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PROBING ENTRY INHIBITORS' ACTIVITY ON HIV AND DEVELOPMENT OF NEW FUSION INHIBITORS:

INTEGRATING EVOLUTIONARY BIOLOGY WITH VIROLOGY

Pedro José Vieira Borga Martins Borrego

DOUTORAMENTO EM FARMÁCIA

(MICROBIOLOGIA)

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Tese orientada pelo Professor Doutor Nuno Eduardo Moura dos Santos da Costa Taveira e co-orientada pela Professora Doutora Maria Helena de Sousa Barroso e pelo Professor Doutor José Moniz Pereira

A presente dissertação foi realizada na Unidade de Retrovírus e Infecções Associadas da Faculdade de Farmácia, Universidade de Lisboa, sob a orientação do Professor Doutor Nuno Eduardo Moura dos Santos da Costa Taveira e co-orientação da Professora Doutora Maria Helena de Sousa Barroso e do Professor Doutor José Moniz Pereira.

Este trabalho foi desenvolvido no âmbito de uma Bolsa de Doutoramento atribuída ao autor pela Fundação para a Ciência e Tecnologia.

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ACKNOWLEDGEMENTS

Many people have contributed to this work and therefore I would like to acknowledge them for making this project possible.

Thank you Professor Nuno Taveira, for all your guidance through this journey and for all the knowledge and expertise you shared with me. Thank you for showing me the passion for research and for all the straightforward motivation, reminding me that there are no unreachable boundaries. You will always be a reference to me. Thank you for your friendship.

Thank you Professor Helena Barroso, for all the support given during my years in the lab. You have been amazingly available for questions and thoughts about my work and influenced my learning in a most incredible positive way. Thank you for your friendship.

Professors Nuno and Helena, I am very grateful for the opportunities you have given me to work in several projects in the lab, which have been so important in my scientific learning process and helped me find new research interests.

I would like to thank Professor José Moniz Pereira, director of the *Unidade de Retrovírus e Infecções Associadas – Centro de Patogénese Molecular*, for allowing me to developed my work at this unit. In addition, I would like to thank all the teachers that work in this unit, particularly: Prof. José Azevedo Pereira, Prof. João Gonçalves, Prof. Isabel Portugal, Prof. Madalena Pimentel, Prof. Elsa Anes and Prof. Aida Duarte.

To my laboratory colleagues Rita, Cheila, Marcelino, Inês, Andreia and Claudia Palladino, thank you for making it such a great working environment, for always being there and helping me whenever I needed. Importantly, thank you for standing all my crazy talk, music taste and singing! Thank you for all the lab meetings that were not only an incentive to conviviality but also a stimulation to my belly! I will always be available for as many Italian lunches you want me to join! Thank you for your friendship.

Especially to Rita, thank you for your collaboration and endless devotion that has contributed to my work in a most precious way.

I would also like to thank the good spirit of other colleagues with whom I've shared the working bench in the past: Vânia, Filipa, Sofia, Daniela Moutinho and Hugo.

This work wouldn't be possible without the great collaboration provided by Prof. Alexandre Quintas. Thank you for your expertise and for keeping the doors of your lab always open

for me. Thank you Carlos, Luís, Patrícia, Ana, Lena and Sara, for always being so welcome and available.

Thank you to all the patients and doctors that provided the samples needed for analysis, without your participation none of this work could have been done.

In the backstage of science there are always indispensable people that work hard every day so that students and teachers are provided with all they need. Thank you Lena Brás, Teresa and Lídia for being there whenever we need.

My daily work in the laboratory would not be possible without the help of D. Fátima, Sr. Augusto, Kleida and D. Noémia, providing us proper working conditions.

To Ofélia and Vera thank you for taking care of all the paperwork, for your availability and contagious good mood.

To all the other lab colleagues at URIA-CPM (Quirina, João, Cláudia, Carla, Marta Calado, Maria, Paula, Daniela, Marta, Camila, Emilie, Sylvie, Paula Brito, Andreia, Ana Catarina, Catarina, Mariana, Acilino, Inês, Sara, Luís, André, Patrícia, Leonor, Diana, Joana, David, Paulo, Nuno), with whom I've shared not only the P3 but also good companionship and positive vibes, many thanks!!

Thank you Prof. Jorge Vítor and Joana Vital for your availability and good will.

I'm also very fortunate to have contacted with several people along the past few years who have so kindly given me the opportunity to collaborate in other activities outside the lab. For different reasons, thank you Luís Proença, Jorge Félix and Filipa Duarte Ramos.

Finally, and most importantly, I would like to thank all my family, godparents and close friends for believing in me, for always supporting my decisions and for all the effort in fulfilling my dreams.

Obrigado mãe, pai, mano e avós, por serem uma fonte de inspiração e orientação inesgotáveis.

Raquel, obrigado pela ajuda, pelo carinho e compreensão, sem os quais teria sido impossível realizar este trabalho. A vida é melhor contigo ao meu lado.

... to my family

LIST OF PUBLICATIONS

This thesis is based on the following publications:

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2011 – Borrego P, Calado R, Marcelino J, Oliveira L, Pereira P, Quinta A, Barroso H, Taveira N. Design and evaluation of an ancestral peptide with potent and broad HIV fusion inhibitor activity. Manuscript submitted, July 2011.

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2011 – Borrego P, Barroso H, Bártolo I, Marcelino JM, Família C, Quintas A, Taveira N. Evolutionary and structural features of the C2, V3 and C3 envelope regions underlying the differences in HIV-1 and HIV-2 biology and infection. In: Keystone Symposia - HIV Evolution, Genomics and Pathogenesis, March 20 – 25, 2010, Whistler Conference Centre, Whistler, British Colombia, Canada (Abstract no. 115).

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2011 – Bártolo I, Abecasis AB, Borrego P, Barroso H, McCutchan F, Camacho F, Taveira N. Origin and epidemiologic history of HIV-1 CRF14_BG. Submitted, PLoS One, March 2011.

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RESUMO

O Vírus da Imunodeficiência Humana do tipo 1 e do tipo 2 (VIH-1 e VIH-2) são os agentes etiológicos do Síndrome de Imunodeficiência Adquirida (SIDA). Embora sejam semelhantes na sua organização estrutural e genómica, estes lentivírus humanos apresentam características antigénicas distintas e partilham uma semelhança genética de apenas 50%. Enquanto o VIH-1 é responsável pela pandemia mundial, a infecção pelo VIH-2 localiza-se sobretudo na África Ocidental, em alguns países europeus como Portugal e França, e na Índia. A infecção pelo VIH-2 tem melhor prognóstico, a progressão para a doença é mais lenta e há melhor controlo imunológico do que na infecção pelo VIH-1.

Ao contrário do VIH-1, o arsenal terapêutico actualmente disponível para tratar a infecção por VIH-2 é reduzido. Os fármacos antiretrovirais em uso foram especificamente desenvolvidos para o VIH-1 e, consequentemente, a sua actividade pode ser reduzida ou nula no VIH-2. Este é o caso concreto dos inibidores não nucleosídicos da transcriptase reversa e de alguns inibidores da protease. Neste contexto, os inibidores de entrada poderão ser úteis para tratar a infecção por VIH-2. Contudo, a susceptibilidade dos isolados primários de VIH-2 aos inibidores de entrada é actualmente desconhecida.

A susceptibilidade do VIH aos inibidores de entrada é determinada pela qualidade da interacção do vírus com os receptores celulares. O VIH-1 e VIH-2 são substancialmente diferentes a este nível. Por exemplo, o VIH-2 pode ligar-se ao co-receptor CCR5 independentemente do receptor CD4 e da região V3 do invólucro. Por outro lado, as regiões C2, V3 e C3 do VIH-2 são substancialmente diferentes do VIH-1 a nível antigénico. Colectivamente, estes dados indicam que a estrutura e conformação das glicoproteínas de superfície do VIH-1 e VIH-2 são substancialmente diferentes e sugerem que a susceptibilidade e resistência dos dois tipos de vírus aos inibidores de entrada podem também ser diferentes.

Os principais objectivos desta tese foram: 1) analisar as características moleculares, estruturais e evolutivas das regiões C2, V3 e C3 no VIH-1 e VIH-2; 2) comparar a susceptibilidade do VIH-1 e VIH-2 aos inibidores de entrada e avaliar o seu potencial terapêutico na infecção por VIH-2; 3) produzir um novo inibidor de fusão para o VIH-2.

Para melhor compreender as potenciais diferenças destes dois vírus na resposta aos inibidores de entrada começámos por analisar as características moleculares, estruturais e evolutivas da região V3 e as regiões circundantes C2 e C3, num número significativo de vírus VIH-1 e VIH-2 isolados em Portugal e noutras regiões do globo, com recurso a diferentes metodologias de biologia evolutiva e computacional (Capitulo 2). Apesar da

menor variabilidade das 3 regiões no VIH-2, verificámos que a região C3 está sob forte selecção positiva e encontra-se exposta à superfície sugerindo que, tal como no VIH-1, esta região poderá constituir um domínio neutralizante. No entanto, ao contrário do VIH-1, a maioria das mutações adaptativas no VIH-2 são prejudiciais e levam à extinção das linhagens virais pelo que o efeito final é um forte constrangimento à variabilidade das regiões analisadas. Ao contrário do VIH-1, verificámos que a ansa V3 do VIH-2 se encontra oclusa no complexo glicoproteico do invólucro, numa conformação que parece ser estabilizada por interacções que mantém com alguns resíduos da regiões C2 e C3. Estes resultados são consistentes com o facto de a V3 não ser imunodominante no VIH-2, ficando assim mais protegida da resposta imunitária e das eventuais mutações que dela resultam. A forte conservação da V3, da C2 e da C3 também é consistente com a sua potencialmente importante actividade imunosupressora. Em conclusão, este primeiro estudo permitiu caracterizar algumas das características estruturais e funcionais que distinguem as glicoproteínas do invólucro do VIH-1 e do VIH-2 e que estão associadas às diferentes características biológicas e fenotípicas destes dois vírus. Estes dados podem ter impacto na resposta dos dois vírus aos inibidores de entrada (analisado no Capítulo 3) e no desenvolvimento de novas vacinas.

No segundo estudo (Capítulo 3) comparámos a actividade antiviral dos antagonistas dos co-receptores (AMD3100, TAK-779 e maraviroc) e dos inibidores de fusão (T-20 e T-1249) entre um grupo de 20 isolados de VIH-2 (19 isolados primários + um isolado laboratorial) e nove isolados de VIH-1 (sete isolados primários + dois isolados laboratoriais). Verificámos que a sensibilidade ao AMD3100 e ao TAK-779 é semelhante no VIH-1 e o VIH-2. No entanto, o perfil da curva dose-resposta do maraviroc (MVC) obtido para os isolados R5 foi diferente nos dois tipos de vírus. No VIH-2 os valores de IC_{90} foram significativamente mais elevados do que no VIH-1; por outro lado, os declives da curva dose-resposta foram mais baixos no VIH-2 do que no VIH-1. Colectivamente, estes resultados sugerem que poderão ser necessárias concentrações mais elevadas de MVC para tratar os doentes infectados pelo VIH-2. Adicionalmente, encontrámos uma correlação forte e de sentido inverso entre as susceptibilidade do VIH-2 ao MVC e o número de células T $CD4^+$ dos doentes quando os vírus foram isolados. Vírus isolados em doentes em fase de SIDA foram menos susceptíveis ao MVC do que os vírus isolados em doentes com uma contagem de células T $CD4^+$ superior a 200 células/μl. Ao contrário do VIH-1 não encontrámos qualquer correlação entre a carga da V3 e a susceptibilidade dos isolados R5 de VIH-2 ao MVC. De um modo geral, os nossos resultados sugerem que são necessários ensaios clínicos para avaliar a efectividade do MVC na infecção pelo VIH-2, determinar a dose terapêutica mais adequada e esclarecer se é

necessário fazer um ajuste de dose de acordo com a fase da doença. Adicionalmente, e uma vez que isolados VIH-2 X4 e populações duplas/mistas são totalmente ou parcialmente resistentes ao MVC, é de extrema importância o desenvolvimento de um ensaio de tropismo (genotípico e/ou fenotípico) para o VIH-2 de modo a determinar o tropismo antes do início da terapia com MVC. Sem o conhecimento prévio do tropismo viral, o tratamento com MVC poderá seleccionar espécies X4 minoritárias que estão associadas a maior resistência à neutralização e uma progressão mais rápida da doença.
No que diz respeito aos inibidores de fusão, verificámos que o T-20 tem actividade reduzida no VIH-2, confirmando estudos anteriores realizados com dois isolados laboratoriais. Por outro lado, observámos uma elevada susceptibilidade deste vírus ao T-1249, indicando que os inibidores de fusão são potencialmente eficazes na infecção pelo VIH-2. Assim, o desenvolvimento de um novo inibidor de fusão do VIH-2 foi o objectivo do último estudo desta tese (Capítulo 4).

No Capítulo 4, desenvolvemos novos péptidos inibidores de fusão a partir da reconstrução de sequências ancestrais da glicoproteína gp36 do invólucro de VIH-2 e de Vírus de Imunodeficiência dos Símios (VIS). Com esta abordagem inovadora pretendemos incorporar a história evolutiva dos vírus na sequência dos péptidos e desta forma melhorar a tolerância destas moléculas aos polimorfismos naturais da sua região alvo bem como às mutações de resistência seleccionadas na sua presença. Obteve-se um péptido ancestral (P3) constituído por 34 aminoácidos, cuja sequência corresponde às posições homólogas 628 – 661 da proteína Env do isolado VIH-1 HXB2 (ou 623 – 656 do isolado VIH-2 ROD). A sequência do P3 difere em 21 aminoácidos da sequência consenso de VIH-1, 14 aminoácidos da sequência do T-20 e 6 aminoácidos da sequência consenso de VIH-2. Ao contrário da natureza não-estruturada do T-20, o P3 tem uma conformação típica em hélice-α, o que lhe poderá conferir maior a estabilidade contra a degradação proteolítica, bem como maior afinidade para a região alvo. Por outro lado, o P3 foi facilmente solúvel em soluções aquosas o que é uma vantagem num futuro desenvolvimento de uma fórmula farmacêutica.
O P3 demonstrou ter uma forte actividade antiviral contra isolados primários e laboratoriais de VIH-1 e VIH-2 (IC_{50} médio, 11 nM para o HIV-1 e 63.8 nM para o HIV-2), incluindo variantes resistentes ao T-20 (IC_{50}, 0.15 – 11.8 nM). Através da passagem consecutiva de vírus em cultura na presença do péptido, foi seleccionada uma mutação de resistência na região HR1 da gp41 (VIH-1), a qual é responsável pela redução da susceptibilidade do VIH-1 ao P3 em 120x. Nas mesmas condições, e após 60 dias em cultura, não foi possível seleccionar mutações de resistência ao P3 no VIH-2. Estes resultado, em conjugação com a sua forte ligação à glicoproteína transmembranar de um

isolado de VIH-2, indicam que, tal como outros péptidos baseados na região HR2 (T-20, T-1249), o P3 inibe a entrada do VIH pela interacção com a região HR1 da gp41 e sugerem que a barreira genética para a resistência ao P3 é significativamente superior no VIH-2 do que no VIH-1. Neste estudo demonstrámos ainda que o P3 é significativamente menos antigénico do que o T-20 nos doentes infectados pelo VIH-1 o que poderá traduzir-se numa maior duração da eficácia clínica do P3 em comparação com o T-20. Os resultados obtidos com o P3 demonstram pela primeira vez que é possível desenvolver péptidos com actividade antiviral significativa utilizando metodologias de biologia evolutiva, pelo que esta abordagem poderá ser explorada no futuro para a produção de medicamentos peptídicos e, eventualmente, de vacinas.

ABSTRACT

The general aims of this thesis were: 1) to examine the C2, V3 and C3 envelope regions of HIV-1 and HIV-2 at the molecular, evolutionary and structural levels; 2) to compare HIV-1 and HIV-2 susceptibility to entry inhibitors and assess their potential value in HIV-2 therapy; 3) to produce a new fusion inhibitor peptide using evolutionary biology based strategies.

In the first study (Chapter 2), HIV-1 and HIV-2 were compared at the molecular, evolutionary and structural levels in the C2, V3 and C3 envelope regions. We identified significant structural and functional constrains to the diversification and evolution of C2, V3 and C3 in the HIV-2 envelope but not in HIV-1. In particular, we found that V3 in HIV-2 is less exposed and more conserved than in HIV-1, suggesting fundamental differences in the biology and infection of these viruses as well as in their susceptibility to entry inhibitors.

In the second study (Chapter 3) we measured the baseline susceptibility of HIV-1 and HIV-2 primary isolates to different fusion inhibitors and coreceptor antagonists, including enfuvirtide (T-20) and maraviroc (MVC). MVC inhibited HIV-2 R5 variants at significantly higher IC_{90} concentrations than HIV-1 variants. Moreover, as previously found in HIV-1, susceptibility of HIV-2 R5 variants to MVC was inversely related with CD4+ T cell counts at time of virus isolation. These results suggest that the structure of the envelope complex of R5 variants changes along the course of infection. More importantly, the results call for new clinical studies to evaluate the efficacy of MVC in HIV-2 infection and to determine its best therapeutic dosage in early and late stage disease. We also provide definitive evidence demonstrating that T-20 is not useful for HIV-2 therapy.

In the final study (Chapter 4), we designed a new HIV fusion inhibitor peptide (P3) based on the ancestral sequences of the HIV-2 and SIV envelope genes. P3 has an α-helix structure as demonstrated by circular dichroism. It has broad antiviral activity at the nanomolar range against HIV-1 and HIV-2 primary isolates, including HIV-1 variants resistant to T-20. Binding ELISA assays and selection of resistant mutants suggest that P3 prevents viral fusion by binding to the transmembrane protein in the HR1 region. These studies provide proof of concept that viable antiviral peptides can be constructed using evolutionary biology strategies. Such strategies should be explored to enhance the production of peptide drugs and vaccines.

