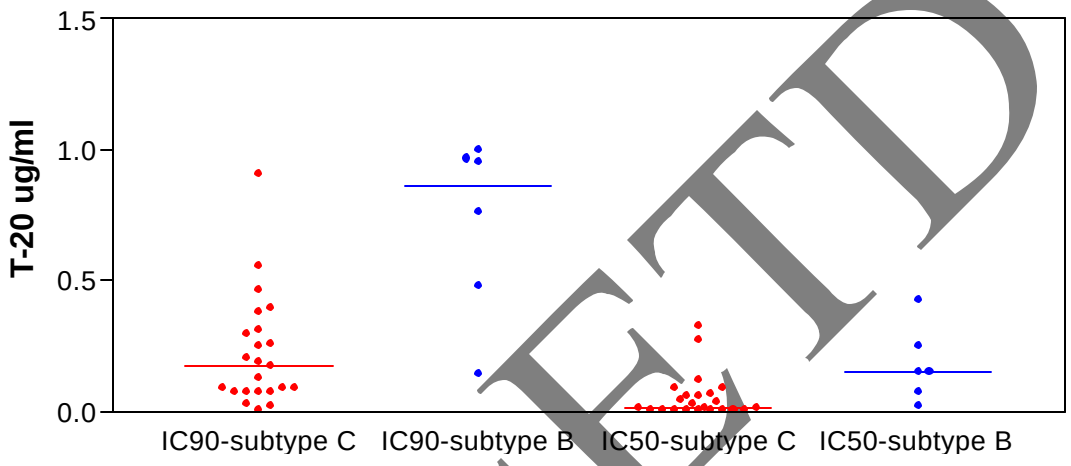


Figure 5.4: Comparison of IC_{90} and IC_{50} values between subtype C and B isolates. Differences between HIV-1 subtypes B and C were found to be not significant

5.4 Discussion

These data show that HIV-1 subtype C isolates are highly sensitive to T-20 *in vitro* and do not harbour naturally occurring polymorphisms that might confer resistance to T-20. This includes isolates from patients failing other anti-retroviral therapies who had resistance mutations in the *pol* gene. All isolates were sensitive at 1 µg/ml, which is the physiological concentration at which T-20 is effective (Kilby *et al*., 1998). This suggests that T-20 would be highly effective in HIV-1 subtype C infected patients including those failing other anti-retroviral regimens.

Sequence analysis revealed that the HR-1 region was considerably more conserved than HR-2, which also showed a high degree of variation between subtypes. While the precise interaction between HR-1 and HR-2 and the mechanism whereby T-20 inhibits the formation of the 6-helical bundle remains unclear, these interactions can nevertheless accommodate significant genetic changes. This may favour the broad applicability of T-20 as an antiviral agent as it is likely to function against very diverse HIV strains. However, there does appear to be a limit to the degree of variation as T-20 is not effective against HIV-2 isolates, which shows 22 amino acid differences in the T-20 region (Wild *et al*., 1994). The large amount of variation may also facilitate multiple pathways to developing T-20 resistance including changes at distal sites, as has been seen among some patients failing therapy. This includes changes in HR-2 as well as other regions of envelope (Derdeyn *et al*., 2000; Derdeyn *et al*., 2001; Reeves *et al*., 2002). The conservation of the GIV motif in the majority of HIV-1 isolates suggests that T-20 binds to the same region in HR-1 of all HIV-1 subtypes.

There is some debate regarding the efficacy of T-20 against isolates using different coreceptors with some studies showing that CCR5-using isolates were more resistant to T-20 (Derdeyn *et al*., 2000; Derdeyn *et al*., 2001; Reeves *et al*., 2002). However other studies have shown no differences in sensitivity based on coreceptor usage (Greenberg *et al*., 2001). While some of the CXCR4-using viruses in this study were more sensitive to T-20 compared to R5 isolates there was no significant differences between these two groups. The IC_{50} values for our subtype C isolates appeared to be lower than published IC_{50} values for subtype B isolates (Derdeyn *et al*., 2000; Derdeyn *et al*., 2001; Reeves *et al*., 2002; Wild *et al*., 1994). However the majority of these studies used cell lines or fusion assays and in some cases cloned envelopes and is therefore not directly comparable to ours, which used primary PBMC and primary viruses. Further comparative studies with larger numbers of subtype B isolates are needed to determine whether subtype C isolates are indeed more sensitive to T-20.

HIV-1 subtype C is the most rapidly expanding viral subtype worldwide and it is therefore important to ensure that new agents function effectively against these viruses. Our data suggest that therapeutic or preventative agents based on T-20 would be effective in countries where subtype C predominates. While T-20 is unlikely to be widely used in developing countries because of the complexities of administration and cost, its development may have particular significance for such countries where the burden of infection resides and where new strategies are needed. Given that entry inhibitors block viral infection at the cell surface, they may serve as potent preventative agents for use as

microbicides or to prevent mother-to-child transmission. Thus further studies on the sensitivities of subtype C isolates to new entry inhibitors, particularly those with different phenotypic and genotypic properties are warranted.

CHAPTER 6

IN VITRO GENERATION OF HIV-1 SUBTYPE C ISOLATES RESISTANT TO T-20

6.1 Introduction

Treatment of HIV-1 infection has focused mostly on antiretroviral therapies targeting the two major viral enzymes, reverse transcriptase and protease. While these therapies have proven highly effective in the treatment of HIV infection they have shown limitations due to intolerability and resistance (Carpenter *et al*., 2000; Bucher, Young, and Battegay, 2004). T-20 represents a new treatment option for patients and is the first entry inhibitor to have received Food and Drug Administration approval (Kilby *et al*., 1998; Lalezari *et al*., 2003b). T-20 has shown efficacy in human trials and proven to be safe and well tolerated in treatment-experienced patients (Lalezari *et al*., 2003a; Kilby *et al*., 2002). .