LIST OF ACRONYMS

95%CI	confidence interval at 95 percent
AIC	Akaike's information criterion
AIDS	Acquired Immunodeficiency Syndrome
BSA	bovine serum albumin
ºC	Celsius degree
CA	conic shaped viral capsid
CD	circular dichroism spectroscopy
CDC	Centres for Disease Control
CO_2	carbon dioxide
COT	center-of-the-tree
CRF	circulating recombinant form
CTL	cytotoxic T lymphocyte
DC	dendritic cells
D/M	dual/mixed population
DMEM	Dulbecco's minimal essential medium
dN	rate of non-synonymous substitutions
DNA	deoxyribonucleic acid
dS	rate of synonymous substitutions
ECLs	extracellular loops
ECL2	second extracellular loop
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	fetal bovine serum

FDA	Food and Drugs Administration
FEL	fixed effects likelihood
FP	fusion peptide
GARD	Genetic Algorithm Recombination Detection
GM	growth medium
GTR	General Time Reversible model
h	hour
HAART	highly active anti-retroviral therapy
HIV	human immunodeficiency virus
HIV-1	HIV type 1
HIV-2	HIV type 2
hLRT	hierarchical likelihood ratio test
HPLC	high-pressure liquid chromatography
HR	heptad repeat
HTLV	human T-cell leukaemia viruses
IC_{50}	50% inhibitory concentration
IC_{90}	90% inhibitory concentration
ICAM1	intercellular adhesion molecule 1
IFEL	internal fixed effects likelihood method
IN	integrase
kDa	kilodalton
LBD	lipid-binding domain
LFA1	lymphocyte function-associate antigen 1
LTR	long terminal repeats

MA	matrix protein
mg	micrograms
µl	microliters
µM	micromolar
ml	milliliters
mm	millimeters
MP	maximum parsimony
MRCA	most recent common ancestor
mRNA	messenger RNA
ML	maximum likelihood method
MSM	men who have sex with men
MVC	maraviroc
NC	nucleocapsid proteins
NK	natural killer cells
nM	nanomolar
nm	nanometers
NNI	nearest-neighbor interchange
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NRTIs	nucleoside reverse transcriptase inhibitors
NtRTIs	nucleotide reverse transcriptase inhibitors
OD	optical density
PBD	pocket-binding domain
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline

PBS-T	PBS containing 0.05% of Tween-20
PCR	polymerase chain reaction
PDB	protein data bank
PFD	pocket-forming domain
PHA	phytohemagglutinin
PIC	pre-integration complex
PIs	protease inhibitors
PR	protease
PS	positively selected
PT	Portuguese patients
RAL	raltegravir
REL	random effects likelihood method
RNA	ribonucleic acid
RRE	rev responsive element
rsCD4	recombinant soluble CD4 molecule
RT	reverse transcriptase
SBP	Simple Breakpoint Recombination
SCH-D	vicriviroc
SD	standard deviation
SIV	simian immunodeficiency virus
SIVcpz	SIV from *Pan troglodytes troglodytes* chimpanzees
SIVgor	SIV from Western lowland gorillas
SIVsmm	SIV from *Cercocebus torgnatus atys* sooty mangabeys
SLAC	single-likelihood ancestor counting

SPR	subtree pruning and re-grafting
STIs	sexually transmitted infections
SU	surface glycoprotein
T-20	enfuvirtide
TAR	transactivation response region
TBR	tree bisection and reconnection
$TCID_{50}$	50% tissue culture infectious dose
TM	transmembrane glycoprotein
TNX-355	ibalizumab
TVM	transversion model
U	units
USA	United States of America
VSV	vesicular stomatitis virus

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I

Introduction

GENERAL INTRODUCTION

The discovery of HIV

The Acquired Immunodeficiency Syndrome (AIDS) was first described in 1981. Symptoms of immune suppression were observed in young homosexual men developing Kaposi´s sarcoma and *Pneumocystis carinii* pneumonia [1,2]. These cases were initially reported in individuals from the United States of America (USA), but shortly after similar observations were made in patients from Haiti [3], Europe [4] and Africa [5]. In 1982, the Centres for Disease Control (CDC), USA, coined the term "acquired immunodeficiency syndrome" [6], and by 1983 the risk groups for contracting AIDS already included homosexuals, injection drug users, haemophiliacs [7], women maintaining sexual contacts with infected men [8,9], and infants (vertical transmission) [10].

Luc Montagnier and Françoise Barré-Sinoussi at Pasteur Institute (France) isolated the first virus from a patient with AIDS in 1983 [11]. It was reported to be a retrovirus belonging to the family of the human T-cell leukaemia viruses (HTLV), but distinct from each previous isolate. In the following year, a similar retrovirus (HTLV-III) was isolated by a group of American investigators [12]. The evidence produced confirmed that this retrovirus, later classified as Human Immunodeficiency Virus type 1 (HIV-1), was the causative agent of AIDS [13].

In 1986, a new retrovirus distinct from HIV-1 was isolated in patients from Guinea-Bissau and Cape Verde Islands (West Africa) interned at a Lisbon (Portugal) hospital. They presented a clinical syndrome similar to AIDS [14,15]. The isolation and characterization of the second HIV virus, HIV type 2 (HIV-2), resulted from a successful collaboration between Pasteur Institute and the pioneer work of Maria Odette Santos Ferreira at Faculty of Pharmacy of Lisbon.

The Nobel Foundation has recently acknowledged the discovery of HIV by rewarding Luc Montagnier and Françoise Barré-Sinoussi with the 2008 Nobel Prize for Medicine.

The HIV/AIDS pandemic

Since the beginning of the epidemic, more than 60 million people have been infected with HIV worldwide and almost 30 million people have died of AIDS-related causes [16]. At the end of 2009, there were an estimated 33 million people living with HIV, including 2.5 million children with less than 15 years of age. Indeed, the number of people living with HIV tended to rise since the late 1990s due to high rates of HIV transmission, but also to the significant scale up of successful antiretroviral therapy. Nonetheless, the latest reports

indicate that the overall growth of the epidemic has now stabilized and the number of new infections and AIDS-related deaths are decreasing [17].

Over the last decade, the incidence of HIV infection has decreased by more than 25%, even in countries from sub-Saharan Africa. This region represents 68% of the global HIV prevalence, has the highest number of new infections and is still the only region, besides the Caribbean, where girls and women are significantly more affected than male individuals. There are, however, a few selected countries in Eastern Europe and Central Asia that escape this global trend. In these countries the incidence has increased by 25% and the HIV epidemics involves a complex association between injection drug users, sex workers, their sexual partners and men who have sex with men (MSM). Noticeably, there is also evidence of a re-emergence of HIV infection among MSM in North America and Western Europe [17].

In contrast to HIV-1 pandemic, HIV-2 infection is mainly restricted to West African countries, such as Guinea-Bissau [14,18], Gambia [19], Senegal [20] and Ivory Coast [21]. Notably, an increasing number of dual infections of HIV-1 and HIV-2 have been documented in HIV-2 endemic countries, and no evidence has been found of a protective effect of HIV-2 against HIV-1 infection [22,23,24]. However, recent data indicates that HIV-2 prevalence is now decreasing, particularly in regions where the number of cases used to be particularly high [23,25,26].

In Portugal, there were a total of 37201 notified cases of HIV/AIDS infection by the end of 2009, the majority of which are associated to injection drug usage and heterosexual transmission. Over the last five years, the number of new infections is decreasing and heterosexual transmission is becoming the most frequent route of infection. Portugal is one of the few countries outside West Africa with a significant number of HIV-2 infection cases. Indeed, it represents 3.2% of the total notified cases of HIV/AIDS in Portugal [27].

The origin and genetic diversity of HIV

Despite being considered human lentiviruses, humans are not the natural hosts of either HIV-1 or HIV-2. Compelling evidence has demonstrated that both viruses were introduced in human population by the zoonotic transmission of distinct lentiviruses naturally infecting non-human primates. While HIV-1 descends from the Simian Immunodeficiency Virus (SIV) infecting *Pan troglodytes troglodytes* chimpanzees (SIVcpz) [28], HIV-2 descends from SIVs endemic in *Cercocebus torgnatus atys* sooty mangabeys (SIVsmm) [29,30]. In fact, it seems that SIVs have entered the human population on 12 separate occasions, resulting in 12 distinct phylogenetic (evolutionary) lineages (groups) of HIV.

So far, HIV-1 has been classified into four groups (M, N, O and P). HIV-1 groups M and N descend from SIVcpz infecting West Central African chimpanzees communities, particularly the ones from Cameroon [31], while HIV-1 groups O and P are more closely related to SIVs infecting western lowland gorillas (SIVgor) from the same region [32,33]. Concerning HIV-2, a total of 8 groups (A to H) have been described [30,34,35,36]. They all descend from SIVsmm endemic in sooty mangabeys inhabiting the West African region [37,38].

The development of sophisticated phylogenetic analysis together with the availability of increasing number of well-characterized viral sequences, have allowed the possibility to estimate, with a reasonable level of confidence, the time when SIV zoonoses occurred [39]. Several studies point to the early 20^{th} century as the time for the origin and initial spread of epidemic HIV strains [40,41,42,43]. Still, the first documented case of HIV-1 infection was identified in a blood sample from 1959 stored in the Democratic Republic of Congo [44]. Noticeably, a recent study indicates that SIVs have been present in African primates for more than 32 000 years, suggesting that SIV transmission to humans may have occurred repeatedly over the ages [45]. Exposure to primate blood by bushmeat trade of wild animals (hunting, consumption as food source or other related activities) is one plausible route for the cross-species transmission [39], and even nowadays constitutes a risk for potentially new transmissions [46].

Changes in human behaviour like social disruption, urbanization and prostitution, or the use of non-sterilized needles might have significantly contributed for the establishment of the nascent HIV epidemics [39,43]. The epicentres for the initial spread of HIV-1 and HIV-2 were probably the West Equatorial Africa and West Africa (respectively), due to the greatest diversity of HIV strains that have been co-circulating in these regions over the years [39].

The dissemination of HIV in humans has resulted in the emergence of highly genetic diverse HIV strains. Indeed, extensive genetic heterogeneity is one of the key characteristics of HIV. Apart from the epidemiological patterns described above, the major mechanisms contributing for such variability are the lack of proofreading activity of the reverse transcriptase, high rate of replication, host selective immune pressures and recombination events during replication. Notably, these variants are unevenly distributed around the globe [47,48,49,50].

The majority of HIV-1 strains found worldwide and responsible for the pandemic belong to group M [50]. These strains seem to have efficiently adapted to the new host, spreading around the world and generating multiple subtypes. In contrast, HIV-1 group O variants are restricted to the West Central African region, particularly in Cameroon, the country where also a limited number of group N and group P viruses have been identified [33,47].

HIV-1 group M can be divided into 9 subtypes or clades (A, B, C, D, F, G, H, J and K), representing phylogenetically linked strains of HIV-1 that are approximately at the same genetic distance from each other [48,49] and have arisen from just one cross-species transmission event [28,39]. Subtypes A and F can be further separated into sub-subtypes A1 – A5, and F1 – F2, respectively [48,49,51]. There are also numerous recombinant forms of HIV-1, which have a mosaic genome composed of regions from different subtypes. A recombinant form is classified as circulating recombinant form (CRF) if it is documented in at least three people without direct epidemiologic linkage. Otherwise it is classified as a unique recombinant form (URF) [48]. So far, there were already identified 49 CRFs and multiple URFs (Los Alamos Sequence Database, http://www.hiv.lanl.gov).

The identification of subtypes and CRF is a useful strategy to track the dissemination of HIV in the worldwide pandemic [48,49]. Globally, the most prevalent HIV-1 genetic forms are subtypes A, B, C, CRF01_AE and CRF02_AG. Subtype A is primarily found in Central and Eastern Africa and in Eastern Europe, and subtype B is the main genetic form in Western and Central Europe, the Americas and Australia. Subtype C is responsible for 50% of the global prevalence and is predominant in India, China, Eastern and Southern Asia. Regarding the CRF01_AE and CRF_AG, each account for 5% of all HIV-1 infections worldwide and while the former circulates mainly in Southeast Asia, the latter is found in West Africa [47,48,49]. In Portugal, the most prevalent HIV-1 genetic forms are subtypes B and G and CRF14_BG [52,53,54].

As mentioned above, HIV-2 infection is primarily restricted to West Africa. Of the 8 phylogenetic clades, only HIV-2 groups A and B are considered as endemic [36,39,55], with group A being frequent in the western part of West Africa (Senegal and Guine-Bissau and Cape Verde) and group B in Ivory Coast [21,56,57,58]. For all other HIV-2 clades, only a few cases have been documented, mostly in Sierra Leone and Liberia (groups C–F) [30,34] or Ivory Coast (groups G and H) [35,36]. The first recombinant form identified for HIV-2 was an A/B recombinant isolated in a patient from Ivory Coast [30]. More recently, three additional HIV-2 A/B recombinants were identified in Japan [59]. Altogether, these findings culminated in the determination of the first CRF for HIV-2, the HIV-2 CRF01_AB [59].

HIV-2 group A infection have also been documented in countries sharing socio-historical links to West Africa, such as, for example, Portugal and France [55,56,60]. It has been proposed that the independence war of Guinea-Bissau against Portugal (1963-1974), and the associated blood transfusions and sexual activities at that time, might have facilitated the spread of the virus out of West Africa [41,61]. HIV-2 group A is also found in other countries with historical and socio-economical ties to Portugal, like Brazil and India [26,55,62].

HIV Genome and structure

HIV-1 and HIV-2 are classified as belonging to the *Retroviridae* family, the *Orthoretrovirinae* subfamily and the *Lentivirus* genus [63].

HIV is a spherical enveloped virus with a diameter of approximately 110 nm (Figure 1). The envelope consists of a lipid bilayer spanned by the transmembrane glycoprotein (TM), which is anchored to the outer surface glycoprotein (SU). In the mature virion these heterodimers are associated as trimers. The envelope is surrounding internally by a matrix protein (MA). Inside the conic shaped viral capsid (CA) there are two identical copies of a positive sense single stranded RNA bound to nucleocapsid proteins (NC). The CA also encloses the viral enzymes reverse transcriptase (RT), integrase (IN), and protease (PR) and the four accessory proteins Nef, Vif, Vpr and Vpu (HIV-1) or Vpx (HIV-2) [64].

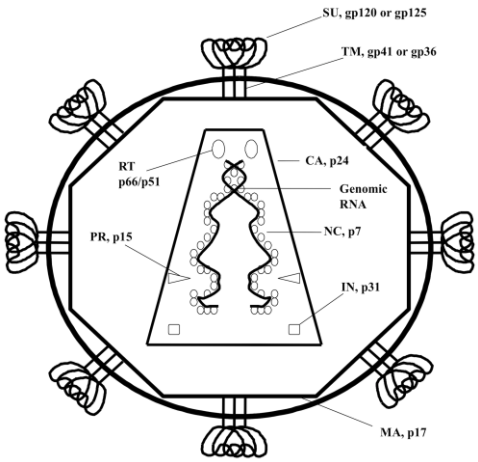

Figure 1. Schematic structure of the HIV particle. (Adapted from Taveira N, Borrego P, Bártolo I (2008) Biologia molecular de VIH. In: Antunes F, editor. Manual sobre SIDA. 3th ed. Lisbon: Permanyer Portugal. pp. 27-50.)

Each RNA molecule is about 9800 nucleotides long and is delimited by long terminal repeats (LTR) at both 5' and 3' ends (Figure 2). It combines nine genes by the use of all three open reading frames. Three genes encode for structural or enzymatic proteins (*gag*, *pol* and *env*), two for regulatory proteins (*tat* and *rev*) and four for accessory proteins (*nef*, *vif*, *vpr* and *vpu*/*vpx*). The *gag* gene encodes the polyprotein precursor $Pr55^{Gag}$ that is then cleaved by the PR enzyme into the MA (with a molecular weight of 17 kDa, p17), CA (p24), NC (p7) and p6 proteins. This process generates the additional p1 and p2 spacer peptides. The *pol* gene encodes for the RT (p66 and p51 subunits), IN (p31), and PR (p15) enzymes. They are produced after PR processes the $Pr160^{GagPol}$, a polyprotein precursor that is synthesized when the reading frame is shifted during the transcription of $Pr55^{Gag}$. The *env* gene encodes for the glycosylated polyprotein precursor $Pr160^{Env}$ (or $Pr140^{Env}$ in HIV-2),

which is cleaved by a cellular protease into the SU (gp120 in HIV-1 or gp125 in HIV-2) and TM (gp41 or gp36) glycoproteins [64].

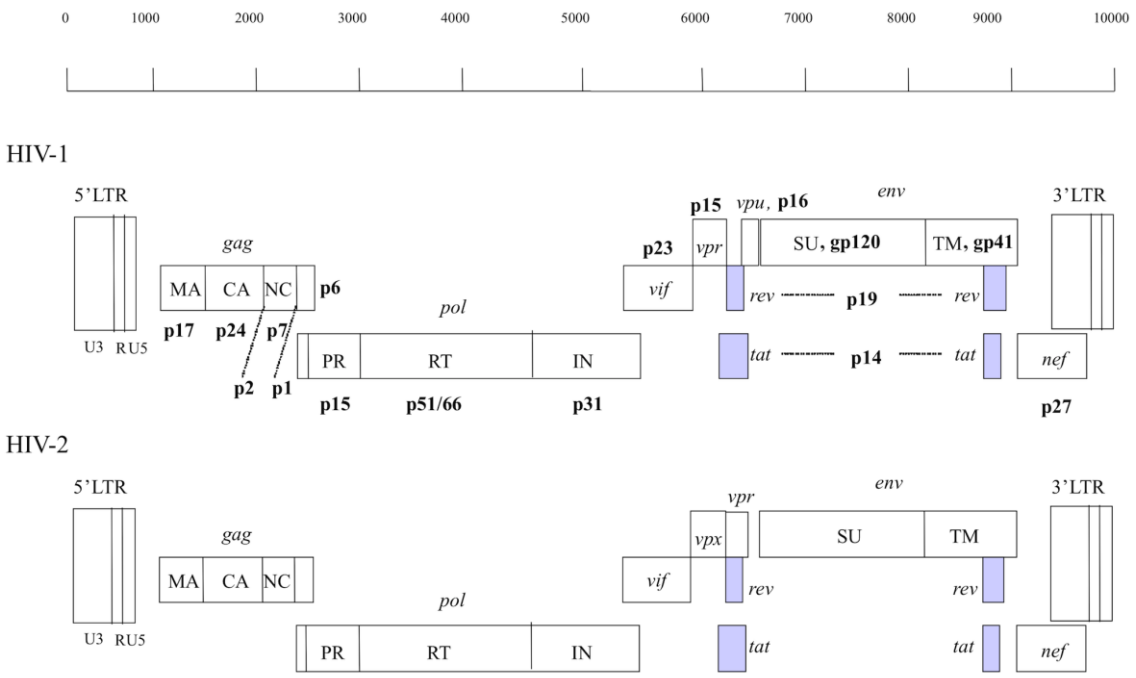

Figure 2. Genomic organization of HIV-1 and HIV-2. (Adapted from Taveira N, Borrego P, Bártolo I (2008) Biologia molecular de VIH. In: Antunes F, editor. Manual sobre SIDA. 3th ed. Lisbon: Permanyer Portugal. pp. 27-50.)

HIV Life cycle

Generally, the viral life cycle of HIV starts when the SU glycoprotein binds to the main receptor, the CD4, present in the cellular surface of the host cell (T-lymphocytes, monocytes, macrophages and dendritic cells). This interaction induces conformational changes in the SU, whereby the site for binding to a second receptor (co-receptor) becomes exposed. *In vivo*, the major co-receptors of HIV are the CCR5 and CXCR4 chemokine receptors. Both CD4 and co-receptor binding leads to conformational changes in TM glycoprotein that result in the insertion of the fusion peptide of TM into the host cellular membrane and, consequently, on the fusion of the viral envelope with the host cell. Thereafter, the viral capsid is release into the cytoplasm (reviewed in [64]) (Figure 3).

After HIV uncoating, the RT enzyme starts the reverse transcription of viral RNA. In the first stage, a single DNA strand is synthesised using one of the two RNA molecules as a template and the tRNAlys molecule as a primer. Once the first complementary DNA strand (negative strand) is transcribed, the ARNase H subunit of RT enzyme (p51) degrades the

RNA template. A new positive DNA strand, complementary to the negative one, is then synthesized by the p61 subunit of RT. The double stranded DNA, together with the MA, NC, IN, RT and Vpr proteins (plus the Vpx in HIV-2), make the pre-integration complex (PIC), which is transported to the nucleus using the cytoplasmatic microtubules network. This process is mediated by the IN and Vpr (and Vpx in HIV-2). Still outside the nucleus, the IN enzyme digests the 3' LTR of both DNA strands creating two recessive ends. The IN will later use these ends to unite (integrate) the viral DNA into an open region of the host chromosomal genome, thus generating a provirus. Proviral DNA can either remain latent (silent) in the host cell or be transcribed by the cellular machinery, progressing with the viral life cycle [64].

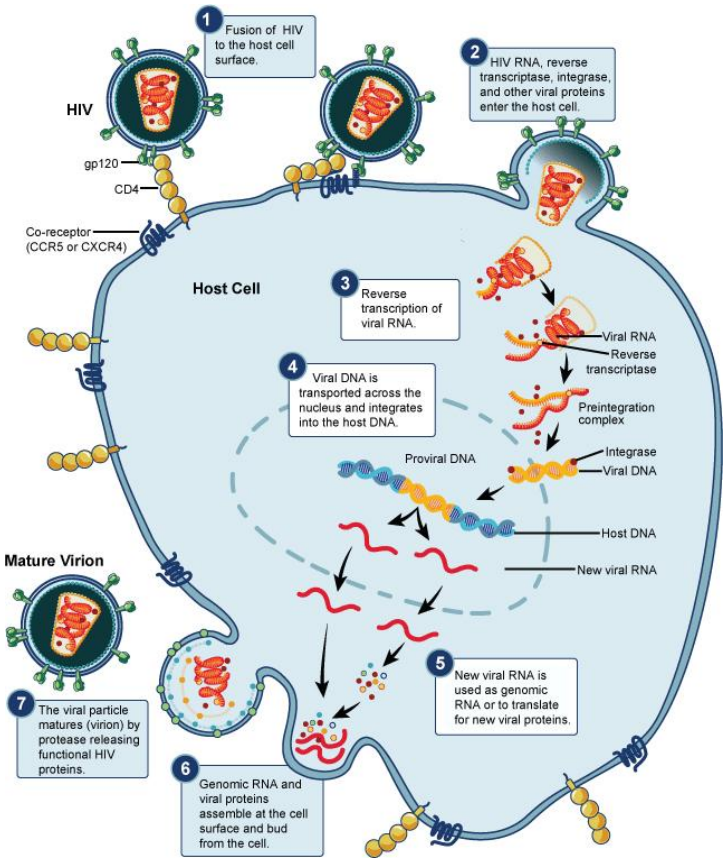

Figure 3. The life cycle of HIV. (Adapted from http://www.niaid.nih.gov/topics/HIVAIDS/)

The promoter region within the 5' LTR mediates the transcription of the proviral DNA. Three classes of RNA are obtained: (1) completely spliced mensager RNA (mRNA) translating for Rev, Tat and Nef (early transcripts); (2) incomplete spliced mRNA encoding for Env, Vif, Vpr and Vpu/Vpx (late transcripts); (3) unspliced and complete mRNA molecules that translate for polyprotein precursors $Pr55^{Gag}$ and $Pr160^{GagPol}$ (late transcripts) and will be incorporated in the nascent viral particles as genomic RNA. Indeed, proteins from early transcripts (Tat and Rev) are required to complete the expression of the later

transcripts. Binding of Tat protein to the transactivation response region (TAR), a secondary structure downstream the LTR of the nascent RNA, is important for stable and efficient elongation of mRNA. The transport of unspliced and incompletely spliced mRNA outside the nucleus is dependent on Rev, which binds to the Rev responsive element (RRE) in the RNA *env* region before carrying them to the cytoplasm to be translated [64].

Once the Env precursor poliproteins are translated, they are glycosylated in the Golgi apparatus before they oligomerize in trimers. The polyproteins are, then, cleaved into the SU and TM glycoproteins and transported to the cytoplasmatic membrane, where the assembly of the viral particles takes place. These particles include the genomic RNA and the polyprotein precursors $Pr55^{Gag}$ and $Pr160^{GagPol}$. They bud from the cell by gemulation of the cytoplasmatic membrane, thus acquiring the lipid envelope already containing the TM/SU trimers (and some cellular membrane proteins). Finally, the $Pr55^{Gag}$ and $Pr160^{GagPol}$ polyproteins are processed into the functional proteins by the PR enzyme [64]. This final maturation of the viral particle (virion) occurs outside the host cell (Figure 4).

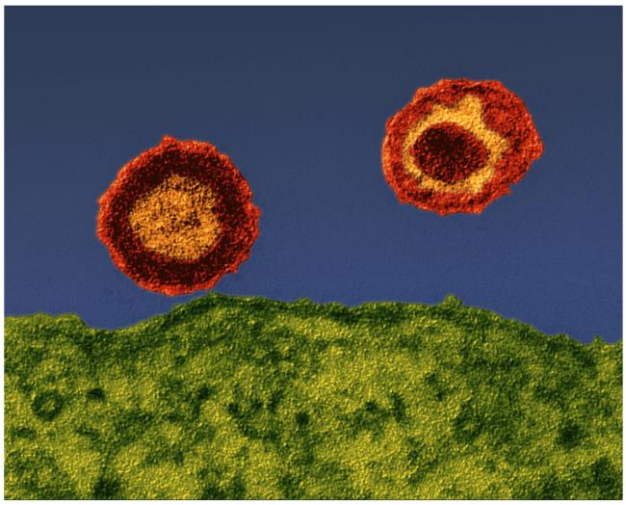

Figure 4. Maturation of the virus particle. False-colored image of two HIV virus particles budding from a human T cell: (left) the CA protein is still associated with the viral membrane in the immature particle, whereas (right) the mature particle has a condensed core inside the virus shell. (Image by Klaus Boller, Paul-Ehrlich-Institute, Germany; http://www.cell.com/Cell_Picture_Show-hiv)

Several host restriction factors can hinder the retroviral replicative cycle [65]. Among these factors are APOBEC3G, TRIM5-α and tetherin proteins. APOBEC3G, a member of the family of cytidine deaminases that is packaged within viral particles, induces G-to-A hipermutation and degradation of the nascent proviral DNA [65,66]. However, the viral protein Vif impairs the activity of this enzyme [65,66,67]. TRIM5-α is a member of the tripartite motif protein family [65,68]. TRIM5-α interacts with the viral capsid and blocks

uncoating, but its activity is highly dependent on species-specific compatibility [66,68,69]. It has been reported that, when compared to HIV-1, HIV-2 is more susceptible to TRIM5-α but more resistant to APOBEC3G [70]. Tetherin is a recently identified host restriction factor that inhibits the release of new viral particles [71]. Vpu and Env proteins can neutralize tetherin's activity in HIV-1 and HIV-2, respectively [71,72].

HIV Transmission

The most common routes of HIV transmission include sexual contacts, contaminated blood or blood products (medical injections, blood transfusions, injection drug usage) and mother-to-child transmission (before, during and after birth or through breast feeding) [69]. Still, heterosexual transmission is the most frequent route of HIV-1 infection worldwide [17,66]. Several human- and HIV-specific determinants are required for efficient viral transmission.

There is evidence that HIV-1 transmission is directly correlated with the level of virus in circulation [73,74]. Moreover, the concentration of HIV-1 in blood and genital secretions varies depending on the stage of disease [69,75]. Indeed, increasing rates of HIV-1 transmission occur during the very early (acute) and later stages of infection (advanced disease), the periods when intense viral replication is observed and the highest levels of viral load are detected [66,69,75]. Notably, up to 50% of new HIV-1 infections are acquired from recently infected patients [76].

The risk of HIV infection is also influenced by the presence of other sexually transmitted diseases, such as syphilis and herpes simplex virus-2. The erosion of skin or mucosa resulting from genital inflammation and ulceration can enhance HIV-1 sexual transmission [66,69,77], or even increase the concentration of HIV-1 in the genital tract of the infecting partner [69]. On the other hand, male circumcision offers a degree of protection against HIV-1 acquisition, probably because removing the penile foreskin eliminates an easily breached entry portal containing many cellular targets of HIV [66,69,76]. Successful antiretroviral treatment also has the potential to prevent HIV transmission, by reducing the levels of HIV in blood and genital secretions [78,79]. In addition, research for new HIV prevention strategies led to the development of microbicides as topical agents to be applied on the vagina or rectum in order to protect from sexually transmitted infections (STIs) [80,81]. The impact of this approach in the prevention of HIV transmission has been highlighted by the results from the recent CAPRISA trial, which reported the use of tenofovir (an antiretroviral agent) in a vaginal gel formulation as a safe and effective method that can reduce HIV acquisition by 54% [81]. Nevertheless, on a global perspective, better access to healthcare services and behaviour changes (like adoption of safer sex

practices), are key strategies to reduce the risk of HIV infection and have a significant impact in the shape of the current epidemics [17].

Despite using similar routes of transmission, the prevalence rates of HIV-2 are much lower than HIV-1 [26,55,62]. As in HIV-1, heterosexual transmission is the most common route of HIV-2 infection [27,82], but at significantly lower rates [83]. Mother-to-child transmission is a rare event in HIV-2 with rates below 5% when compared to almost 25% in HIV-1, in the same untreated population [26,84]. The reduced transmissibility of HIV-2 is probably linked to the markedly lower plasma viremia [25,84] and reduced viral shedding in the genital tract [62,79].

A number of genotypic and phenotypic evidence support the active selection of specific variants during HIV-1 transmission [76]. Newly infected individuals acquire only a limited number of variants (1-10) circulating in the source donor (bottleneck effect), the majority of which are only able to use the CCR5 coreceptor [85]. This is observed either in sexual or percutaneous routes of infection. Although the mechanisms underlying these observations are not totally clear, it seems that the availability, infectability and spatial distribution of early target cells might severely limit the variability of the initial viral population (reviewed in [76]). This should be particularly true in mucosal transmission where a small, focal infected founder population of cells expands locally before posterior dissemination and systemic infection [86]. HIV then evolves away (diverge) from the founder virus as soon as anti-HIV humoral and cellular immune responses arise after exposure (usually takes several weeks) [66].

HIV Pathogenesis

The course of HIV infection can be divided into four stages: the acute phase (primary infection), the chronic asymptomatic phase, the early symptomatic phase and AIDS [87]. The acute phase is characterized by intense viral replication and massive loss of $CD4^+$ T cells that takes place mainly in mucosal tissues, particularly in the gut [88]. At the early stages of infection, HIV transmission across the mucosal epithelial layers is enhanced by dendritic cells (DC) present at the *lamina propria*. This is where productive viral replication initially occurs mostly in memory CD4+ T cells. DCs also seem to contribute for HIV dissemination to draining lymph nodes and secondary lymphoid tissue throughout the organism (e.g. the gut-associated lymphoid tissue), where high levels of activated $CD4^+$ T cells are present (reviewed in [66,89,90]). $CD4^+$ T cell depletion is a combination of direct viral infection, activation-induced cell death and host-derived cytotoxic responses [91]. The integrin $\alpha_4\beta_7$ mediates the migration of T cells to the gut-associated lymphoid tissue and is coexpressed with the CCR5 coreceptor in a small subset of metabolically activated

$CD4^+$ T cells, in which it appears in a complex with CD4 [92,93]. Notably, this integrin is also an HIV-1 receptor [93]. Binding of HIV-1 gp120 to integrin $\alpha_4\beta_7$ seems to facilitate cell-to-cell spread of HIV-1 and may enhance viral propagation following mucosal transmission [92,93].

The majority of HIV-infected individuals develop flu-like symptoms (acute HIV syndrome), approximately two to four weeks following the transmission of the virus. Seroconversion, with detection of specific anti-HIV antibodies, usually occurs within 3 to 12 weeks after exposure. Among the first antibodies detected are those directed against the viral capsid (p24) [87,94]. Plasma viremia (or viral load) typically peaks at three to four weeks after infection and then decreases to a steady state (viral set-point) [66], due to HIV-specific cytotoxic T lymphocyte (CTL) responses and humoral responses (neutralizing antibodies) [89]. The viral set-point marks the beginning of the chronic stage and is an important determinant on the rate of disease progression in untreated patients [66,95] (Figure 5).

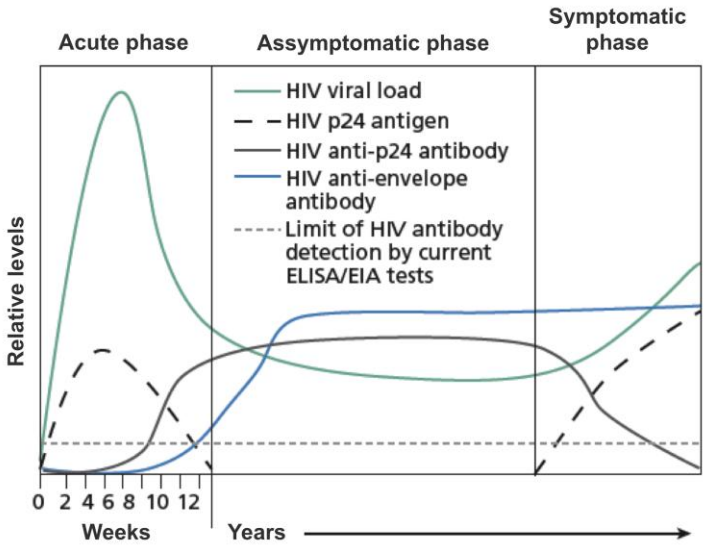

Figure 5. The clinical and laboratorial course of untreated HIV-1 infection. (Adapted from Daskalakis D (2011) HIV Diagnostic Testing: Evolving Technology and Testing Strategies. Top Antivir Med. 2011;19(1):18-22)

The chronic phase is the asymptomatic stage of HIV infection that lasts on average between 8 to 10 years in HIV-1 (it can be much longer in HIV-2) [70,96]. It is a period of clinical latency (silent infection) characterized by low levels of viral replication in the lymphoid tissue (viral reservoir) and constant antigen stimulation of the host immune system (immune activation) [96]. Persistent immune activation is manifested by increased turnover of T cells, monocytes and natural killer (NK) cells, high levels of $CD4^+$ and $CD8^+$ T cell apoptosis and polyclonal B cell activation which leads to generalized hipergammaglobulinemia (reviewed in [91]). It should be noted that in HIV-2 patients IgA

levels are not increased suggesting a selective B cell activation [97]. Chronic immune activation, which is a strong predictor of HIV disease progression, will eventually lead to the exhaustion of the immune system and occurrence or reactivation of opportunistic infections (e.g. candidiasis, pneumonia and tuberculosis) and development of neoplasic diseases (Epstein-Barr virus-related lymphomas, Kaposi's sarcoma, etc) [66,87,96]. Clinical manifestations of these co-infections mark the onset of the early symptomatic phase [87]. In untreated patients, progression to AIDS occur by continuous loss of $CD4^+$ T cells and rising viremia, as a consequence of intensifying viral replication from viral reservoirs and latently infected $CD4^+$ T cells [66,96]. Ultimately, the level of $CD4^+$ T lymphocytes drops below 200 cells/ml, defining the beginning of the AIDS stage [87,96].

Despite having similar proviral loads (n. of proviral DNA copies in PBMCs), at the same disease stage [70,98,99], HIV-1 and HIV-2 infections lead to very different immunological and clinical outcomes. Compared to HIV-1 infected patients, the majority of HIV-2 infected individuals have reduced general immune activation, normal $CD4^+$ T cell counts, low or absent plasma viremia and absence of clinical disease [55,70,99,100,101,102]. Indeed, HIV-2 infection is characterized by slow disease progression, long survival and reduced mortality rates [55,70,100,103,104,105,106]. These observations might be a consequence of the lower replication capacity of HIV-2 [107,108] and more effective immune response produced against HIV-2. In fact, most HIV-2 infected individuals have strong cytotoxic responses to Env and Gag proteins and raise autologous and heterologous neutralizing antibodies [55,109,110,111,112,113]. The lower state of immune activation in HIV-2 patients may be related with the immunosuppressive activity of the C2, V3, and C3 envelope regions of HIV-2 [19,20,21]. Nevertheless, with disease progression $CD4^+$ T cell depletion becomes similar in HIV-1 and HIV-2 infections [102,114], most of the immunological differences are lost and the mortality risk is equivalent [55,70,105,106].

HIV ENVELOPE

Molecular and structural organization of the viral envelope

Viral entry into host cells is mediated by the envelope SU and TM glycoproteins, which are encoded by the *env* gene. These glycoproteins are attached by a noncovalent association and are assembled as trimers [3x(SU/TM)], representing up to 14 functional spikes on the surface of the mature virion [115,116].

The SU glycoprotein is composed by five hypervariable regions, V1 to V5, separated by five more conserved regions, C1 to C5 (Figure 6). Hypervariable regions tend to form loops, stabilized by disulfide bridges. In its native trimeric conformation, SU has two domains,

one internal, hydrophobic in nature, and one external. After binding to the CD4 receptor, a major structural change occurs and a bridging sheet is formed between the V1/V2 stem and β20/β21 in C4. While both the external domain and bridging sheet are involved in the interaction between the SU and the cellular receptors (CD4, CCR5 and/or CXCR4), the internal domain is essential for SU–TM association [64,115,117,118,119]. Also, interaction between SU and the integrin $\alpha_4\beta_7$ gut-homing receptor is mediated by a conserved motif in the V2 loop of the bridging sheet [93]. Numerous glycosylation sites as well as major antigenic determinants, including neutralizing epitopes, can be found on the external domain [64,115,118,120].