T-20 is a peptide consisting of 36 amino acids that mimics a corresponding area in the second heptad repeat region (HR-2) in the gp41 envelope glycoprotein of an HIV-1 subtype B isolate (Wild *et al*., 1994). The T-20 peptide binds to the first heptad repeat region (HR-1), thereby preventing the binding of HR-1 to HR-2 and fusion of the host and virus cell membranes (Wild *et al*., 1994; Chan *et al*., 1997). Genetic analyses of published sequences in Genbank have shown very little variation in the HR-1 region. This led to the assumption that T-20 will be effective against most genetic subtypes (Hanna *et al*., 2002; Villahermosa *et al*., 2003; Xu *et al*., 2002), which was confirmed for subtype C isolates (Cilliers *et al*., 2004). The high level of genetic variation seen in HR-2, the region from which T-20 was derived, among different genetic subtypes appears not to compromise the efficacy of T-20, most likely due to the low variation in the HR-1 region to which T-20 binds (Hanna *et al*., 2002).

Both *in vitro* and *in vivo* studies have shown that resistance to T-20 is associated with changes in the GIV motif at positions 36-38 in the HR-1 region (Kilby *et al*., 2002; Wei *et al*., 2002) particularly with changes at G36D/S and V38M/A (Lalezari *et al*., 2003b; Rimsky, Shugars, and Matthews, 1998). Less frequent resistance mutations include G36E, G36V, I37T and to a lesser extent I37V (Rimsky, Shugars, and Matthews, 1998; Roman *et al*., 2003; Lu *et al*., 2004). Resistance-associated amino acid substitutions outside this region have also been reported including Q39H, Q40H, N/S42T and N43D (Roman *et al*., 2003; Sista *et al*., 2004). Changes in the HR-2 region (Q110Q/E, E119E/Q and R122R/K) have been described in a patient participating in a clinical study (Poveda *et al*., 2002). Regions in gp120 have also been implicated in influencing sensitivity to T-20 with changes found in some X4 viruses exposed to T-20 (Reeves *et al*., 2002; Reeves *et al*., 2004).

Although resistance to T-20 among subtype B HIV-1 isolates has been well characterized, there is at present little information about T-20 resistance among non-B viruses. It is known that T-20 is effective against subtype C viruses, and that background genotypic resistance to T-20 is low (Chapter 5). The majority of new infections worldwide are now caused by subtype C viruses, therefore alternative treatment options and characterization of resistance to novel drugs among these viruses is of importance. This study therefore aimed to investigate resistance to T-20 among isolates of HIV-1 subtype C.

6.2 Materials and Methods

6.2.1 Generating T-20 resistant isolates

Eleven HIV-1 subtype C isolates, previously shown to be sensitive to T-20 (Chapter 5), were cultured in the presence of increasing concentrations of T-20 to select for resistant viruses. Peripheral blood mononuclear cells (PBMC) were CD8 depleted using RosetteSep CD8 depletion cocktail (StemCell Technologies, Vancouver, Canada) (as described in Chapter 2) and plated at a concentration of $2x10^6$ cells/ml in 24 well plates. The cultures were maintained in RPMI 1640 containing glutamine, 10% fetal calf serum (FCS) and IL-2 (Chapter 2, Chapter 5). Virus was added at 1000 $TCID_{50}$ and incubated for an hour before the addition of 0.005 µg/ml T-20. Cultures were monitored for viral replication every second day using a p24 antigen assay as previously described (Cilliers *et al*., 2003; Trkola *et al*., 2001). Cultures with low (<1 ng/ml) p24 antigen were re-fed using the same concentration of T-20. Cultures with more than 1 ng/ml p24 antigen were cultured with an increased concentration of T-20. Once cultures reached more than 50 ng/ml p24 antigen they were maintained with between 5 and 10 µg/ml T-20. The cultures were fed weekly with fresh CD8 depleted PHA stimulated donor PBMC. Two sets of resistance experiments were performed, the first (S1) used T-20 obtained from American Peptide Company, Inc, Sunnyvale, California and the second experiment was performed with T-20 obtained from Roche, Palo Alto. The second experiment (S2) was set up using day 29 culture supernatants from S1 starting with 5 µg/ml of T-20, increased later to 10 µg/ml. This was to eliminate any virus that might be sensitive to T-20 and to obtain pure cultures of T-20 resistant viruses. Once isolates grew in a concentration of 10 µg/ml they were considered to be resistant to T-20.

6.2.2 Phenotypic sensitivity assay

The level of phenotypic resistance to T-20 was determined using PBMC with a p24 antigen read-out. Resistant viruses (S2 day 29, 1000 $TCID_{50}$) were incubated with five-fold dilutions of T-20, starting at 25 µg/ml, for 1 hour before the addition of 10^5 cells/well CD8 depleted PBMC in 96 well plates. The levels of p24 antigen were determined on days 4, 6, 8 and 10. IC_{90} and IC_{50} values were determined as previously described (Chapter 2, Chapter 5) (Trkola *et al*., 2001).

6.2.3 Sequencing of gp41

RNA was extracted from 200 µl of culture supernatant using the MagNA Pure LC Isolation station (Roche Applied Science, Penzberg, Germany) and the Total Nucleic Acid isolation kit (Roche Applied Science) as described by the manufacturer. The nucleic acid was resuspended in a final volume of 50 µl. Reverse transcription was performed using Expand High Fidelity Reverse Transcriptase (Roche Applied Science). The reaction was primed using the gp41RO primer (5'-GGCAAGTGCTAAGAATCCGT-3'). Amplification was performed using the Expand High Fidelity PCR system (Roche Applied Science) in a nested PCR. First round amplification was performed using gp41FO (5'-GCCAGTGGTATCAACTCAAC-3') and gp41RO. Amplification was performed as follows: 94°C for 5 min, 25 cycles of 94°C for 30 secs, 55°C for 30 secs and 72°C for 1 min. Primers used for the second round reaction were gp41FI (5'-GAAGGACAACTGGAGAAGTG-3') and gp41RI (5'-GCTCTCCACCTTCTTCTTCG-3'). Cycling conditions were identical to those used in the first round. Amplified samples

were electrophoresed on a 1% agarose gel and yielded a product of approximately 700 bp. PCR product was purified using the High Pure PCR purification system (Roche Applied Science). Sequencing was performed in both directions with the ABI Big Dye terminator system, as described by the manufacturer, using gp41FI and gp41RI. Sequences were joined using the Sequencher 4.1.4 program (GeneCodes, Ann Arbor, MI). Multiple alignments were performed using Clustal X. Phylogenetic analysis of parental, S1 and S2 nucleic acid sequences was performed with Mega version 2.1. A neighbor-joining tree was constructed with distances calculated using Kimura's two-parameter method. Bootstrap values are the result of 1000 resamplings and are given as percentages.