The TM glycoprotein consists of one extracellular ectodomain, one transmembrane region and one intracytoplasmatic domain (Figure 6). The fusion peptide, at the hydrophobic N-terminal end of the ectodomain, is followed by two α-helices containing leucine zippers-like motifs: heptad repeats 1 and 2 (HR1 and HR2, respectively). Separating these heptad repeats, there is a small loop defined by cysteine residues (CC, cysteine bridge). HR1 and HR2 contain repeated patterns of seven residues and are arranged as trimers. The fusion peptide and both HR1 and HR2 play a significant role on the fusion of the viral envelope with the host cellular membrane. On the other hand, the intracytoplasmatic domain mediates the binding of the envelope to the MA protein, during the maturation of new viral particles [64,115,117,121].

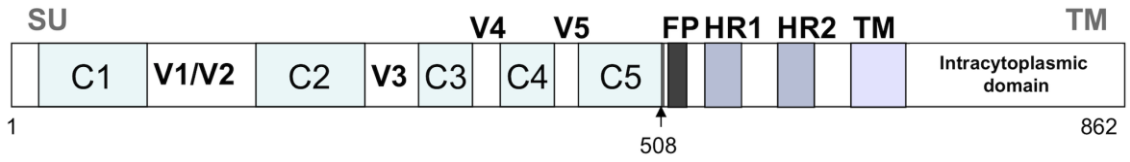

Figure 6. Schematic representation of SU and TM envelope glycoproteins. The SU glycoprotein is composed by five conserved (C1 to C5) and five variable (V1 to V5) domains. The TM glycoprotein contains the N-terminal fusion peptide (FP), two heptad repeats (HR1 and HR2), one transmembrane region (TM) and the intracytoplasmic domain. The figure is numbered according to the HIV-1 JR-FL isolate. (Adapted from Taveira N, Borrego P, Bártolo I (2008) Biologia molecular de VIH. In: Antunes F, editor. Manual sobre SIDA. 3th ed. Lisbon: Permanyer Portugal. pp. 27-50.)

Mechanism of HIV entry

The process of HIV entry generally involves three sequential steps occurring on the surface of the target cell: (1) binding of the SU glycoprotein to the CD4 receptor, (2) binding of the SU to the CCR5 and/or CXCR4 coreceptor and, finally, (3) fusion of the viral envelope with the cellular membrane (Figure 7). The mechanisms underlying these stages will be described in the next sections, and they characterize viral spread driven by cell-free

virions. Alternatively, HIV can disseminate through cell-to-cell contact using either viral synapses or membrane nanotubes [122,123].

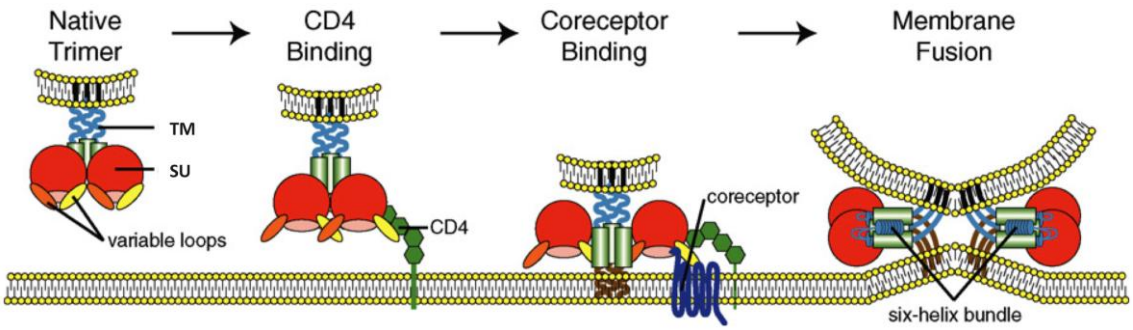

Figure 7. Model of the multi-step process of HIV entry. (Adapted from Tilton JC, Doms RW (2010) Entry inhibitors in the treatment of HIV-1 infection. Antiviral Res 85: 91-100.)

Interaction with the CD4 receptor

The CD4 receptor is a transmembrane protein with 58 kDa that exists on the surface of several cell lines, like T cells, monocytes, macrophages and DCs [64]. As mentioned above, it is often found in a complex with the integrin $\alpha_4\beta_7$ in activated $CCR5^{high}/CD4^{+}$ T cells in the gut compartment [92,93]. Four domains compose the extracellular region of CD4, D1 to D4. Attachment to the viral SU glycoprotein occurs at the CDR2 sub-region, one of the three sub-regions of D1 domain [64].

Electrostatic forces are responsible for the interaction between CD4 (positive charge) and the SU (negative charge), which is stabilized by Van der Walls forces and hydrogen bonds [117]. This interaction promotes conformational changes in the SU, leading, as previously stated, to the formation of the bridging sheet and increasing the exposure of V1, V2, V3 and C4. This results in the approximation of the viral envelope and the cellular membrane and the subsequent interaction of V3 with the coreceptor [64,115,121,124,125].

Interaction with the coreceptor

In vivo, the major coreceptors for HIV entry are the CCR5 and CXCR4 G-protein coupled receptors that function as the natural receptors for α and β chemokines [64,118]. These receptors are integral membrane proteins with seven transmembrane helices, an extracellular N-terminus and three extracellular loops (ECLs) that form a small pocket [118]. CCR5 is predominantly expressed on the surface of memory T lymphocytes, activated T lymphocytes and macrophages, while CXCR4 is mainly found in T lymphocytes, monocytes, DCs and B lymphocytes [126].

Upon SU – CD4 binding, the viral V3 loop is projected into closed proximity to the cellular membrane where it can interact with the coreceptor [125]. Interaction with the viral SU involves two coreceptor regions. Initially, the N-terminal region binds to the SU core and the base of the V3 loop, and then the second extracellular loop (ECL2) binds to the V3 tip [125,127,128]. While both coreceptor regions are necessary for successful cell entry by variants using the CCR5 coreceptor, only ECL2 seems to be critical for CXCR4 usage [129,130,131].

Fusion

Attachment of SU to CD4 and coreceptor promotes the approximation of the viral envelope and the cellular membrane and structural rearrangements of the TM glycoprotein. As a result, the fusion peptide becomes exposed and is inserted into the cytoplasmatic membrane, thus creating a prehairpin intermediate configuration of TM [132,133,134,135,136]. Notably, this intermediate state can be initiated by CD4 binding alone, but binding of a coreceptor enhances the process [137]. Then, the HR2 trimer folds back on an anti-parallel fashion towards the HR1 trimer, forming a six-helix bundle structure (6HB; final hairpin state) stabilised by the hydrophobic interactions between the HR1 domains in the center (central coiled-coil) and the HR2 domains outside. During this process, the viral envelope and the cellular membrane are brought together, leading to the formation of the fusion pore, through which the viral capsid enters the target cell [132,133,134,135,136] (Figure 8).

An alternative model of cell free HIV-1 cell entry is via the endocytic pathway [138]. Time-resolved imaging of single viruses and differential blocking of fusion by site-specific and universal inhibitors revealed that fusion with the cytoplasmatic membrane at the cell surface did not progress beyond the lipid mixing step [139]. Instead, HIV-1 was internalised upon CD4 and coreceptor interaction and complete fusion occurred only in endossomal compartments, leading to productive infection. Nonetheless, further studies are still needed to confirm the incidence and biological relevance of this pathway in HIV infection.

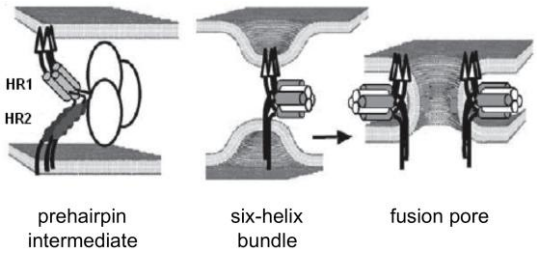

Figure 8. Model of the envelope glycoprotein-mediated membrane fusion. (Adapted from Weiss CD (2003) HIV-1 gp41: mediator of fusion and target for inhibition. AIDS Rev. 2003 Oct-Dec;5(4):214-21.)

Kinetic studies of HIV-1 and HIV-2 envelope glycoprotein-mediated fusion have elucidated some differences observed in the mechanism of entry of these two viruses. Despite, higher affinity of gp120 (HIV-1) than gp125 (HIV-2) to the CD4 receptor [140], Env-mediated fusion seems to be more rapid in HIV-2 than HIV-1 [141]. This difference might be explained by distinct efficiencies of CD4-induced conformational changes of gp120 and gp125. In the context of the trimeric SU/TM glicoproteins, the rate at which coreceptor binding site becomes exposed after CD4 binding is faster in gp125 [141]. In fact, several HIV-2 strains have the ability to infect cells via CCR5 and CXCR4 but independently of CD4 [142], indicating that in its native state the HIV-2 envelope gp125 may adopt a CD4–induced conformation of gp125 that is stabilized (constricted) by interactions between the cysteine residues of the V1/V2 regions in the hydrophobic cavity of this glycoprotein [140].

Cell-to-cell viral entry

Dissemination through cell-free virions is particularly advantageous for inter-host transmission and for viral spread between different compartments within each host. However, to efficiently infect a new cell, viral particles must overcome a number of biophysical (e.g. distance to the next cell) and immunological barriers (e.g. neutralizing antibodies). Cell-to-cell spread provides a fast and direct route of virus transmission that overcomes these barriers [122,123]. At least *in vitro*, HIV-1 infection by cell-to-cell contacts seems to be more efficient than by cell-free virus [143,144], but coreceptor antagonists and fusion inhibitors can efficiently inhibit both pathways at the nanomolar range [145,146,147,148,149].

Spreading of HIV-1 through cell-to-cell contact occurs via syncytia formation, viral synapses or membrane nanotubes. Syncytia results from fusion of HIV infected cells with adjacent cells and results in the formation of one multinucleated giant cell (see below). However, the relevance of syncytia formation for viral spread *in vivo* is still unclear [122].

In a virologic synapse, the HIV envelope glycoproteins expressed on the surface of the infected cells interact with the CD4 and CCR5/CXCR4 receptors of the target cell. Additional molecules stabilize this adhesive junction; e.g. the intercellular adhesion molecule 1 (ICAM1), on the infected cell, attaches with the lymphocyte function-associate antigen 1 (LFA1) on the target cell. Importantly, the recruitment of the cellular receptors and adhesion molecules requires the remodelling of the actin cytoskeleton. Viral assembly and budding are then polarized towards the viral synapse and virus is released into the synaptic cleft before fusing with target cell plasma membrane [150].

The virologic synapse seems to be an important mechanism of compartmentalized viral amplification [122] and of early viral spread from the mucosal surfaces to secondary

lymphoid tissue (between DCs and T cells) during mucosal virus transmission [151] (Figure 9). Recent data indicates that virologic synapses favours the endocytic entry of HIV [146].

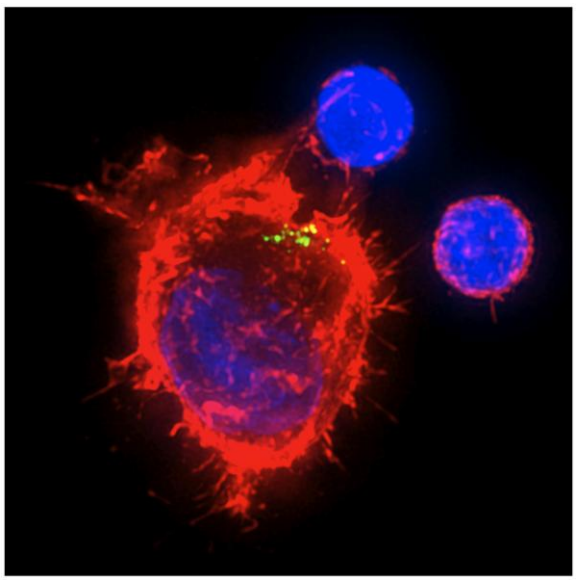

Figure 9. Virologic synapse. A dendritic cell (left) presents HIV (green) to primary T cells (right) in an infectious synapse. (Image by David McDonald and Thomas Hope, Case Western University and Northwestern University; http://www.cell.com/Cell_Picture_Show-hiv)

Alternatively, HIV-1 is also effectively transferred between T cells connected by membrane nanotubes [122,123]. The virus moves along the outside of nanotubes before attachment to the receptors of the target cell [152]. Such mode of transmission might be particularly efficient in secondary lymphoid tissue, which is full of susceptible target T cells [122].
To date, cell-to-cell transmission has only been described to HIV-1. It is still unclear whether HIV-2 uses these mechanisms with the same efficiency as HIV-1.

Coreceptor usage, pathogenesis and disease progression

As mentioned above, CCR5 and CXCR4 are the most important coreceptors in the pathogenesis of HIV infection in humans. R5 HIV viruses use CCR5 as a coreceptor for viral entry, while X4 viruses use CXCR4. Variants using both CCR5 and CXCR4 with equal efficiency are called dual-tropic (R5X4); the term dual/mixed (D/M) is applied to a mixed population of viruses using CCR5 and CXCR4 [126,153]. In contrast to HIV-1, *in vitro* studies show that some HIV-2 isolates may enter into cells using multiple alternative co-receptors besides CCR5 and CXCR4 (CCR1, CCR2b, CCR3, BOB) [126,142,154]. However, such a broader range of coreceptor usage does not appear to be associated with pathogenicity of HIV-2 [155,156].

Generally, R5 viruses are characterized by having slow/low replication rates and by not inducing syncytia [126,153,157,158]. In contrast, X4 strains are syncytia inducing and have rapid/high replication patterns. R5 strains are frequently isolated in the acute and asymptomatic stages of infection, while X4 variants usually emerge at latter stages of infection. The evolution for X4 phenotype occurs in about 50% of HIV-1 infected patients, with decreasing $CD4^+$ T cell counts and progression to AIDS [153]. Nonetheless, R5 strains can still persist in advanced stages of disease and be responsible for $CD4^+$ T cells depletion in the absence of X4 variants [159,160]. Notably, for HIV-1, X4 variants are more sensitive to neutralization than coexisting R5 variants, which may contribute to the late emergence of X4-tropic viruses [161]. In contrast, X4 HIV-2 isolates are more resistant to neutralization and the emergence of the X4 strains *in vivo* seems to be related with escape from the neutralizing antibodies targeting the V3 region [113]. Indeed, besides being an important immunodominant region in HIV, inducing the production of neutralizing and non-neutralizing antibodies [97,113,162,163,164,165,166], the V3 loop is also a major determinant of CCR5 or CXCR4 coreceptor usage. The global charge of the V3 region is a good predictor of CCR5/CXCR4 usage [167]. Increasing the V3 loop charge, by the presence of positively charged residues (R, K or H) at specific positions of the V3, is correlated with CXCR4 usage. In HIV-1 the relevant residues are at positions 11, 24 and 25, whereas in HIV-2 positions 18, 19 and 27 seem to be the most important [111,127,168,169,170,171]. Notably, V3 loop subtype-specific conformation differences or truncations within this region, influence coreceptor interaction and sensitivity to CCR5 inhibitors (reviewed in [172] for HIV-1 and [97,113,169] for HIV-2). The glycosylation pattern of the V3 and V1/V2 regions may also influence the coreceptor use of HIV-1 [173,174,175,176], but its impact on HIV-2 is still unclear [111]. Other regions beyond the V3 loop, and including the fusion peptide and the cytoplasmic tail of the TM glycoprotein, may also be important determinants of R5 to X4 switch in HIV-1 [176,177,178,179].

The CCR5 and/or CXCR4 coreceptor use of HIV-1 can be predicted by genotypic and phenotypic tests, currently available for both research and clinical use (when therapy with maraviroc is planned, see below). Common genotypic tropism inference tools are $Geno2pheno_{[coreceptor]}$ [180], WetCat [181] and WebPSSM [182]; the commercial TROFILE assay (Monogram Biosciences, USA) is the standard phenotypic tropism test used in clinical practice [183,184]. However, these tests are specific for HIV-1; similar tools designed for the prediction HIV-2 coreceptor usage haven't been developed yet.

HIV entry inhibitors

The introduction of highly active anti-retroviral therapy (HAART), a combination of three or four anti-HIV drugs, significantly decreased the morbidity and mortality of HIV infected patients. However, despite quick advances in the treatment of AIDS, several factors like drug resistance, limited patient adherence or drug-induced toxicity, have motivated the ongoing research for new molecules and targets of therapeutic intervention [185,186].
One decade ago, the available anti-HIV agents were classified into four groups: nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) [187]. However, with increasing knowledge about the molecular mechanisms underlying the HIV entry process, the inhibition of viral entry has become one of the most attractive approaches in the research for new anti-HIV molecules. Enfuvirtide (FUZEON, Roche, Switzerland; T-20) was approved for clinical use in 2003 and represented the first agent of a new class of anti-HIV drugs, the entry inhibitors [188]. Four years later, maraviroc (SELZENTRY, Pfizer, USA; MVC) received the approval by the Food and Drugs Administration (FDA), USA, and joined T-20 at this new class [189]. As of 2007, the integrase inhibitor raltegravir (ISENTRESS, Merck, USA; RAL) has also been added to the anti-HIV therapeutic arsenal [190].
Entry inhibitor molecules can be classified in four groups according to the step of viral entry that they target: inhibitors of the SU-CD4 interaction, CCR5 antagonists, CXCR4 antagonists and fusion inhibitors [118,191]. T-20 is a fusion inhibitor, while MVC is a CCR5 antagonist.

Inhibitors of gp120-CD4 interaction

Several strategies have been pursued in order to block the interaction between SU and CD4. So far none has resulted in a clinical useful anti-HIV drug. One of the earliest was the development of recombinant soluble CD4 (rsCD4) molecules, which function as molecular decoys inhibiting the ability of SU to attach to cell-associated CD4. Despite good activity *in vitro* against lab-adapted HIV-1 strains, *in vivo* the levels of rsCD4 were too low to inhibit primary isolates [192]. Another example of a molecule that mimics the CD4 receptor is the PRO-542, a tetravalent CD4-IgG2 fusion protein in which the heavy and light chains in the variable domain of IgG2 were replaced by the D1 and D2 domains of the human CD4 receptor [193,194]. Preliminary results supported the development of PRO-542 for salvage therapy of advanced HIV-1 disease [195], but only modest reductions in HIV-1 viremia were

observed in phase I and II clinical trials. No further studies are currently ongoing for this molecule [191].

BMS-488043 is a small-molecule that binds with great affinity to SU and seems to prevent the CD4-induced conformational changes in SU [196,197]. It has strong antiviral activity against HIV-1 but not against HIV-2 or SIV [197,198] and the development of this molecule stopped at phase II trials.

Ibalizumab (TNX-355) is an anti-CD4 monoclonal antibody that binds to the D2 domain of CD4 [199]. It acts as a post-attachment inhibitor such that instead of preventing SU-CD4 binding it seems to decrease the flexibility of CD4 and hinder the access of CD4-bound SU to HIV coreceptors [191]. Promising results were obtained *in vivo*, leading to significant decrease of viremia and increase of $CD4^+$ T cell counts in combination with an optimized background therapy [200]. Additional studies for this compound are being prepared [191].