6.2.4 Sequencing of V3

The V3 region was amplified using primers BF (5'-TAACACAAGCCTGTCCAAAGG-3') and BR (5'-AATTCTAGGTCCCCTCCTGA-3'). RNA was reverse transcribed with AMV Reverse Transcriptase (Roche Molecular Biochemicals) and amplified with Super-Therm Polymerase (Southern Cross Biotechnologies); the PCR product was cleaned with a High Pure PCR Product Purification kit (Roche Applied Science). Sequencing was performed in both directions with the BF and BR primers as described in Chapter 3.

6.3 Results

6.3.1 Generation of T-20 resistant isolates

Eleven HIV-1 subtype C isolates, previously shown to be sensitive to T-20 (Cilliers *et al.*, 2004), were included in this study. Eight isolates were from adult patients with advanced HIV disease (Cilliers *et al*., 2003), two were from acutely infected adult patients (Du151a, Du422a) (Williamson *et al*., 2003) and one was from a pediatric patient with rapid disease progression (RP1). Seven isolates were R5, two were R5X4 and two were X4 viruses. Two isolates were able to utilize minor coreceptors: CM9 (CXCR6, CCR3, Bob) and PCP1 (CXCR6) (Chapter 2). One of the X4 isolates from a drug-treated patient (DR28) had existing reverse transcriptase drug resistance mutations K103N, M184V, T215Y and M230L (Chapter 5). All other isolates were from drug naïve patients. The subtype B isolates consisted of three R5 and one R5X4 virus from patients with acute infection (SM1 and SM2) and patients with AIDS (DS3 and DS12).

Isolates were grown in the presence of increasing concentrations of T-20 (from 0.005 µg/ml up to 10 µg/ml) and virus growth was monitored using a p24 antigen assay for 36 days (S1). Most isolates showed only low-level replication during the first 2 weeks of culture when T-20 concentrations ranged from 0.05 to 1 µg/ml, but by day 18 all isolates replicated at varying but detectable levels. Two isolates (Du422a and SW38) showed higher levels of replication producing more than 100 ng/ml p24 antigen within 11-15 days at 1 µg/ml T-20. By day 29 viral replication for all isolates at 10 µg/ml was high (>25 ng/ml p24 antigen), stable and unaffected by the addition of fresh T-20. Virus

populations from day 29 cultures (S1) were sequenced to determine the resistance profiles in gp41.

In order to enrich for T-20 resistant virus the S1 day 29 cultures were used to infect a fresh batch of PBMC at a starting concentration of 5 µg/ml T-20 (S2). These cultures were continuously cultured in the presence of 10 µg/ml T-20 from day 4 onwards until day 29. Three isolates (SW38, SW7 and RP1) were able to grow from outset of culture although most isolates had a lag phase before virus replication was noted. However, by day 29 all isolates replicated to high levels (>25 ng/ml p24 antigen) in 10 µg/ml of T-20. These viruses were sequenced on day 29 (S2) to determine if new resistance mutations had developed and how these compared to the subtype B isolates and previously reported mutations.

6.3.2 Changes in HR-1 associated with T-20 resistance

The gp41 region of virus cultures from T-20 naïve parental viruses, S1 and S2 was sequenced and the HR-1 region analysed to determine if genotypic resistance to T-20 developed. Parental viruses were all genotypically sensitive to T-20 and, with the exception of SW7 (GVV), all had the GIV motif (Chapter 5). The cultures from the first passage (S1) had changes mostly in the GIV motif with more changes at position 36 (7/11) followed by position 38 (3/11) (Table 6.1). Some changes were also noted outside the GIV motif at positions 42, 43, 45 and 50 in the HR-1 region. No resistance mutations were noted for the subtype B isolates in S1 even though they were able to replicate in the

presence of 10 µg/ml T-20. Further analysis on day 36 revealed resistance mutations not evident at day 29 (data not shown).

Cultures in S1 contained mixed populations with both T-20 sensitive and resistant viruses present at day 29 (as observed in sequencing chromatograms and indicated in Table 6.1). To obtain pure resistant cultures a second experiment (S2) was set up using 5 µg/ml T-20 to exclude T-20 sensitive viruses. The cultures were sequenced in the gp41 region on day 29. Sequence data confirmed that viruses genotypically resistant to T-20 were present largely as pure populations in S2 cultures. Phylogenetic analysis of parental, S1 and S2 sequences was performed to eliminate the possibility of contamination during virus culture. All sequences from the same isolate clustered together with high bootstrap support, and consensus sequences supported subtype designations of isolates (Figure 6.1). Mutations were noted in the GIV motif of 9/11 subtype C isolates and 3/4 subtype B isolates (Table 6.2). Some isolates also had mutations outside the GIV motif at positions S42D (SW29), N43K/S (Du422a, RP1, DS12), L45R (SW5), R46M (DS3) and A50T/V (Du151a, CM7 and RP1). For three viruses (Du422a, RP1 and DS12) high–level resistance was conferred by mutations in these regions as all had wild-type GIV sequences at S2. In some cases, resistant virus in S2 contained the major resistance mutations observed for that virus in S1 eg. SW29. However, interestingly, in some cases the predominant resistant population observed in S1 was not the same population present in S2, with new mutations emerging (eg. CM9). This observation reflects the dynamic nature of T-20 resistance mutations reported *in vivo* (Baldwin *et al*., 2004) and may be due to differences in fitness (presumably determined by virus entry) in a system where T-

20 concentrations are increased gradually as in S1, as opposed to S2 where virus was exposed to immediate and high (5 μg/ml) levels of T-20. Most of the resistance mutations observed occurred at the same positions as previously reported for subtype B isolates, further demonstrating the importance of the GIV motif in resistance to T-20.

Table 6.1: Changes in the HR-1 region of subtype B and C isolates exposed to increasing concentrations of T-20 for 29 days (S1).

	Biotype	HR-1 amino acid sequences															
SubtypeC																	
Consensus		G	I	V	Q	Q	Q	S	N	L	L	R	A	I	E	A	Q	Q
Du151a	R5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**A/T**	.	.
Du422a	R5	**G/D**	.	**M/V**	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SW5	R5	.	.	.	.	.	.	.	.	.	**L/R**	.	.	.	.	.	.	.
CM7	R5	.	.	.	.	.	.	N	.	.	**L/M**	.	.	.	.	**A/T**	.	.
SW29	R5	**G/S**	.	.	.	.	.	**N/D**	.	.	.	.	.	.	.	.	.	.
SW38	R5	**G/S/D**	.	.	.	.	.	.	.	.	.	K	.	.	.	T	.	H
PCP1	R5/X6	**G/E**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RP1	R5/X4	**G/D**	.	.	.	.	.	.	**N/K**	.	.	.	.	.	.	**A/V**	.	.
CM9	R5/X4/X6/R3/Bob	**G/D**	.	**V/M**	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SW7	X4	.	V	**V/A**	.	.	.	.	.	.	**L/M**	.	.	.	.	.	.	.
DR28	X4	**G/S/D**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Subtype B																	
Consensus		G	I	V	Q	Q	Q	N	N	L	L	R	A	I	E	A	Q	Q
SM1	R5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SM2	R5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DS3	R5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DS12	R5/X4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

6.3.3 Changes in the HR-2 region

The HR-2 region of resistant subtype C and B isolates was sequenced and analyzed to investigate compensatory changes, which may arise as a result of T-20 exposure. These changes, compared with parental and S1 viruses, were noted only for the second experiment (S2). Four of the 11 subtype C viruses had changes in the HR-2 region with one isolate containing three changes in the region corresponding to the T-20 peptide: SW38 had D129N, D148E and K152S mutations, Du123 a D137E mutation, CM9 an E151K mutation and RP1 a K147Q mutation (Table 6.3). Changes in the HR-2 were also noted for three of the four subtype B isolates. SM1 had a G129N mutation, SM2 a V130F mutation and DS12 a G129D mutation.

6.3.4 Analysis of V3 amino acid sequences

The V3 region of X4 viruses has previously been noted to undergo changes when T-20 interacts with the gp120 region (Yuan *et al*., 2004). V3 sequencing was therefore performed on all resistant isolates to determine if changes in the V3 region might arise from exposure to T-20. Two of the 11 subtype C isolates had changes in the V3 region: Du151a had a Threonine (T) to Isoleucine (I) change at position 2 and SW38 had a Lysine (K) to Glutamine (Q) at position 32 (Table 6.3). These changes were close to the stem of the V3 region, which has been implicated in T-20-related changes within gp120 (Yuan *et al*., 2004). For SW38 this change resulted in a decreased charge in the V3 loop from +4 to +3.

6.3.5 Determination of T-20 IC_{90} and IC_{50} for resistant isolates.

The IC_{90} and IC_{50} values were determined for the T-20 resistant isolates (S2 day 29) to establish if there was any correlation between the degree of phenotypic resistance and the type or number of resistance mutations. The IC_{50} values had a wide range from 9.52 µg/ml to more than 25 µg/ml, equivalent to a 7 to 234-fold increase in resistance to T-20 compared with T-20-naïve parental viruses (data not shown). The IC_{90} values ranged from 14.1 µg/ml to more than 25 µg/ml. Three isolates had IC_{50} values higher than 25 µg/ml T-20: SW7 (V38A), PCP1 (G36E and V38A) and RP1 (N43K and A50V). The subtype B T-20 resistant isolates had an IC_{50} between 17.12 µg/ml and 22.31 µg/ml, equivalent to an 8 to 30-fold increase in resistance to T-20 compared with parental viruses (data not shown). One subtype B isolate (DS3) did not replicate sufficiently to obtain an IC_{50} value. No clear association was noted between the resistance profile and the inhibitory concentration of T-20.

Table 6.2: The IC_{50} and IC_{90} values with corresponding resistance sequences for subtype C and B isolates from S2 day 29.

	Biotype	Inhibitory concentration (ug/ml)		HR-1 amino acid sequence															
		IC_{50}	IC_{90}																
SubtypeC Consensus				G	I	V	Q	Q	Q	S	N	L	L	R	A	I	E	A	Q	Q
Du151a	R5	9.52	14.08	**S/G**	.	.	.	.	.	.	.	.	.	.	.	.	.	**T**	.	.
Du422a	R5	15.24	23.55	.	.	.	.	.	.	.	**K**	.	.	.	.	.	.	.	.	.
SW5	R5	24.99	>25	.	.	L	.	.	.	.	.	.	**R**	.	.	.	.	.	.	.
CM7	R5	15.75	23.87	**S**	.	.	.	.	.	N	.	.	.	.	.	.	.	**T**	.	.
SW29	R5	19.79	>25	**S**	.	.	.	.	.	**D**	.	.	.	.	.	.	.	.	.	.
SW38	R5	19.89	>25	.	.	**A**	.	.	.	.	.	.	.	K	.	.	.	T	.	H
PCP1	R5/X6	>25	>25	**G/E**	.	**A/V**	.	.	.	.	.	.	.	.	.	.	.	.	.	.
RP1	R5/X4	>25	>25	.	.	.	.	.	.	.	**K**	.	.	.	.	.	.	**V**	.	.
CM9	R5/X4/X6/R3/Bob	22.62	>25	.	.	**E**	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SW7	X4	>25	>25	.	V	**A**	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DR28	X4	16.69	23.7	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Subtype B Consensus				G	I	V	Q	Q	Q	N	N	L	L	R	A	I	E	A	Q	Q
SM1	R5	17.21	24.91	.	.	**M/E/K**	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SM2	R5	22.31	>25	**D**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DS3	R5	NR	NR	**S**	.	.	.	.	.	.	.	.	.	**M**	.	.	.	.	.	.
DS12	R5/X4	17.12	>25	.	.	.	.	.	.	.	**N/K/S**	.	.	.	.	.	.	.	.	.