CCR5 antagonists

The observation that Δ32-CCR5 mutation confers resistance to HIV-1 infection in homozygous individuals (or delayed rates of disease progression in heterozygous patients) without significant clinical impact, has encouraged different approaches of pharmacological blockade of the SU-CCR5 interaction in an effort to inhibit HIV infection [118].

CCR5 antagonists can be divided in three groups according to the size of the molecule: large molecules, such as the PRO-140, an anti-CCR5 monoclonal antibody; medium size molecules, e.g. AOP-RANTES and PSC-RANTES, derivatives of RANTES, a CCR5 natural ligand; and small-molecules, like TAK-779, MVC and vicriviroc [118,201]. PRO-140 is a strong inhibitor of HIV-1 B and non-B subtypes and is currently on phase II clinical trials [201]. Natural occurring ligands of CCR5 receptor block HIV infection but have a potential undesirable agonist activity on CCR5. RANTES derivatives have been developed in order to maintain anti-HIV activity while reducing the agonistic effects on CCR5. PSC-RANTES is now being tested as a microbiocide [118]. Small molecules block the CCR5 coreceptor, hindering the SU-CCR5 interaction, and have demonstrated potent inhibition of HIV-1 replication *in vitro* [191]. TAK-779 was one of the first compounds of this group [202]. It binds to residues lining a cavity formed by the 1, 2, 3 and 7 transmembrane helices of CCR5 ECLs [148]. It is highly selective to CCR5 and is a potent antiviral agent, but its clinical development was discontinued due to high toxicity [201,203]. Vicriviroc (SCH-D) is an orally bioavailable second-generation compound (based in a previous molecule, SCH-C) highly active against a large spectrum of HIV-1 primary isolates, that has progressed to phase III clinical trials [118,204,205]. However, vicriviroc didn't achieve the primary

efficacy endpoint of these studies and it was decided not to pursue for regulatory approval. Further development of vicriviroc was suspended.

As mentioned above, MVC is the only coreceptor antagonist approved for clinical use in HIV infection. MVC acts as a functional antagonist of CCR5. It inhibits the binding of the CCR5 natural ligands (like, MIP-1α, MIP-1β and RANTES), blocks chemokine-induced signal transducing events and once bound to CCR5 it doesn't trigger the release of intracellular calcium and fails to induce CCR5 internalization [145]. MVC interacts with residues lining a cavity formed by the by the 2, 3, 6 and 7 transmembrane helices of CCR5 ECLs [206]. MVC has potent antiviral activity (in the nanomolar range) against HIV-1 groups M and O [145] and occupies physically and functionally the coreceptor for a prolonged period, which might explain the delayed recovery of viral replication once the drug is discontinued in MVC treated patients [207,208].

MVC is administered orally twice daily and in combination with other antiretroviral agents for the treatment of HIV-1 infection in treatment-experienced patients infected with CCR5-tropic viruses. Twice daily dosing regimens include 2x150 mg (when co-administrated with potent CYP3A inhibitors, e.g. PIs, except Tripanavir/Ritonavir), 2x300 mg (with drugs that are not potent CYP3A inhibitors or CYP3A inducers, e.g. NRTIs), and 2x600 mg (with potent CYP3A inducers, e.g. efavirenz) [209]. MVC is only active against viruses using exclusively the CCR5 coreceptor [145]; therefore it is necessary to test for coreceptor usage before starting therapy [210]. The efficacy of MVC was confirmed in a pair of phase III clinical trials, MOTIVATE 1 and 2 [211,212]. Only patients infected exclusively with R5 viruses, as screened by the phenotypic Trofile assay, with viremia above 5000 copies/ml and failing previous triple-class treatment were enrolled in these studies. Significant increase of CD4+ T cell counts and reduction of plasma viral load to undetectable levels (< 50 copies/ml) were observed in the MVC arms when compared to the placebo arms of the studies. MVC has also shown equivalent activity to efavirenz in treatment-naive patients (MERIT clinical trial) [213].

Resistance to CCR5 antagonists

Escape from or resistance to CCR5 antagonists usually occur through two primary pathways: emergence of X4-tropic variants or the adaptation of viruses to use the CCR5 coreceptor in the presence of the antagonist [214].

In vivo, the most common mechanism associated with treatment failure is the expansion of pre-existing CXCR4-using variants, which were not previously identified by the tropism test at baseline nor completely suppressed by other drugs of the regimen [214]. Close to 55% of the subjects failing MVC therapy in the MOTIVATE 1 and 2 trials, presented D/M or X4

viruses [211]. Rapid shifts in the viral population towards CXCR4-using strains were also associated with high levels of resistance against vicriviroc [215]. Nevertheless, the circulating variants tend to revert to R5-tropic viruses upon MVC's therapy cessation [216], suggesting that those transitional X4 strains and D/M populations carry a fitness cost *in vivo* [217]. The emergence of CXCR4-using variants promoted by CCR5 antagonists could, at least theoretically, accelerate disease progression. However, there is not enough evidence presented to date by clinical studies that would support such concern [214,217].

Evolution to MVC resistance in R5 variants has also been documented. These viruses can entry into cells by using the antagonist-bound form of CCR5 as well as the free coreceptor (reviewed in [214,218]). This fact results in a plateau effect of the dose-response curve, since increasing drug concentrations has no impact on viral replication, and is consistent with a non-competitive mechanism of resistance. Additionally, there is evidence that altered CCR5 use may evolve during the course of HIV-1 infection such that R5 variants isolated from late-stage disease (and with lower levels of CD4+ T cell counts) have reduced susceptibility to inhibition by CCR5 natural ligands and entry inhibitors, like the CCR5 antagonists [219,220]. Similar results were obtained with RANTES in HIV-2 [221]. These observations have motivated new clinical studies designed to evaluate the potential clinical benefit of starting therapy at earlier disease stages [118].

The resistance to CCR5 antagonists is usually, but not exclusively, dependent of the accumulation of amino acid changes on the V3 region, although with no consistent pattern between patients (reviewed in [214]). In addition, V3 loop truncations leads to resistance to CCR5 antagonists in both HIV-1 and HIV-2 [222,223]. Sequence changes can directly or indirectly modify the conformation of the V3 loop; resistance seems to result from an altered use of the CCR5 coreceptor, in a way that the V3 loop crown may no longer be needed to interact with the ECL2 of CCR5 to mediate infection [214]. Noticeably, however, R5 HIV-1 isolates resistant to CCR5 antagonists can exhibit enhanced sensitivity to neutralizing antibodies [222,224], hence one can expect that there might be additional selective pressures *in vivo* (e.g. the humoral immune response) that limit the sequence changes that can be tolerated during escape from these entry inhibitors, without compromising viral fitness or replication capacity [214].

CXCR4 antagonists

While there are numerous CCR5 antagonists with different structures, the number of CXCR4 antagonists available is more reduced and their structure is similar to AMD3100, one of the first small molecules of this group to enter in clinical trials [225,226]. Despite strong activity against X4 strains *in vitro*, the clinical development of AMD3100 was discontinued

due to cardiac abnormalities [225,227]. Interestingly, however, one side effect noted was increased release of $CD34^+$ pluripotent stem cells from the bone marrow into the blood. This led to the development of AMD3100 as a hematopoietic stem cell mobilizer (plerixafor; MOZOBIL, Genzyme, USA) [118,191].

Subsequent studies resulted in the identification of AMD070, a compound orally bioavailable with similar antiviral activity to AM3100 [228]. However, this molecule is no longer being developed due to results of abnormal liver histology in preclinical studies. Toxicities of these two compounds have raised the concern of the long-term safety of targeting CXCR4, since this coreceptor seems to be important for multiple physiological processes [118,191]. Like SDF-1, the CXCR4 natural ligand, these antagonists are positively charged and of basic nature. They bind strongly to the negatively charged surface of the coreceptor, hampering its' interaction with the SU glycoprotein [229]. AMD3100 and AMD070 bind to the ECL2 of CXCR4 [201,230].

Noteworthy, the blockade of CXCR4 results in the shift from X4 to R5 phenotype in HIV-1 primary isolates [231].

Fusion inhibitors

Peptides derived from HR1 and HR2 sequences in the TM glycoprotein can inhibit HIV infection by competitive binding to their complementary regions. T-20 (former DP-178) and C34 were among the first fusion inhibitors developed in the 1990s [147,232,233], and since then peptide fusion inhibitors have been extensively exploited [149]. T-20 (36 amino acids) and C34 (34 amino acids) are linear peptides that mimic the HR2 sequence and inhibit virus entry by binding to the HR1 core, exposed at prehairpin intermediate state of TM, thereby blocking the subsequent formation of the six-helix bundle structure and viral fusion [132,233,234,235] (Figure 10). They inhibit both virus-cell fusion and cell-to-cell contact at the nanomolar range [147,232,233]. Although C34 displays stronger antiviral activity than T-20, the poor solubility of C34 under physiological conditions hindered its potential as a drug candidate [149]. The sequence of T-20 corresponds to amino acids 638-673 of the HIV-1 LAI isolate [147,232]. T-20 binds to HR1 but, in contrast to C34, it doesn't form a 6HB with the HR1 regions. The mechanism of action of T-20 seems to involve also the interaction with lipids of the target cell membrane [234,236]. T-20 inserts into the external layer of the cell plasmalemma, preferentially in fluid phase lipid membranes, and can reach local high concentrations. This way, cell membranes act as T-20 reservoirs, enabling direct contact of the peptide with its gp41 target region, thereby favouring its inhibitory activity. Noticeably, cell membranes also play a role in the mode of action of

second (e.g. T-1249) and third generation fusion inhibitor peptides (e.g. Sifuvirtide) developed latter [237,238].

As mentioned above, T-20 was the first entry inhibitor approved for clinical use, under the generic name enfuvirtide. It is indicated, in combination with other antiretroviral agents, for the treatment of HIV-1 infection in treatment-experienced patients with evidence of viral replication despite ongoing HAART [239]. The recommended dosage is 90 mg twice daily by subcutaneous injection. Phase III clinical trials (TORO 1 and 2) confirmed the efficacy of T-20 when combined with an optimized based regimen in HIV-1 infected patients failing previous treatment [240,241]. Co-administration of T-20 has even significantly improved the response rates to newer agents like MVC [191,211]. There is, however, considerable variability (up to 500 fold) in the T-20 sensitivity of HIV-1 primary isolates within subtypes B and non-B [242,243,244]. This variability can be explained by the genetic heterogeneity of Env in the HR1/HR2 regions of TM and also in the V3 region of SU [244]. In fact, it has been suggested that the genetic variability of HR1/HR2 regions between HIV-1 and HIV-2, especially between the 36-45 codons of HR1, is responsible for the lack of activity of T-20 observed on HIV-2 [245,246].

Second generation peptides were developed based on the consensus sequences of HIV-1, HIV-2 and SIV strains. In addition, these new peptides were designed to include different functional domains of HR2 region: the pocket-binding domain (PBD), the HR core (3HR) and the lipid-binding domain (LBD) [235] (Figure 10). T-1249 is a representative second generation peptide with 39 amino acids that contains the three regions (PBD-3HR-LBD) and has potent activity against both HIV-1 and HIV-2 [247,248]. The stronger antiviral activity of T-1249 over T-20 and C34 is attributed to the inclusion of the multiple functional domains, since C34 only contains the PBD-3HR segment and T-20 the 3HR-LBD [234]. It is even active against most T-20- resistant variants [248,249]. However, the disadvantage of longer peptides like T-1249 might be the production cost and reduced delivery efficiency [235]. In fact, the clinical development of T-1249 was halted after phase I/II clinical trials apparently due to formulation issues [250].

The main limitations of peptide fusion inhibitors are their high production cost and poor bioavailability. T-20 has a short half-life because it is easily degraded by proteolytic enzymes in the blood. Consequently, it must be administered subcutaneously twice a day, causing adverse reactions at the site of injection in the majority of the patients [239]. Additionally, being therapeutic proteins, one can speculate that anti-drug antibodies might develop during peptide therapy and reduce its clinical efficacy [251,252]. Though, the antigenicity of T-20 has not been characterized to date.

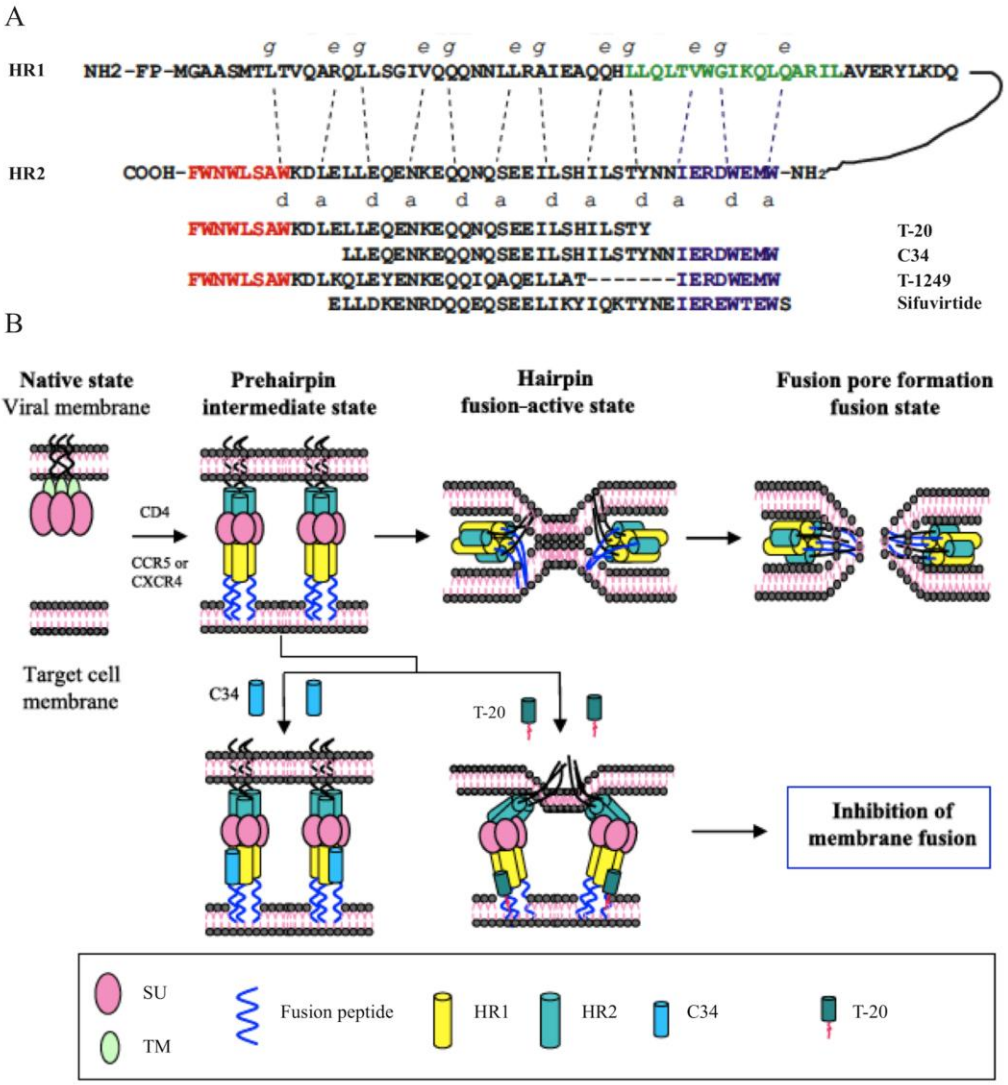

Figure 10. Schematic representation of the functional domains in HR1 and HR2 regions of the TM glycoprotein and the target sites of HIV fusion inhibitors. (**A**) The HR2 region contains tree functional domains: 1, pocket-binding domain (PBD, in purple); 2, HR core in the center (3HR, black); 3, lipid-binding domain (LBD, in red). The HR1 region contains one 3HR and one pocket-forming domain (PFD, in green). Interaction between the 3HRs results in the hairpin structure, which is stabilised by the connection of the PDB and the PFD. Dashed lines represent the interactions between the residues located at *e* and *g* positions in HR1 and the *a* and *d* positions in HR2, respectively. Sequences of selected peptide fusion inhibitors, and their corresponding functional domains, are presented. (**B**) Model of the envelope glycoprotein-mediated membrane fusion and the proposed mechanism of action for T-20 and C34 antiviral activity. (Adapted from (**A**) Pan C, Liu S, Jiang S (2010) HIV-1 gp41 fusion intermediate: a target for HIV therapeutics. J Formos Med Assoc 109: 94-105.; (**B**) Liu S, Jing W, Cheung B, Lu H, Sun J, et al. (2007) HIV gp41 C-terminal heptad repeat contains multifunctional domains. Relation to mechanisms of action of anti-HIV peptides. J Biol Chem 282: 9612-9620.)

Rational design strategies are being used to improve the stability of the peptide helix, leading to the development of the third generation of fusion inhibitors. Peptides with increased α-helix content and reduced random coiled conformation are less susceptible to proteolytic degradation in the serum and have higher binding affinity to its target [149,235]. This stabilization can be achieved by the introduction of charged residues to create salt-bridges between the turns of the helix. Sifuvirtide, a peptide based in C34, is a good example of the third generation fusion inhibitors [253]. It has 93% of α-helical content, while T-20 and T-1249 only have 12-20% and 49%, respectively [149,235,253,254]. Sifuvirtide is now under development and showed promising results in phase II clinical studies being active against a broad range of HIV-1 isolates, including T-20- resistant strains. Although Sifuvirtide shows a better pharmacokinetic profile than T-20, it is still administered as a subcutaneous injection [253]. To our knowledge, Sifuvirtide hasn't been tested on HIV-2, but since its sequence is solely based on HIV-1, it is highly likely that it won't be active against this virus [245].

Alternative approaches have also resulted in the development of short peptides composed of D-amino acid residues (D-peptides). They bind to the hydrophobic pocket presented on HR1 trimers and are resistant to proteases, thereby having the potential for oral bioavailability. The clinical efficacy of these peptides is yet to verify, since they are still under the early stages of development [118,250].

Research for HR1 based peptides that target the HR2 region has been much more limited. Generally, monomeric HR1 peptides such as N36 and N46 are highly hydrophobic, have tendency to aggregate under physiologic conditions and are less active than HR2 based peptides. As an alternative, chimera peptides were designed by fusing N-peptides with a highly trimerized model peptide (e.g. IQN17, N28Fd). These chimeras form stable uniform trimers under physiological conditions and show enhanced antiviral activity. However, due to their large size, these chimeras are more expensive and difficult to produce than HR2-based peptides [149,235,255].

Another example is targeting the fusion peptide instead of the heptad repeats: VIRIP is a natural peptide corresponding to the C-proximal region of α1-antitrypsin and inhibits a wide variety of HIV-1 strains [256]. It hasn't reached clinical stage development yet [250].

Despite recent advances in peptide modification to improve potency and stability, research efforts have also focused on the development of small molecule fusion inhibitors. They should have better bioavailability and lower costs of production. One example of such a compound is ADS-J1, which prevents membrane fusion by binding to the hydrophobic pocket of gp41, thereby hampering six-helix bundle formation. It inhibits HIV-1 infection at

the low μM range. None of these molecules have progressed to clinical studies yet [149,235,250].

Resistance to fusion inhibitors

Since T-20 is the only fusion inhibitor approved for clinical use, most of the resistance data regarding fusion inhibitors is available only for this drug. The majority of the mutations associated with T-20 resistance are found within the 36-45 positions of HR1 region (Table 1), and especially in the GIV motif (codons 36-38) [235,257].

Table 1. Mutations associated to T-20 resistance in the HR1 region of gp41 *

gp41 position	36	37	38	39	40	41	42	43	44	45
HIV-1 HXB2	G	I	V	Q	Q	Q	N	N	L	L
Mutations[1,2]	**D**/**E**/V/S	V/T	**E**/**A**/M/G	G	**H**		T/Q/H	**D**/**K**/S/Q	M	M

[1]Mutations in bold represent the most common high-level resistance mutations.

[2]Other less frequently observed mutations include Q32/H/R, L33S/T, Q56K/R and L54M.

*(Adapted from Eggink D, Berkhout B, Sanders RW (2010) Inhibition of HIV-1 by fusion inhibitors. Curr Pharm Des 16: 3716-3728.)

T-20 has a low genetic barrier to resistance, since a significant decrease in viral susceptibility (range, 2- to 1100-fold) can be caused by a single mutation. Double mutations frequently increase the level of resistance expected from the effects of individual substitutions [235,258]. Mutations in the HR2 region also contribute to T-20 resistance, and seem to represent secondary or compensatory mutations [259]. These substitutions coexist with the mutations of HR1 and increase the affinity of HR2 for the mutated HR1, thereby favouring HR1-HR2 association over HR1- T-20 binding [235]. T-20-resistant variants display lower biological fitness than original isolates [260,261] and seem to be more susceptible to antibody neutralization [260].

Additional mutations associated with T-20 resistance can also occur in the V3 loop of the SU glycoprotein [242]. In fact, coreceptor specificities can modulate HIV-1 susceptibility to T-20 and other fusion inhibitors [242,262,263]. In the TORO clinical trials, the sensitivity of D/M viral populations to T-20 was higher than CCR5- or CXCR4-using viruses [264]. Some authors suggested a kinetic model in which the susceptibility to T-20 is directly proportional to the length of time during which HR1 is exposed and accessible to T-20 and inversely proportional to coreceptor binding affinity and coreceptor density (reviewed in [265]).

It has been found that natural polymorphisms in T-20- resistant positions are more frequently observed in HIV-1 non-B subtypes than on subtype B viruses [244,266]. Some of these mutations are even related to specific subtypes. One of the most common

polymorphisms in these studies is N42S (>60% in non-B subtypes), which is associated with enhanced susceptibility to T-20 [267], and is frequently found subtypes A, G and C, but not in F. Other examples include Q56R/K in subtype A and L54M in the CRF14_BG recombinant form. Nonetheless, whether T-20 susceptibility is dependent on HIV-1 subtype is still not entirely clear, since T-20 has only been tested against very few highly divergent non-B isolates [244,245].

The majority of mutations associated to second and third generation fusion inhibitors' resistance are also mapped to the HR1 regions [268,269,270]. Although some of these mutations are cross-resistant to T-20, they often require different substitutions at the same positions. For instance, at position 38 only charged amino acids (E/R/K) cause resistance to T-1249; this particular finding suggests that T-20 and T-1249 might exhibit similar inhibition modes that trigger comparable but not identical escape routes [269].

HIV EVOLUTION

Evolutionary biology is a fast-evolving field that has been fuelled over the last couple of decades by escalating amounts of sequence data. Along with the increasing availability of such data, numerous methodologies have been developed to describe and quantify the processes underlying the genealogical relationships among a set of genes or organisms (phylogenetic analyses) [271,272].

RNA viruses, which are responsible for many emerging diseases, have become an important area of study in this field. Accordingly, there is more genomic sequence data for HIV than for any other virus and this has supported our current knowledge of HIV origin, evolution and molecular epidemiology. The recent development of high throughput sequencing methods promises even greater opportunities to study HIV biology and infection, as genome sequencing of single DNA/RNA molecules is now much faster [271,273].

Basic principles of evolutionary biology and phylogenetic analysis will be briefly introduced in the following sections, and the focus will be on the methodologies applied in Chapters 2 and 4.

Mechanisms of viral evolution

RNA viruses share common characteristics that make them particularly good models to study evolution: high mutation rates, small genomes, large population sizes and short generation times with high number of offspring. All these factors contribute for the exceptionally high mutation rate observed in HIV-1, 2.4×10^{-5} mutation/replication [274]. It

is worth mentioning that the rate of nucleotide substitutions in the *env* gene is higher in HIV-2 ($10.2x10^{-3}$ nucleotides/year) than in HIV-1 ($6.4x10^{-3}$ nucleotide/year) [275]. These mutations include substitutions, duplications, deletions and insertions. Reverse transcription is the process that contributes the most for the introduction of point mutations, due to the lack of 3'-5' exonuclease activity of the viral RT enzyme. Another potential error-prone step is the synthesis of viral genomic RNA (from proviral DNA) by the host RNA polymerase II, which also lacks proof reading activity [273,276]. Hypermutation, with high-density accumulation of specific nucleotide substitutions, can also contribute to the mutation spectrum; in particular, the G-to-A hypermutation is frequently induced by the host APOBEC3 family cytidine deaminases [277]. Polymorphisms are generated when these mutations are passed to the offspring and coexist with the original form of the gene. At a polymorphic site, two or more variants of a gene circulate in the population simultaneously [278]. In HIV, the viral population infecting a single individual is composed of a group of variants often referred to as quasispecies.

Together with the elevated mutation rates, recombination between different viral variants is equally responsible for the abundant genetic variability of HIV. In fact, between $8.3x10^{-4}$ and $1x10^{-5}$ recombinations per site can occur during each replication cycle in HIV-1 [279,280], exceeding the estimated genomic mutation rate. Recombination occurs when a cell is infected by two different strains and leads to the production of virions that pack an RNA molecule from each strain (heterodimeric DNA). Once these virions infect a new cell, reverse transcription will produce a mosaic genome by exchange of genes or gene fragments during the minus DNA strand synthesis, due to template switching of the viral RT between the two RNA strands [272,276].

Although events like mutation and recombination are significant sources of genetic variation, there are other evolutionary forces that affect the frequency of the mutant in the population, namely natural selection and genetic drift [281]. Whenever a new mutation is generated, it either becomes fixed or is eliminated from the population depending on (1) the degree to which the mutation increases or decreases (respectively) a virus ability to survive/reproduce in the current environment (fitness) and on (2) the size of the actual population. One mutation is beneficial or advantageous if it increases virus fitness relative to wild-type, deleterious if it decreases relative fitness or neutral if it has no significant fitness effect (as measured by the selection coefficient). The process of natural selection will favour the fixation of beneficial mutations by increasing their frequency in the population (positive selection) and promote the elimination of deleterious mutations by reducing their incidence (negative selection). Selection does not affect the frequency of neutral mutations. In HIV infection, the importance of natural selection as

driver of molecular evolution is typically illustrated by the strong selective pressure imposed by neutralizing antibody responses over the envelope gene [282,283,284,285].
In contrast to the highly deterministic evolutionary pattern of natural selection, random genetic drift is a stochastic process in which mutation frequencies fluctuate randomly through time, with no tendency towards increase or decrease, until the mutations either become fixed or eliminated. Mutation frequencies are affected by natural selection and genetic drift at the same time, but the rate at which a mutation becomes fixed through deterministic or stochastic forces depends on the distribution of the selective coefficients and on the effective population size (size of an idealized population which would have the same capacity for genetic variation as the population being studied). The smaller the effective population size, the larger the effect of chance events and, as such, more important is the role of genetic drift in determining the frequency and fate of mutations. If a mutation under negative selection is not entirely deleterious it can also become fixed due to random genetic drift, although this requires more generations than expected for a neutral mutation [273,278,281].

An introduction to phylogeny reconstruction

Multiple alignment of sequences

In order to study the relationship between a group of genes or gene fragments and infer about their evolutionary history, phylogenetic methods consider the similarity between those genes, assuming that they are homologous (share a recent common ancestor). Therefore, these sequences (nucleotides or amino acids) need to be aligned appropriately, so that each of their homologous sites can be compared in the same alignment column (positional homology). This means that sequences are arranged in rows in a way that similar nucleotides or amino acids are placed above each other at the same vertical position. Gaps are inserted in positions where there are insertions and/or deletions, in order to optimize the alignment [272,278]. The alignment procedure can be performed automatically with a number of multiple alignment algorithms incorporated in several software and web-based tools, like ClustalX [286]. It is critical for phylogenetic inference that sequences are unambiguously aligned. Therefore it is good practice to visual inspect the alignment produced, especially for highly variable sequences, and if necessary do some manual editing, like correction of obvious alignment errors, deletion of ambiguously aligned segments and removal of overly gapped columns. In the presence of nucleotide sequences coding for proteins it is often useful to generate the nucleotide alignment based

on a previous codon/amino acid alignment in order to maintain the reading frame. Doing otherwise would invalidate further codon-based analysis [272,287].

Accounting for homoplasy and recombination events

Reliable estimates of the phylogenetic relationship between genes are only possible if they share a common evolutionary history. However, some events like recombination and homoplasy might affect phylogenetic inference and should be carefully considered when performing this type of analysis. Homoplasy is defined by the sharing of identical character states that cannot be explained by inheritance from the common ancestor of a group of taxa. One example is the independent evolution of a similar feature in separate lineages, starting from either a different ancestral state (convergent evolution) or from a similar ancestral state (parallel evolution). This can result in two sequences that have higher similarity than it would be expected by chance but that are not homologous (evolutionarily related) to each other [272,288].

A recombination event also violates the homology assumption since it allows sites to move freely between different genetic backgrounds, swapping the evolutionary histories within the gene under study. This can have a profound effect on the models of evolution and phylogenetic tree inferred from the alignment (see below) and on several features of molecular evolution, like detection of selection pressures [289]. Numerous approaches have been developed to identify the molecular footprint of recombination. One way to handle with the recombination is to identify potential recombination breakpoints within the dataset, quantify the level of support for their locations, and finally identify sequences or clades involved in putative recombination events. This is methodology is implemented in the Simple Breakpoint Recombination (SBP) and Genetic Algorithm Recombination Detection (GARD) methods [290]. Hence, the alignment can then be split into non-recombinant sequence fragments, which are allowed to evolve independently from each other and according to their own phylogenetic tree [289].

Models of evolution

The evolutionary relationship among genes and organisms are best illustrated in typically structured diagrams called phylogenetic trees. These trees are generally reconstructed under a specified model of sequence evolution (or model of substitution). Instead of making a direct (observational) comparison between sequences to calculate their evolutionary divergence or dissimilarity (genetic distance), models of evolution produce more realistic estimates of genetic distance using a statistical approach to describe the

stochastic (or probabilistic) process of substitution in nucleotide or amino acid sequences; importantly, they can account for multiple substitutions at the same site (multiple hits, like, A → G → C), including reverse mutations (A → G → C → A) [272,291]. In the case of nucleotide sequence, these models make basic assumptions about the base composition, rate, and frequency of base substitutions among different sites and the nature of these substitutions. The process of nucleotide substitution can be generalized into a Markov process, which uses a Q matrix that specifies the relative rates of change of each nucleotide along the sequence in a total of eight free parameters.

$$Q = \begin{array}{c} \begin{matrix} \quad\mathbf{A} & \quad\mathbf{C} & \quad\mathbf{G} & \quad\mathbf{T} \end{matrix} \\ \begin{pmatrix} -\mu(a\pi_C + b\pi_G + c\pi_T) & a\mu\pi_C & b\mu\pi_G & c\mu\pi_T \\ g\mu\pi_A & -\mu(g\pi_A + d\pi_G + e\pi_T) & d\mu\pi_G & e\mu\pi_T \\ h\mu\pi_A & i\mu\pi_C & -\mu(h\pi_A + j\pi_C + f\pi_T) & f\mu\pi_T \\ j\mu\pi_A & k\mu\pi_C & l\mu\pi_G & -\mu(i\pi_A + k\pi_C + l\pi_G) \end{pmatrix} \end{array}$$

Figure 11. Instantaneous rate matrix *Q*. Each entry represents the instantaneous substitution rate from nucleotide i to j; μ, mean instantaneous substitution rate; a - l, relative rate parameters describing the relative rate of each nucleotide substitution to any other; π_A - π_T, frequency parameters corresponding to the nucleotide frequencies; the sum of each row is equal to zero. (From Strimmer K, von Haeseler A (2009) Genetic distances and nucleotide substitution models. In: Lemey P, Salemi M, Vandamme AM, editors. The Phylogenetic Handbook: A Practical Approach to Phylogenetic Analysis and Hypothesis Testing. Cambridge: Cambridge University Press. pp. 111-125.)

The simplest model of evolution, the Jukes-Cantor model, specifies that the equilibrium frequencies of all four bases are 25% each and that all substitutions are equally likely [292]. Assuming, for instance, unequal transition and transversion substitution frequencies, will increase the complexity of the models. If all parameters are specified, then the General Time Reversible (GTR) model is derived [293,294]. Besides the parameters of the Q matrix, one must take into consideration the heterogeneous rate of nucleotide substitutions for different positions in a sequence. For instance, in protein coding genes the third codon positions mutate usually much faster than the first or second positions because, in general, it doesn't lead to amino acid change (silent substitution). A common approach is to use a gamma distribution (+G) to model this heterogeneity and/or include class of invariant sites (+I) [272,291,295].

These Markov nucleotide models are nested models. This means that by starting with the most-complex model (GTR+G+I), one can derive all other models by restricting the possible values of one or more parameters. So, in order to choose the best-fit model of evolution for a particular dataset, nested models can be compared using statistical approaches like

the hierarchical likelihood ratio test (hLRT) or the Akaike's information criterion (AIC); both procedures for model comparison are implemented in the software jModeltest [296].

A measure of the genetic diversity in the alignment can be obtained by averaging all genetic distances between the sequences, as estimated by the model of evolution. An alternative and much simpler approach to access the diversity (of nucleotides or amino acids) is to calculate the Shannon Entropy, which is a quantitative measure of uncertainty in a dataset [297]. It should be noted, however, that phylogenetic history of the sequences is not considered in this procedure. Instead, Shannon entropy analyses the variability of each column of the alignment independently, by assigning a score that incorporates both the observed number of different nucleotides (or amino acids) and their frequencies (http://www.hiv.lanl.gov). It is, thus, possible to study the variability along the alignment, or to have global measure of the diversity by averaging the scores from all the positions.

Phylogenetic tree reconstruction

A phylogenetic tree consists of external nodes and internal nodes connected by branches. External nodes (terminal nodes or tips) represent the taxa under study (organisms or individuals belonging to the same or different species). A group of taxa that share the same branch is a "cluster" or "clade", and have a monophyletic origin. An internal node is the most recent common ancestors (MRCA) of all the branches and tips arising from that node. The branching-pattern, or the order of the nodes, defines the topology of the tree, and the length of the branches can be proportional to genetic distances or to a time unit [278].

Phylogenetic trees are inferred by two main approaches: distance methods or character-state (discrete) methods. Distance based methods are usually more rapid and compute pairwise genetic distances between all sequences into a distance matrix. These methods can either use an optimality criterion to search for different tree topologies (e.g. Fitch-Margoliash) or stepwise clustering algorithms to construct one "best" tree (ex. UPGMA and neighbor-joining). In character-state methods, each position in the alignment is a "character" and the nucleotide or amino acid at that position is a "state". Under this method, each alignment column is analysed independently and an optimality search criterion is used to evaluate different tree topologies. One advantage of discrete character methods is that they retain the original character status of the taxa, making it possible to reconstruct the character state of the internal (ancestral) nodes [278].

One of the most commonly used character-state methods is Maximum Likelihood (ML). For a given number of taxa, ML examines different tree topologies under an optimal search criterion to find the tree that maximizes the probability of observing the data (character states), given a tree topology and a specified model of evolution. Hence, the likelihood of a particular tree giving rise to the dataset is calculated for all trees, and the one with the highest (maximum) likelihood score is chosen [272,278]. Since the exhaustive search of all possible trees is usually too computational intensive, several heuristic search strategies are used to explore the "tree space" for only a subset of trees. Examples of these strategies are the nearest-neighbor interchange (NNI), subtree pruning and re-grafting (SPR) and tree bisection and reconnection (TBR). However, there is no guarantee that the best tree for the data is found using the heuristic approaches [298]. Additional character-state methods include Maximum Parsimony (MP) and Bayesian methods, the latter being increasingly popular in evolutionary biology and phylogenetic analysis.

The bootstrap method is a statistical technique frequently used to access the robustness (confidence) of the phylogeny inferred by ML [299]. This procedure involves repeated sampling of alignment columns (at random and with replacement) from the original alignment until a new alignment of the same length is produced (replica). The bootstrap is repeated multiple times, producing in general 1000 replicates (user-defined), and for each replicate a new tree is constructed. Ate the end, the bootstrap values can be annotated in the original final tree or in a majority-rule consensus tree of all the replicates; they indicate the level of confidence of each internal node in the tree and represent the percentage of replicates that show the same clade under that node in the final phylogeny. A minimum bootstrap value of 70% is usually considered necessary for a significant support of a given clade in the inferred phylogeny [272,299].

Estimating selection pressure

As discussed above, the fixation of point mutations can be determined by the effect of natural selection has on the viral population. Due to the degeneracy of the genetic code, nucleotide substitutions in protein-coding regions can be classified as synonymous and non-synonymous. While non-synonymous substitutions alter the encoded amino acid, synonymous (or silent) substitutions leave the amino acid unchanged. One of the methods to study natural selection is to compare the rate of non-synonymous substitutions (dN) to the rate of synonymous substitutions (dS) by determining the dN/dS ratio (ω). If the ratio is significantly inferior to one (dN/dS<1), the rate of non-synonymous substitutions is slower, indicating that these mutations are deleterious; hence, the coding region is under negative (or purifying) selection, and structural/functional motifs at the protein level are

more conserved. If the ratio is significantly superior to one (dN/dS>1), there is a faster fixation of non-synonymous substitutions, indicative of adaptive protein evolution through a positive (or diversifying) selection. If dN and dS are equal (dN/dS=1), mutations have no effect on viral fitness (there is no influence of natural selection) and evolution is neutral. Currently, ω is estimated within a ML framework by fitting a codon model of evolution that can additionally be crossed with a nucleotide substitution model [281,300].