NR = no replication

Table 6.3: Summary of T-20-associated changes in the HR-2 and V3 regions S2 viruses.

	HR-2	V3
Subtype C		
SW38	D129N	K32Q*
	D148E	
	K152S	
Du123	D137E	-
CM9	E151K	-
RP1	K147Q	-
Du151	-	T2I*
Subtype B		
SM1	G129N	-
SM2	V130F	-
DS12	G129D	-

* V3 numbering: position 1 corresponds to residue 294 in NL4.3 gp120
- indicates no change compared with parental virus

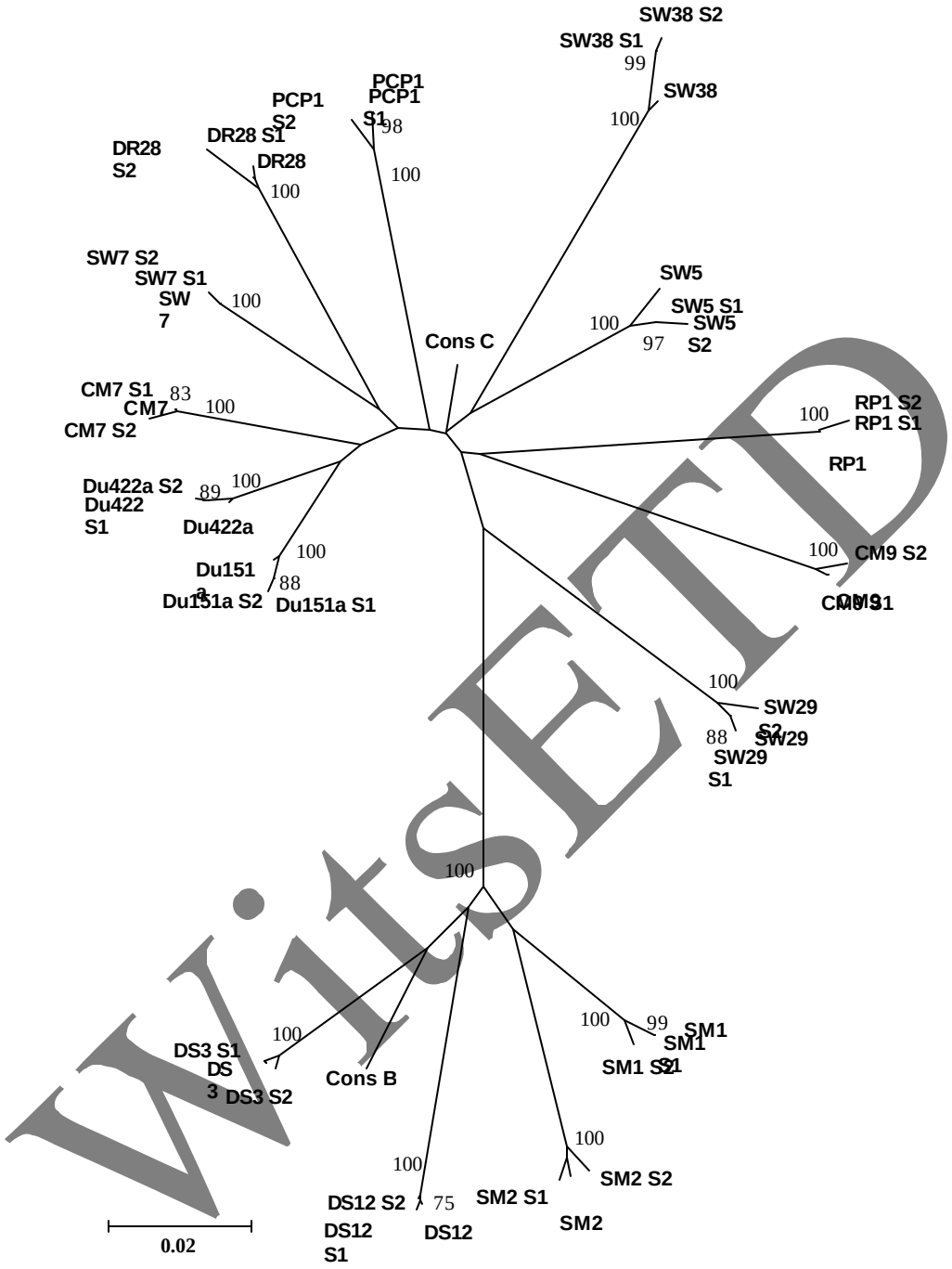


Figure 6.1: Phylogenetic analysis of nucleic acid sequences from parental, S1 (day 29) and S2 (day 29) sequences (Mega version 2.1). Consensus sequences for subtype B and subtype C are included. Bootstrap values exceeding 75% are shown. This data indicates that no contamination by another virus or different strains were responsible for the appropriate T-20 resistance mutations (Courtesy of Dr Penny Moore).

6.4 Discussion

The development of resistance appears to be an inevitable result of exposure to T-20, regardless of the subtype of HIV-1. Here we describe the development of *in vitro* resistance to T-20 among subtype C isolates from patients at different stages of disease including one with pre-existing reverse transcriptase mutations. Mutations occurred predominantly in the HR-1 region, mostly at the G36 and V38 positions, which have previously been shown to be associated with resistance to T-20 in subtype B viruses (Rimsky, Shugars, and Matthews, 1998). Occurrence of T-20 associated resistance mutations at the same positions as subtype B emphasizes the likelihood that the mechanism of action for T-20 remains the same for HIV subtype C isolates.

Analysis of amino acid changes in the HR-1 region of isolates selected to replicate in 10 µg/ml T-20 (S2) revealed mutations associated with T-20 resistance in all subtype C and B isolates. Most of these changes occurred in the GIV motif with some isolates having additional changes outside this region. However, in many cases these mutations were not those observed in the earlier selection (S1). The reasons for this are not clear but indicate a dynamic interplay between populations of mutant viruses under T-20 selection *in vitro*. A number of isolates that had changes at the G36 motif at S1 reverted to the wild-type glycine in S2. The relative reduction in the number of G36 mutants in S2 may simply be a result of exposure of a mixed population of viruses (selected gradually in S1) to immediate high levels of T-20. This inability of G36 mutants to persist at S2 suggests that for some viruses, mutations at this residue may not confer the highest level of T-20 resistance. Alternatively, perhaps the fitness of these variants was sufficiently

compromised to restrict their infection of fresh PBMC in the presence of high concentrations of T-20, resulting in the infection of PBMC in S2 with sub-populations of T-20-resistant viruses with increased fitness compared with G36 variants. The three isolates (Du422a, RP1 and DS12) that showed high level resistance in the absence of mutations in the GIV motif all had the N42K/S mutation suggesting that this mutation may cause high level resistance on its own. Further support for this comes from the observation that for 2 of these isolates a mutation at G36D was lost between S1 and S2 suggesting that N42K has increased fitness compared to G36D. Thus, mutations in GIV as well as those outside GIV can confer high-level resistance to T-20 for HIV-1 subtype C and B isolates.

T-20-associated mutations in HR-1 have been reported to result in reduced affinity for HR-2 (and concomitant reduction in fusion activity). Mutations within HR-2 have been shown to result in a hyperfusinogenic Env which compensated for the initial reduction in fusogenicity associated with T-20 resistance (Baldwin *et al*., 2004). In this study, compensatory changes in the HR-2 region were noted in four HIV-1 subtype C isolates. These changes occurred in the region corresponding to that from which T-20 was derived and were spread evenly across this region. Interestingly, three of the four HIV subtype B isolates had changes in the HR-2 and these were mostly in the beginning of the T-20 region, although not at the SNY motif implicated as a compensatory mutation in a T-20-resistant and -dependent virus (Baldwin *et al*., 2004). Whether the changes observed in HR-2 among T-20 resistant subtype C isolates have any significance for development of T-20 resistance among subtype C isolates still needs to be determined.

Changes outside the gp41 have previously been shown to influence the efficiency with which T-20 binds to the HR-1 region (Reeves *et al*., 2002; Reeves *et al*., 2004). The position next to the ß21-bridging sheet of gp120 has been shown to influence coreceptor binding and thus impact on how well T-20 interacts with the gp41 region (Reeves *et al*., 2002; Reeves *et al*., 2004). Coreceptor usage has also been implicated in sensitivity to T-20, with X4 variants shown to be more susceptible than R5 isolates (Derdeyn *et al*., 2000; Derdeyn *et al*., 2001). In this study no difference was noted in T-20 related mutations between R5 and X4 isolates suggesting that the mechanism of resistance is independent of coreceptor usage. When T-20 resistant viruses were retested in U87.CD4.CCR5 and CXCR4 cells the cell tropism remained unaffected compared with parental viruses (data not shown). T-20 has been shown to interact with gp120 of subtype B X4 viruses and the V3 loop has been reported to undergo changes close to the cysteine bonds at the stem of the loop in the presence of T-20 (Yuan *et al*., 2004). In this study we noted changes within the V3 region of two subtype C isolates. The significance of these changes still needs to be determined, particularly as both were R5 isolates. We cannot exclude the possibility that the changes were simply culture artifacts or selection of minor populations either in culture or during sequencing.

Selection of resistance was most pronounced for one isolate in both S1 and S2. This isolate, SW38, had one change in the HR-1, three changes in the HR-2 as well as a change in the V3 region. SW38 has three polymorphisms in the HR-1 region to which T-20 binds, which may contribute to the rapid development of resistance to T-20 in S1 and

S2. This may indicate the possible role of compensatory changes at different positions in the envelope region to accommodate primary drug resistance mutations that allow the virus to replicate in the presence of T-20, despite possible reduced fitness compared with parental viruses (Lu *et al*., 2004). To fully understand the mechanism of T-20 resistance future work will need to examine the role of regions other than gp41 to investigate what influence these regions have on T-20 resistance and how the gp160 compensates for potentially deleterious T-20-associated mutations.

In a previous study we showed that T-20 is effective against HIV-1 subtype C isolates *in vitro* suggesting that it would prove useful in the treatment of individuals infected with these viruses (Chapter 5). Here we demonstrate *in vitro* development of resistance to T-20 among subtype C isolates. Virus resistance developed rapidly with continued exposure to T-20, and was genotypically comparable to resistance among subtype B HIV-1 isolates, suggesting a common mechanism of action of the drug in both subtypes. These data may prove useful in the management of resistance in T-20 treated patients infected with subtype C viruses.

CHAPTER 7

CONCLUSION

While considerable progress has been made in the last eight years in understanding how HIV interacts with human cells, one of the major advances has been the discovery of HIV-1 coreceptors and how they function. The major coreceptors are CCR5 and CXCR4 of which CCR5 is used more frequently. CXCR4 is used less frequently and at later stages. The significance of coreceptor switching and expanded coreceptor use and how this impacts on HIV pathogenesis remains unclear. The rarity of coreceptor switching among HIV-1 subtype C infected persons indicates that it is not a pre-requisite to developing AIDS. In the few reported cases the use of CXCR4 in HIV-1 subtype C was accompanied by changes mostly in the V3 region with increased positive charges as well as insertions or deletions similar to subtype B. Since HIV-1 subtype C accounts for most infections worldwide it is important to understand what allows this subtype to undergo a coreceptor switch albeit at a very low frequency.