By estimating the global dN/dS ratio, one is studying the selection pressure averaged over all codon positions (entire sequence) of the alignment. However, one could expect to detect site-by-site and branch-by-branch variation in these rates, meaning that positive selection has only occurred in certain specific codons (sites) of the gene or in different lineages across the phylogenetic tree [272,281,289]. There are two different approaches to estimate site-specific rates: (1) rates are estimated directly from each site independently, using for instance the single-likelihood ancestor counting (SLAC) and fixed effects likelihood (FEL) methods, or (2) by using a distribution of rate classes and assigning each site to a rate class, using the random effects likelihood (REL) models. Distributions of rate classes are also useful to compare selection pressure in different genes or datasets. All these methods have their strengths and weaknesses. While counting methods like SLAC are very efficient and quick, particularly in large datasets, FEL and REL methods are much more sensitive but far more time-consuming. Hence, the best approach would be to apply several methods and compare the results [301]. Site-by-site variation of dS can bias estimations of codon's diversifying selection. Therefore it is also good practice to test for this in the dataset [302]. All the tree methods, described above, model for this variation.

On another perspective, by using models that allow substitution rates to vary across the tree branches, it is possible to compare non-synonymous substitutions between terminal and internal branches. This could be of interest on a population study, to verify if selection on a virus population within a host is distinct from selection among hosts. In such a case, an internal fixed effects likelihood method (IFEL) can be applied to identify positively selected sites along the internal branches and compare the results with the ones detected by SLAC/FEL/REL on the tips of the tree [289,303]

Ancestral state reconstruction

Current knowledge on molecular synthesis allows the production of proteins and peptides designed by computational methods and directly access the properties/functions of such molecules in the laboratory. In the virology field, these methods have been applied in the rational design of new vaccine immunogens for HIV, Influenza virus and Epstein Barr Virus [304,305,306,307,308]. Of the several approaches taken, ancestral state reconstruction has

attracted some attention in variable viruses such has HIV [304,307,308] due to the potential to capture more conserved features of viral immunogens than any contemporary natural strain [309].

Methods for ancestral state reconstruction include consensus, ancestral sequences and center-of-the-tree (COT) approximations [309]. Consensus is a sequence that has at each position the most frequent nucleotide or amino acid residue across an alignment of homologous sequences. Ancestral sequences are computationally derived and involve the reconstruction of ancestral states in the internal nodes of a phylogenetic tree; in this sense, they represent the MRCA [304]. Finally, the COT approach is also a computational method that derives a sequence from a point called center-of-the-tree (on an unrooted phylogeny) that minimizes the average evolutionary distance to all sequences in the dataset [310]. In fact, all of these approaches attempt to generate a "centralized" sequence that minimizes the amount of sequence divergence between contemporary strains. The major advantage of ancestral and COT sequences is that they are reconstructed by tracing the most likely evolutionary path along a phylogeny, conserving any site co-variation that has arisen as a consequence of evolutionary history (such co-variation might be biologically relevant) [309]. In addition, they are less likely to change as new sequences are added to the sample. Nonetheless, these methods are all subject to different biases, and in optimized conditions the derived (artificial) sequences might even be relatively similar to each other. In fact, the biological function of HIV centralized sequences is yet to be successfully demonstrated *in vivo* [306,308,311].

The first methods developed to derive ancestral sequences were based on a maximum parsimony (MP) criteria [309]. MP is a character-state method that assigns characters to internal nodes of the tree in order to minimize the number of character-state changes required [312]. It performs well when sequence divergence is low [313], but as it doesn't use an explicit model of evolution it can't take into account biased substitution patterns (ex. multiple substitutions in a single site) and cannot distinguish between equally parsimonious reconstructions [310]. Since then, new methods of ancestral state reconstruction have been developed. One possible approach is based on ML. In contrast to MP, ML relies on an explicit model of evolution, uses branch length information, and assigns a probability to each alternative character. This means that sites that have ambiguous ancestral state assignments under MP can be explored in the likelihood approach by making use of specific probabilities associated with particular ancestral reconstructions. Selection of a best-fit model of evolution and a well supported phylogeny are critical elements for ancestral state reconstruction under ML [314].

To my knowledge, reconstruction of ancestral sequences has never been applied on the design of new antiviral agents.

Homology modelling

Knowledge of the three-dimensional structure of a protein can often provide invaluable information for biomedical research. Rational drug design and the study of the biological role of protein molecules in cellular processes illustrate the interest on such data [315,316,317,318]. Currently, there are millions of sequenced proteins but only a small fraction of structures have been experimentally solved [315,316]. Protein structure determination using experimental methods (X-ray crystallography or NMR spectroscopy) is time consuming and not successful with all proteins, especially membrane proteins [317]. Hence, computational modelling methods have been developed to predict protein structure.

Homology modelling builds the structure of a protein by aligning its amino acid sequence to the homologous sequence of a protein with known structure [318]. The rational behind this methodology is that an amino acid sequence carries all the information needed to guide protein folding and that the evolutionary relationship between protein structures is more conserved than between sequences [316]. The most common and accurate approach is made through template-based modelling and this typically involves four steps: (1) identification of an homologous sequence with a known structure that can be used as template, (2) alignment of the query sequence to the template, (3) construction of a structural model for the query sequence based on the alignment, and (4) evaluation of the model [315,317,318]. Although the applicability of this method is limited to the cases where is possible to find an appropriate template, more than half of all known sequences have at least one domain that is related to at least one protein of known structure [317]. Moreover, only a limited number of folds are tolerable in nature [316]. For a certain level of similarity between the query and the template (35-40% or greater), homology modelling can accurately generate high-resolution structural models comparable to the ones solved experimentally and with a level of detail suitable for practical applications in medicine and biotechnology [315,316].

AIMS AND WORK PLAN

The etiologic agents of AIDS, HIV-1 and HIV-2, are two distinct human lentiviruses with similar structural and genomic organization but sharing only 50% of genetic similarity. In HIV-1 infection, escape from antibody neutralization is the major driving force of the molecular evolution of the envelope glycoproteins, particularly around the V3 loop. V3 is a region well exposed on the surface of the viral envelope and a major determinant of viral tropism and coreceptor usage. In HIV-2, however, the structure of the envelope complex remains to be determined and the impact of the neutralizing antibody response in the evolution of the envelope is still unknown.
Entry inhibitors are a recent class of antiretroviral drugs specifically designed to prevent HIV-1 viral entry. There is evidence that modified V3 loop conformations result in different levels of susceptibility and resistance to entry inhibitors in HIV-1, although such structural changes might be constrained by increased sensitivity to neutralizing antibodies. The activity of entry inhibitors on primary HIV-2 isolates has not been tested. Moreover, while the development of new fusion inhibitors and other entry inhibitors for HIV-1 is currently a very active field of research no similar efforts are being pursuit to develop peptides or other molecules that act on HIV-2.

The aims of this thesis were: 1) to examine the C2, V3 and C3 envelope regions of HIV-1 and HIV-2 at the molecular, evolutionary and structural levels; 2) to compare HIV-1 and HIV-2 susceptibility to entry inhibitors and assess their potential value in HIV-2 therapy; and 3) to produce a new fusion inhibitor peptide using evolutionary biology based strategies.
In the first study (Chapter 2), HIV-1 and HIV-2 were compared at the molecular, evolutionary and structural levels in the C2, V3 and C3 envelope regions. Next we determined the *in vitro* baseline susceptibility of HIV-1 and HIV-2 primary isolates to fusion inhibitors (T-20 and T-1249) and coreceptor antagonists (AMD3100, TAK-779 and MVC) and related this susceptibility with the time of virus isolation as well as with the genetic and phenotypic characteristics of the isolates (Chapter 3). Finally, we evaluated the antiviral activity of a newly designed ancestral peptide on both HIV-1 and HIV-2 (Chapter 4).

In Chapter 2 we amplified and sequenced the C2, V3 and C3 regions of 60 HIV-1 and 49 HIV-2 samples collected from Portuguese HIV infected patients. As controls to this study we used alignments of HIV-1 and HIV-2 worldwide reference sequences. The sequence variability of each dataset was estimated by the following parameters: inter-patient genetic diversity, amino acidic diversity along the alignment and the number of potential

N-linked glycosylation sites. Selective pressures over the HIV-1 and HIV-2 C2, V3 and C3 regions were examined under different approaches. We were particularly interested in (1) identifying specific codons under selection using site-by-site analysis, (2) understanding if selection pressure within a host is different from selection for transmission among hosts, and (3) comparing the distribution and strength of diversifying selection between the HIV-1 and HIV-2. Structural models of HIV-1 and HIV-2 C2, V3 and C3 regions were produced by homology modelling, using the known structure of HIV-1 gp120 and SIV gp120, respectively, as templates. These models were then used for the calculation of solvent accessible surfaces of these regions.

The baseline susceptibility of HIV-2 to entry inhibitors was evaluated on a panel of 20 HIV-2 isolates, including 19 primary isolates, and compared to that of nine HIV-1 viruses (7 highly divergent primary isolates) (Chapter 3). After virus isolation and genotyping by phylogenetic analysis, CCR5 and CXCR4 tropism was determined using a single-round viral infectivity assay performed with TZM-bl reporter cells ($CD4^+$, $CCR5^+$ and $CXCR4^+$) in the presence or absence of CCR5 and CXCR4 antagonists. Representative dose-response curves for HIV-1 and HIV-2 were obtained using increasing concentrations of fusion inhibitors (T-20 and T-1249) and coreceptor antagonists (AMD3100, TAK-770 and MVC) in TZM-bl reporter cell assays. 50% (IC_{50}) and 90% (IC_{90}) inhibitory concentrations and curve slopes were determined for each drug. We have also investigated the potential association between the time of virus isolation during disease progression and susceptibility to entry inhibitors.

In Chapter 4 we performed the ancestral reconstruction of HIV-2 and SIV gp36 sequences, in order to derive the amino acid sequence of new fusion inhibitor peptides. The antiviral activity of one selected peptide was evaluated *in vitro* against HIV-1 and HIV-2 primary isolates, including HIV-1 variants resistant to T-20. Possible sites of action in HIV-1 and HIV-2 were explored through *in vitro* selection of resistant variants and binding to its predicted target, the ectodomain of the HIV transmembrane glycoprotein. Circular dichroism spectroscopy was used to determine the secondary structure of the peptide. The potential *in vitro* cytotoxicity of the peptide was assessed in primary cells and tissue culture cells. Finally, we measured the antigenic reactivity of the peptide in HIV-infected patients.

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II

Evolution in HIV-1/HIV-2 Env

Evolutionary and structural features of the C2, V3 and C3 envelope regions underlying the differences in HIV-1 and HIV-2 biology and infection

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Research article

PLoS One. 2011 Jan;6(1):e14548.

ABSTRACT

Background: Unlike in HIV-1 infection, the majority of HIV-2 patients produce broadly reactive neutralizing antibodies, control viral replication and survive as elite controllers. The identification of the molecular, structural and evolutionary footprints underlying these very distinct immunological and clinical outcomes may lead to the development of new strategies for the prevention and treatment of HIV infection.

Methodology/Principal Findings: We performed a side-by-side molecular, evolutionary and structural comparison of the C2, V3 and C3 envelope regions from HIV-1 and HIV-2. These regions contain major antigenic targets and are important for receptor binding. In HIV-2 these regions also have immune modulatory properties. We found that these regions are significantly more variable in HIV-1 than in HIV-2. Within each virus, C3 is the most entropic region followed by either C2 (HIV-2) or V3 (HIV-1). The C3 region is well exposed in the HIV-2 envelope and is under strong diversifying selection suggesting that, like in HIV-1, it may harbour neutralizing epitopes. Notably, however, extreme diversification of C2 and C3 seems to be deleterious for HIV-2 and prevent its transmission. Computer modelling simulations showed that in HIV-2 the V3 loop is much less exposed than C2 and C3 and has a retractile conformation due to a physical interaction with both C2 and C3. The concealed and conserved nature of V3 in the HIV-2 is consistent with its lack of immunodominancy *in vivo* and with its role in preventing immune activation. In contrast, HIV-1 had an extended and accessible V3 loop that is consistent with its immunodominant and neutralizing nature.

Conclusions/Significance: We identify significant structural and functional constrains to the diversification and evolution of C2, V3 and C3 in the HIV-2 envelope but not in HIV-1. These studies highlight fundamental differences in the biology and infection of HIV-1 and HIV-2 and in their mode of interaction with the human immune system and may inform new vaccine and therapeutic interventions against these viruses.

INTRODUCTION

Human Immunodeficiency Virus type 1 (HIV-1) infection affects more than 40 million individuals throughout the world. It is caused mainly by isolates belonging to group M. Within this group there are nine different subtypes named A to H, six sub-subtypes (F1, F2, A1-A4) and at least thirty six recombinant forms named CRF01 up to CRF36 [1]. In contrast to the HIV-1 pandemic, HIV-2 is only prevalent in West Africa where it seems to have been present since the 1940s [2]. In Europe infection with HIV-2 remains rare (2-3% of all AIDS cases), being observed mainly in France and Portugal [3,4,5]. Eight different HIV-2 groups named A through H have been reported but only groups A and B cause human epidemics

[6,7,8,9]. Isolates from group A are, however, responsible for the vast majority of HIV-2 infections worldwide [10].

For reasons that are still not clear, HIV-1 and HIV-2 infections lead to very different immunological and clinical outcomes. In contrast to HIV-1 infected patients, the majority of HIV-2-infected individuals have reduced general immune activation, normal CD4+ T cell counts, low or absent viremia and absence of clinical disease [11,12,13,14]. This may be related with a more effective immune response produced against HIV-2. In fact, most HIV-2 infected individuals have strong cytotoxic responses to Env and Gag proteins and raise autologous and heterologous neutralizing antibodies [3,15,16,17,18]. The attenuated course of HIV-2 infection compared to HIV-1 has also been associated to a lower state of immune activation, which may be related to the immunosuppressive activity of the C2, V3 and C3 envelope regions [19,20,21]. Similar immunosuppressive activity has not been found in the homologous C2, V3 and C3 regions in the HIV-1 envelope [19]. Finally, the transmission rate of HIV-2 is also significantly lower than that of HIV-1 and this has been associated with the low or absent viremia found in most HIV-2 patients [22,23].

The HIV-1 Env glycoprotein is a trimer on the virion surface with extensive N-linked glycosylation that effectively shields many conserved epitopes from antibody recognition [24]. It is composed of trimers of a surface (SU) glycoprotein with a molecular weight of 120-125 kDa (gp120-125) that is bound to a transmembrane (TM) glycoprotein with 36-41 kDa (gp36-41). SU can be divided into five hipervariable regions, named V1 to V5, bordered by five conserved regions, named C1 to C5. The C2 and C3 regions associate to form the CD4 binding site such that mutations in amino acid at positions 267Q in C2 and 368R in C3 abrogate gp120 binding to CD4 [25,26]. In HIV-1, V3 is one of the most important determinants of viral tropism and co-receptor usage [27,28]. This region also contains major antigenic and neutralizing epitopes in HIV-1, which are well exposed upon CD4-binding [29,30,31,32,33,34,35]. Although still debatable, the V3 region in HIV-2 may also contain broadly neutralizing epitopes [36,37,38,39,40,41,42]. However, in contrast to HIV-1, the V3 and flanking C2 and C3 regions are not immunodominant in HIV-2 infected patients [43,44,45,46]. Moreover, it remains to be determined whether these regions are exposed or concealed in the envelope complex of primary isolates of HIV-2.

In HIV-1 infection escape from antibody neutralization occurs frequently and is the major driving force of the molecular evolution of the envelope glycoproteins [47,48]. Not surprisingly, codons under diversifying selection (positive selection) seem to be clustered mostly in the hypervariable V1/V2 and V3 regions that contain important and accessible neutralizing targets [49,50]. The impact of the neutralizing antibody response in the *in vivo* evolution of the HIV-2 Env is currently unknown.

The present study was designed to identify molecular and evolutionary features of the C2, V3 and C3 regions in HIV-1 and HIV-2 infected patients that could be related with their different immunological and clinical outcomes. We describe some potentially important differences in the genetic constitution, molecular evolution and conformation of the C2, V3 and C3 regions in HIV-1 and HIV-2 that provide new insights into their function and may inform the design of HIV vaccines.

MATERIALS AND METHODS

Amplification, cloning and sequencing of HIV-1 and HIV-2 viruses from Portugal

Portuguese (PT) samples were collected from HIV infected patients, followed in hospitals in the North and South of Portugal and presenting different clinical stages of infection and CD4+ T-cell counts. HIV-2 samples were collected between 1997 and 2005 from 49 patients, some of whom were infected in late-1970s [51,52]. HIV-1 samples were collected from 60 patients between 1993 and 1998.

Proviral DNA was extracted from uncultured PBMCs, or viral genomic RNA was extracted from plasma and reverse transcribed. A nested PCR technique was used to amplify a 373 bp HIV-2 C2-V3-C3 *env* gene region and a 409 pb HIV-1 C2-V3-C3 *env* region as described elsewhere [53,54]. PCR products were sequenced using the BigDye Terminator Cycle sequencing kit (Applied Biosystems) and an automated capillary sequencer (ABI PRISM 310, Applied Biosystems). Newly derived HIV-1 sequences from Portugal have been assigned GenBank accession numbers: EU335962 - EU335903. Newly derived HIV-2 sequences from Portugal have been assigned GenBank accession numbers: AY913773-AY913794, AY649545-AY649554 and GU591163.

Additionally, 16 HIV-2 consensus sequences from a previous publication [52] were also included in this study. The samples used to obtain these consensus sequences were: 03PTHCC1, 03PTHCC2, 03PTHCC4, 03PTHCC5, 03PTHCC7, 03PTHCC8, 03PTHCC12, 05PTHCC13, 03PTHCC14, 03PTHCC17, 03PTHCC19, 03PTHSM2, 05PTHSM3, 03PTHSM7, 03PTHSM9 and 03PTHSM10.

Control datasets

As Control datasets to this study, HIV-1 group M (all subtypes) reference sequence alignment (94 sequences) was obtained from the Los Alamos HIV database (http://www.hiv.lanl.gov/). HIV-2 group A reference sequence alignment was also obtained from the Los Alamos HIV database. Additional C2-V3-C3 sequences derived from group A primary isolates were retrieved from the Los Alamos Database adding to a total of

59 HIV-2 Control sequences. Both control alignments are available as Supplementary Material (Alignment S1 and S2).

Molecular and phylogenetic analysis

Nucleotide sequences were aligned using ClustalX 1.8 [55]. Maximum likelihood analyses were performed using the best-fit models of molecular evolution estimated by Modeltest [56]. These were GTR+G+I [57] for the PT HIV-2 dataset and TVM+G+I for PT HIV-1 and for HIV-1 and HIV-2 Control datasets [58].