The development of a new class of compounds that can block HIV-1 infection has opened up numerous possibilities for viral control, including their use as microbicides to prevent sexual transmission and in infants to prevent transmission via breast-milk. In general these inhibitors have been well tolerated and effective in limited clinical testing, and their ability to block multiple steps in the entry process makes this an attractive approach. There is limited data available on the effectiveness of these compounds on HIV-1 subtype C. Due to genotypic and phenotypic differences between HIV-1 subtype

B and C studies need to be performed to assess the effectiveness of new and existing inhibitors on HIV-1 subtype C.

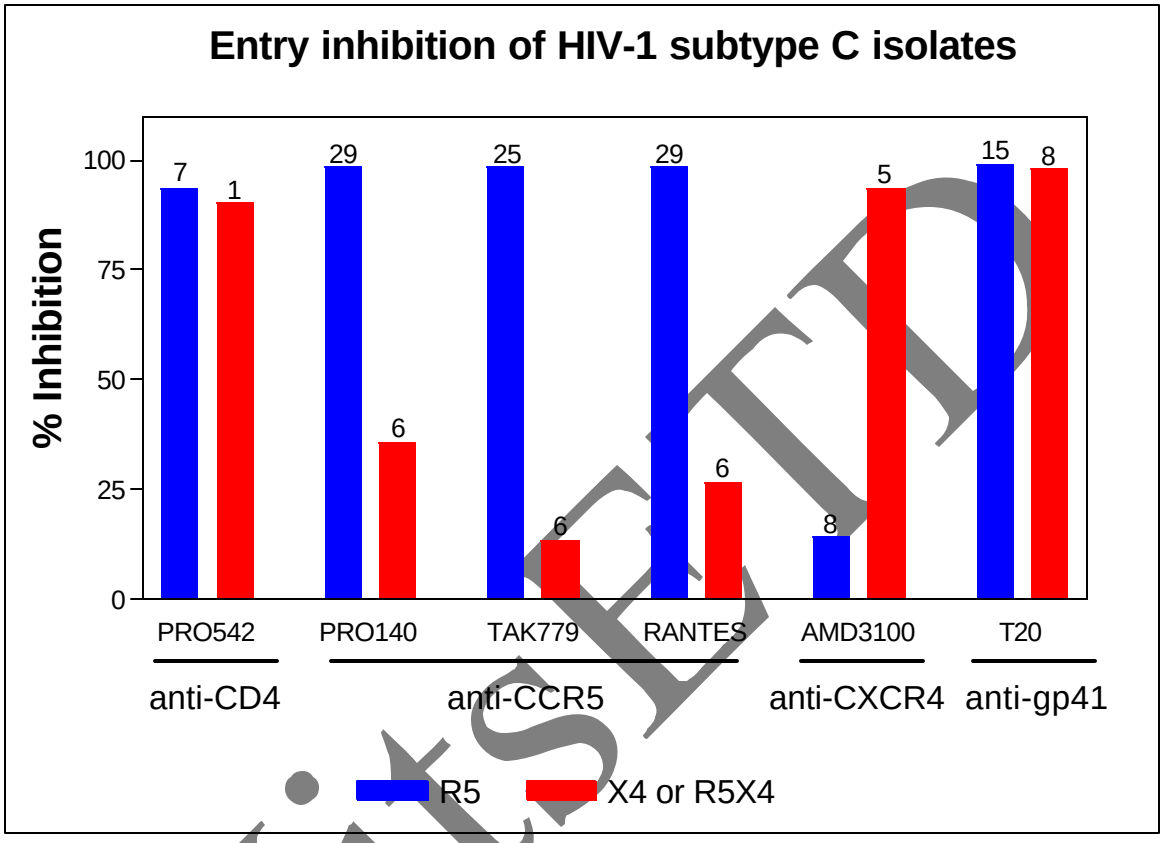


Figure 7.1: A summary of the mean percentage inhibition for the three classes of entry inhibitors for HIV-1 subtype C. The number of isolates tested is shown on top of each bar. The results for the anti-CD4 inhibitor are not discussed in this thesis. The R5 isolates are shown in blue. The X4 or R5X4 isolates are shown in red.

Figure 7.1 represents a summary of data presented in this thesis and additional data (courtesy of Elin Gray). PRO542 is a recombinant tetravalent antibody coupled to the CD4 molecule that binds gp120 (Allaway *et al.*, 1995), inhibited subtype C viruses in a coreceptor independent manner (E. Gray unpublished data). R5 viruses were inhibited effectively by the CCR5 coreceptor inhibitors and the X4 and R5X4 isolates were

inhibited by the CXCR4 inhibitors. The fusion inhibitor was highly effective against all isolates tested irrespective of coreceptor use. All three stages of entry for subtype C are therefore susceptible to entry inhibitors.

A potential obstacle to using coreceptor inhibitors is the emergence of isolates able to use coreceptors other than CCR5 or CXCR4. Although the use of alternate coreceptors is a rare event and whether these receptors are used *in vivo* is unknown. Nevertheless such isolates may pose a threat to the successful use of coreceptor inhibitors as clinical tools (Zhang and Moore, 1999). Two HIV-1 isolates in this study were shown to use alternate coreceptors on primary cells. However, they may be blocked by other agents that can bind to these coreceptors. In one case this was CCR8 for which an inhibitor is available although in the second case the receptor used was unknown. Few HIV-1 isolates are tested for alternate coreceptor use and their occurrence may be more common than current data suggests. Future studies may need to consider blocking alternate coreceptors should their use be more prevalent than currently described.

Collectively these data suggest that the mechanism used by HIV to enter cells will be the same for subtype C as for subtype B and that the same prevention strategies should be successful for HIV-1 subtype C infected populations. However, HIV due to its genetic variability has already shown evidence that it can circumvent some of these new inhibitors irrespective of clade either by using another coreceptor or by having genetic differences in regions that entry inhibitors target (Moore *et al*., 2004). Thus it cannot be assumed that all entry inhibitors will be effective on HIV-1 subtype C and *in vitro* testing

is required to verify this for new agents. The synergistic use of entry inhibitors will be advisable to circumvent coreceptor switching either to CXCR4 or an alternate coreceptor. Combinations of entry inhibitors will also minimize drug resistance to a specific entry inhibitor targeting different phases of the entry process. The development of this new class of compounds and their ability to be used in combination with highly active anti-retroviral therapy expands the opportunities to target multiple points in the viral replication cycle and increases the possibilities for controlling HIV.

7.1 USEFUL OUTCOMES

These data has provided useful information on the susceptibility of HIV-1 subtype C isolates to entry inhibitors as well as resistance profiles for T-20, which is more or less similar to subtype B. Such data are needed prior to the clinical use of these drugs in South Africa. The information obtained may be more broadly applicable to other regions of the world where subtype C circulates. Furthermore the pattern of genetic mutations in the subtype C envelope gene associated with resistance to entry inhibitors will be useful for future application of entry inhibitors. This is important, given that the envelope gene of HIV shows considerable genetic variation and that resistance patterns may differ between subtypes for some inhibitors. Collectively the data obtained here will give an indication as to the potential efficacy of these novel drugs in HIV-1 subtype C infected patients and to their potential use as preventative agents, which are desperately needed in countries where HIV-1 subtype C circulates.

CHAPTER 8

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APPENDIX A

Patient information from whom viral isolates were derived

Sample ID	Ethnicity	Age	Sex	CD4	VL	SI/NSI	Clinical Status
Acutely infected patients							
Du123a	N/A	N/A	F	841	19,331	NSI	acute
Du151a	N/A	N/A	F	367	>500,000	NSI	acute
Du174	N/A	N/A	F	N/A	11,628	NSI	acute
Du422a	N/A	N/A	F	397	67,982	NSI	acute
Pediatric AIDS							
RP1	Black	N/A	N/A	7	178,830	SI	N/A
TM1b	Black	8.4	M	N/A	190,990	SI	severe (C)
TM9	Black	8	M	11	296,865	SI	severe (C)
Drug resistant							
DR10	Black	40	M	43	14,194	NSI	Candida Oesophagus
DR19	Black	6	M	439	15,200	NSI	N/A
DR28	Black	9	M	173	269,000	SI	N/A

Sample ID	Ethnicity	Age	Sex	CD4	VL	SI/NSI	Clinical Status
DR62	Black	6	F	1231	57,100	NSI	N/A
DR100	Black	2	M	716	489,000	NSI	N/A
				Subtype B			
SM1	Caucasian	N/A	M	N/A	N/A	NSI	Acute
SM2	Caucasian	N/A	M	N/A	N/A	NSI	Acute
DS3	Caucasian	39	M	59	N/A	NSI	AIDS
DS8	Caucasian	34	M	99	N/A	SI	AIDS
DS9	Caucasian	39	M	4	N/A	NSI	AIDS
DS12	Caucasian	26	M	51	N/A	SI	AIDS

N/A= not available

APPENDIX B

Amino acid abbreviations

Amino acid	Abbreviation
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

APPENDIX C

Buffers and solutions for p24 assay

10x NaHCO3:

Weigh 42g NaHCO3 and add 500ml double distilled water (ddH_20) and set pH at 8.5.

10x TBS to make up 5 litres:

Weigh 421g NaCl and 51g Tris-Trizma base. Add 5 liters of double distilled water (ddH_2O). Set pH at 7.5 using HCl to adjust.

1x TBS for 20 liters:

Use 2 liters 10x TBS and add 18 liters of distilled water.

10x PBS for 10 liters:

Weigh 800g of NaCl, 20g KCl, 144g NA_2HPO_4, 24g KH_2PO_4

Add 8 liters of distilled water and adjust pH to 7.4 with HCl and adjust to 10 liters

10x TROPIX Wash buffer:

ELISA-Light Kit. For 1-liter use 100ml of 10x concentrate and add 900ml double distilled water.

10x TROPIX Wash buffer

To make up 1 liter weigh out 24.3g of TRIS base add 10ml of $MgCl_2$ (1M). Adjust to 1 liter using distilled water and set pH at 9.8.

APPENDIX D

GenBank accession numbers for the V3 sequences are

AY170657 (SW4), AY170658 (SW5), AY170659 (SW14), AY170660 (SW16), AY170661 (SW22), AY170662 (SW23), AY170663 (SW26), AY170664 (SW29), AY170665 (SW34), AY170666 (SW35), AY170667 (SW38), AF411967 (CM9), AF411966 (SW7), AY230878 (SW12), AY230879 (SW20), AY230880 (SW30).

GenBank accession numbers for gp41 sequences

AY504998 (99ZADu174)
AY504999 (99ZASW5)
AY505000 (99ZASW26)
AY505001 (99ZASW29)
AY505002 (99ZASW38)
AY505003 (98ZACM1)
AY505004 (99ZACM7)
AY505005 (02ZADR10)
AY505006 (02ZADR19)
AY505007 (02ZADR28)
AY505008 (02ZADR62)
AY505011 (99ZASM2)
AF411967 (99ZACM9)
AF411966 (99ZASW7)
AF544007 (ZADu123)
AY043173 (ZADu151)
AY043175 (ZADu422)
AY529667 (00ZAPCP1)
AY529669 (99ZASW20)
AY529673 (99ZASW30)
AY529673 (01ZARP1)
AY529677 (99ZATM1b)

AY505009 (03ZADR100) AY529679 (99ZATM9)

AY505010 (99ZASM1)

Full genome sequences

AF411964 (99ZACM4)

AF411966 (99ZASW7)

AF411967 (99ZACM9)

APPENDIX E

Ethical clearance

Ethical clearance was obtained from the University of Witwatersrand Committee for Research on Human Subjects (Medical) protocol number M040911