Evolutionary distances were estimated under these models using PAUP version 4.0 [59]. Tree searches were also conducted in PAUP version 4.0 using either nearest-neighbor interchange (NNI) or subtree pruning and re-grafting (SPR) heuristic strategies, with bootstrap resampling. All positions containing gaps and missing data were eliminated from the dataset. In the final datasets there were a total of 369 nucleotide positions in PT HIV-2 and 372 positions in PT HIV-1 alignments, and 369 positions in HIV-2 and HIV-1 Control alignments. Both alignments were tested for recombination with the Single Breakpoint Recombination (SBP) tool [60] in the DATAMONKEY web-server [61]; evidence for recombination, inferred by the small sample AIC score, was only found for HIV-1 Control dataset. Thus, when appropriate, a multiple partition dataset was used for HIV-1 Control analysis. Detection of *N*-linked glycosylation sites was performed with Glycosite [62]. The entropy at each position in protein alignment was measured with Shannon's entropy [63].

Tests for codon selection

Selection pressures over the HIV-1 and HIV-2 C2, V3 and C3 regions were examined with the HYPHY software package [64] and the DATAMONKEY web-server [61]. All estimations were performed using the MG94 codon substitution model [65] crossed with the nucleotide substitution model previously selected with Modeltest, GTR for PT HIV-2 and TVM for PT HIV-1 and Control alignments. To understand if selection pressure within a host is different from selection for transmission among hosts, non-synonymous substitutions were compared between terminal and internal branches of the phylogenetic tree, with the TestBranchDNDS.bf batch file in HyPHy, as described elsewhere [66].

Four different approaches were used to identify codons under selection: single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), internal fixed effects likelihood (IFEL) and relaxed-effects likelihood (REL) methods [67,68]. While SLAC, FEL and REL detect sites under selection at the external branches of the phylogenetic three, IFEL identifies such sites only along the internal branches. To classify a site as positively or

negatively selected the cut-off P-value was 10% for SLAC, FEL and IFEL. For REL, codons under selection were detected with a cut-off value for the Bayes factor of 50. Since SLAC, FEL and IFEL can estimate site-specific ratios of non-synonymous and synonymous substitutions rates (dN/dS ratios) as undefined or infinite due to dS = 0, we reported dN-dS values instead, which were scaled by the total codon tree length to allow a better comparison between the two datasets. A multiple partition dataset was used for the identification of codons under selection in HIV-1 Control analysis. Site-by-site variation of synonymous substitution rates can bias estimations of codon's diversifying selection [69]. Although all four methods described above model for this variation, variation of synonymous rates from codon to codon in each dataset was tested with the dNdSRateAnalysis.bf batch file in HyPHy, as described elsewhere [66]. Finally, comparison of the dN/dS distribution rates and the strength of selection between the HIV-1 and HIV-2 alignments, was performed with dNdSDistributionComparison.bf batch file also in HyPHy, as described elsewhere [66].

Molecular modelling and calculation of solvent accessible surfaces

Consensus amino acid sequences were derived for the different HIV-1 and HIV-2 datasets. Structural models of HIV-1 and HIV-2 C2, V3 and C3 were produced with SWISS-MODEL homology modelling server in project mode resorting to Swiss-Pdb Viewer (DeepView) version 4.0, using PDB file 2B4C (from HIV-1 JR-FL gp120) for HIV-1, and PDB file 2BF1 (from SIV gp120) for HIV-2 as templates [70,71,72,73]. Accelrys Discovery Studio Visualizer 2.5 [74] was used to produce three dimensional images of the models obtained. Solvent accessible surface area in $Å^2$ was calculated by Gerstein's calc-surface software on UCSF Chimera [75,76] with a probe size of 1.4 Å. All atoms in the input PDB file were included in the calculation. The solvent accessible surface data was normalized dividing each amino acid residue solvent accessible surface value added by the solvent accessible surface value of the corresponding amino acid residue (X) in the tripeptide Gly-X-Gly. The inter-chain H-Bonds formed by HIV-2 V3 with C2 and C3 were calculated with H-Bond Finder software on UCSF Chimera [75,76] with a probe size of 1.4 Å. All atoms in the input PDB file were included in the calculation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 4.0c for Macintosh (GraphPad Software, 2005, San Diego, California, USA, www.graphpad.com) with a level of

significance of 5%. Non-parametric Mann-Whitney U test was used to compare Shannon's entropy values and nucleotide distances.

RESULTS

HIV-1 is significantly more variable in the envelope C2, V3 and C3 regions than HIV-2

We compared the inter-patient genetic diversity of HIV-1 and HIV-2 in two different datasets: HIV-1 group M (all subtypes) and HIV-2 group A sequences from all over the world (Control dataset composed of reference sequences) and newly derived HIV-1 and HIV-2 sequences obtained from Portuguese (PT) patients. Phylogenetic analysis showed that HIV-1 sequences circulating in Portugal belong to different subtypes and recombinant forms (Figure S1A). Forty five sequences were subtype B and six belonged to the recombinant form CRF14_BG. Subtypes G (4 sequences) and C (2), sub-subtype F1 (2), and CRF02_AG (1) were also found. Regarding HIV-2, all sequences from Portugal clustered together within group A (Figure S1B). Collectively, these results are consistent with previous studies showing a highly complex HIV epidemics in Portugal caused exclusively by HIV-2 group A and different subtypes of HIV-1 group M [52,77,78,79]. Nucleotide diversity between HIV-1 viruses found in Portugal was significantly higher compared to HIV-2 (mean number of substitutions per site, 0.336, 95%CI [0.329; 0.342] *vs* 0.239, [0.236; 0.243], $P<0.0001$). Similar results were found for the HIV-1 and HIV-2 Control datasets (Table S1). Hence, we conclude that HIV-1 is genetically more diverse than HIV-2 in the envelope region comprising C2, V3 and C3.

Amino acid diversity in the C2, V3 and C3 regions of HIV-1 and HIV-2 were compared by calculating Shannon's entropy [63]. Mean entropy values for the three regions were significantly higher in HIV-1 than in HIV-2 both in PT (0.794 *vs* 0.409, $P<0.0001$) and Control datasets (0.702 *vs* 0.353, $P<0.0001$) confirming that these regions are more variable in HIV-1 than in HIV-2. Entropy was also significantly higher in HIV-1 than in HIV-2 in each separate region (C2, $P<0.05$; V3, $P<0.005$; C3, $P<0.0005$) of PT sequences. The region with higher mean entropy was C3 in both viruses (1.031, 95%CI [0.845, 1.217] for HIV-1 *vs* 0.534, 95%CI [0.378, 0.689] for HIV-2, $P<0.0005$) followed by V3 (0.674, [0.506, 0.841]) and C2 (0.574, [0.427, 0.721]) in HIV-1 and C2 (0.326, [0.175, 0.477]) and V3 (0.304, [0.176, 0.433]) in HIV-2 (Figure 1). Comparable results were obtained for the Control datasets but in this case V3 was the least entropic region both in HIV-1 and HIV-2 (Table S1 and Figure S2). Not surprisingly, amino acids with higher entropy (values above 1) were primarily located in the C3 region of both viruses and there were more highly entropic amino acids

in C3 in HIV-1 than in HIV-2 both in the PT and Control datasets (PT dataset: 51.9% in HIV-1 *vs* 24.5% in HIV-2; Control dataset: 35.3% in HIV-1 *vs* 20.8% in HIV-2). Notably, the amino acids in V3 that are related with co-receptor usage, positions 11/25 in HIV-1 (codons 306/320) [80,81] and possibly positions 18/19/27 in HIV-2 (codons 319/320/328) [17,82], had a high entropy score in both viruses.

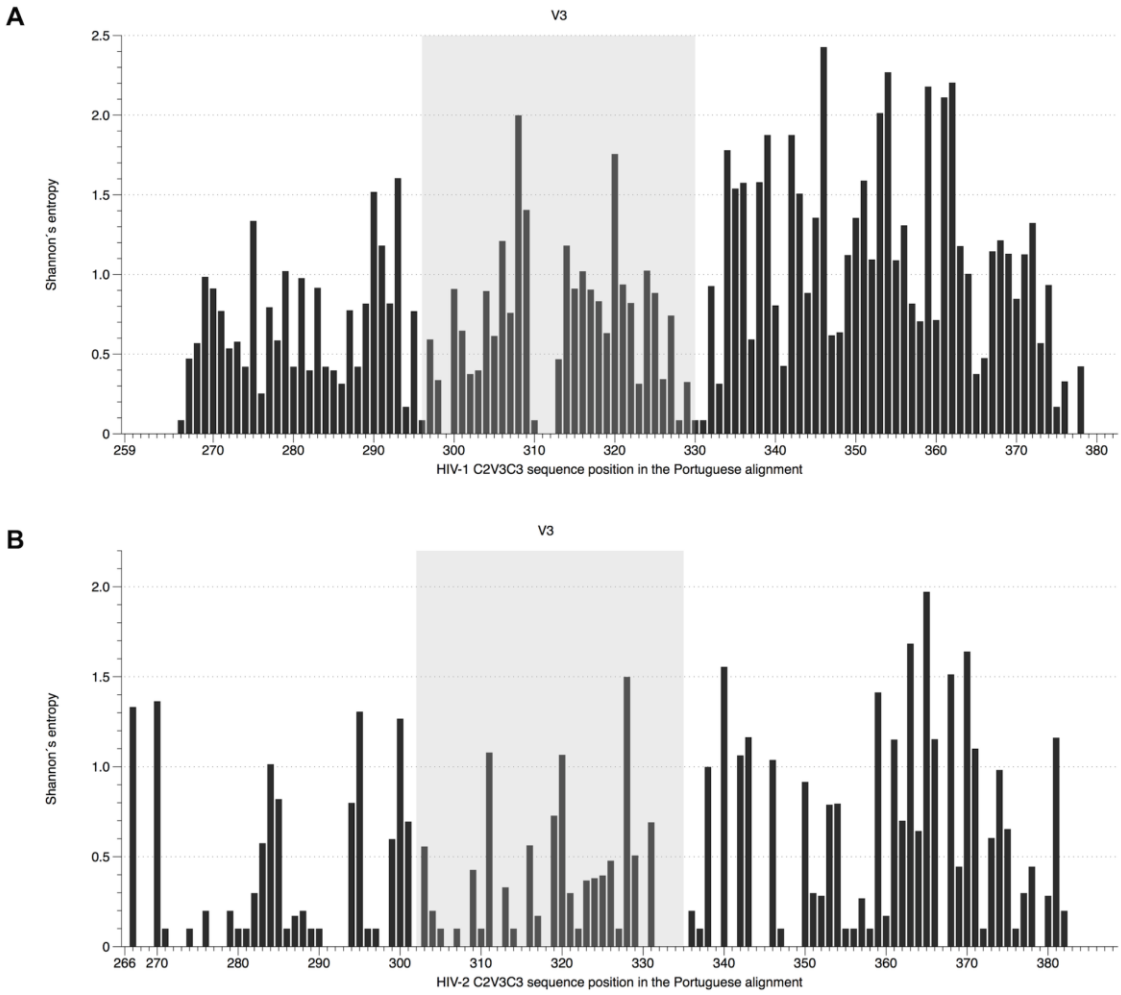

Figure 1. Shannon's entropy of individual amino acids in the C2, V3 and C3 envelope regions in HIV-1 and HIV-2. (**A**) HIV-1 alignment (PT dataset), sites were numbered according to codon *env* position of HIV-1 HXB2 reference strain; (**B**) HIV-2 alignment (PT dataset), sites were numbered according to codon *env* position of HIV-2 ALI reference strain.

The mean number of potential *N*-linked glycosylation sites both in HIV-1 and HIV-2 sequences from Portugal was 7 (range: 4-9 in HIV-1; 5-9 in HIV-2). The most conserved glycosylation sites were located in C2 in both viruses (Figure 2). Nonetheless, in this region, there were four highly conserved glycosylation sites in HIV-2 (present in ≥ 80% of strains) and only two such sites in HIV-1. With the exception of the highly conserved site

located in the beginning of C3 in HIV-1, glycosylation sites found in C3 varied from strain to strain in number and location, this being more evident in HIV-1 than in HIV-2. In V3 there were two highly conserved glycosylation sites in both viruses. Similar observations were made for HIV-1 and HIV-2 sequences in the Control datasets (Table S1 and Figure S3).

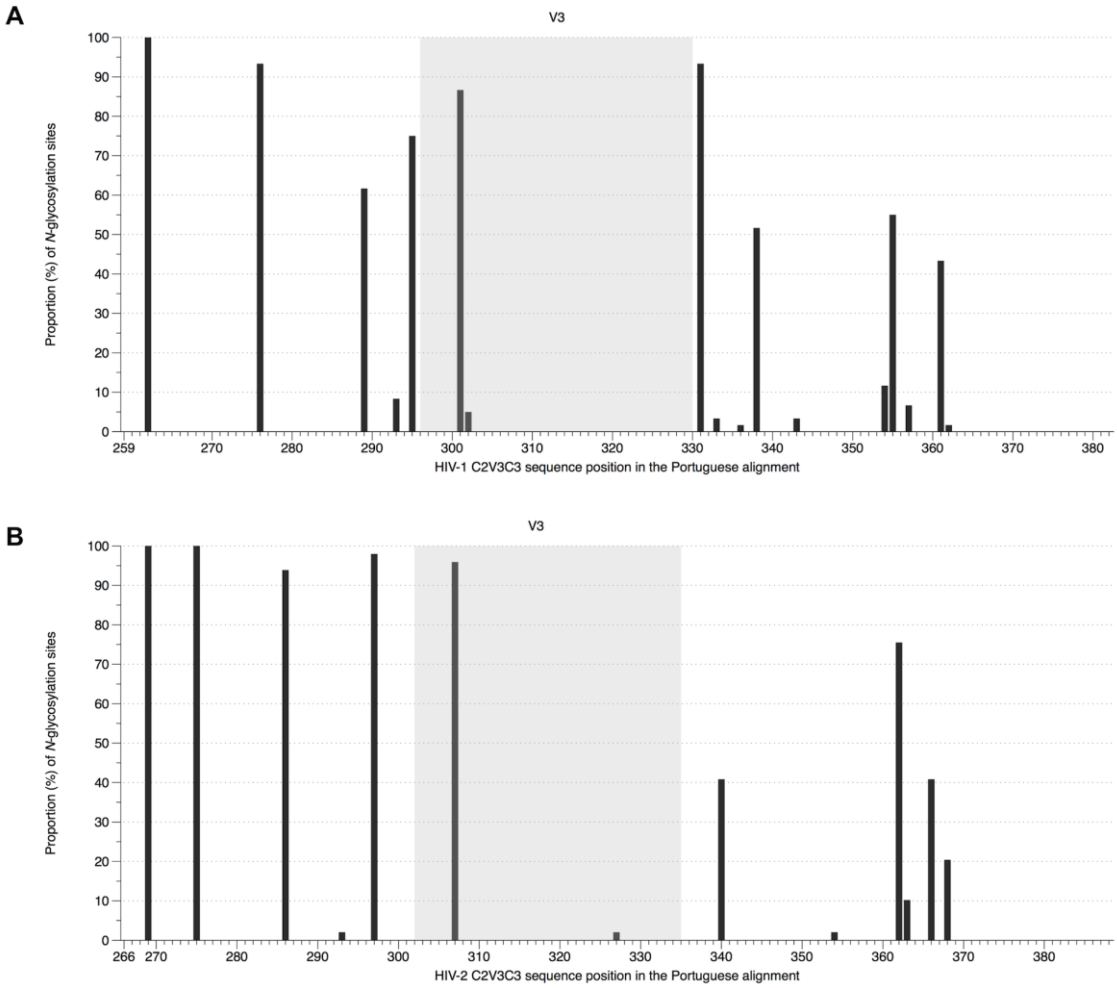

Figure 2. Frequency of *N*-glycosylation sites in the C2, V3 and C3 envelope regions in HIV-1 and HIV-2. (**A**) HIV-1 alignment (PT dataset), sites were numbered according to codon *env* position of HIV-1 HXB2 reference strain; (**B**) HIV-2 alignment (PT dataset), sites were numbered according to codon *env* position of HIV-2 ALI reference strain.

Selective pressures act differently in HIV-1 and HIV-2

We have recently found that HIV-2 displays a faster evolutionary rate in the envelope gp125 and C2, V3 and C3 regions than HIV-1 in patients with chronic and advanced disease [52,83]. The faster evolutionary rate in HIV-2 was more pronounced in synonymous sites than in non-synonymous sites suggesting a weaker positive selection in HIV-2 than in HIV-1.

To investigate this possibility, we analysed diversifying selection in the C2, V3 and C3 regions of both viruses using codon-based models of molecular evolution. Firstly, we estimated the ratio of non-synonymous and synonymous substitution rates (dN/dS ratio) averaged over all sites. For HIV-1 sequences from Portugal dN/dS ratio was 0.703, 95%CI [0.668, 0.740]; for HIV-2 it was 0.451, [0.419, 0.484]. Similar values were obtained for the Control alignments (Table S1). These results are consistent with the higher degree of genetic conservation of the C2, V3 and C3 regions in HIV-2.

Site-by-site analysis revealed that diversifying selection is unevenly distributed along the studied region between the two viruses (PT, $P<0.001$; Controls, $P<0.001$) (Figures 3 and S4). For HIV-2 sequences from the PT dataset, there were between 7 and 9 positively selected (PS) sites depending on the method that was used (SLAC/FEL/REL) while for HIV-1 the number of sites ranged from 7 to 17 (Table 1). Taking into account only sites that were selected by at least two methods, HIV-2 had a total of 7 PS sites whereas in HIV-1 there were 9 sites. The sites were distributed as follows: in C2 there were 3 sites in HIV-2 and 2 in HIV-1; in V3 there were 2 sites in HIV-1, and no sites in HIV-2; in C3 there were 4 sites in HIV-2 and 5 in HIV-1, including one codon within the CD4 binding site (codon 378 in HIV-1) and two in the α2-helix (codons 343 and 346) [84]. In Control data sets the number of PS sites was slightly lower but they were similarly distributed, with the exception of the V3: 1 PS site in HIV-2, but no sites in HIV-1 (Tables S1 and S2). Importantly, we found that when compared to HIV-1, positive selection was stronger in HIV-2 in most sites (Tables 1 and S2).

The comparison of diversifying selection between terminal and internal branches of the phylogenetic trees revealed two distinct profiles for HIV-1 and HIV-2. Firstly, non-synonymous substitution rates were significantly different between the internal nodes and the tips of the tree in all datasets: PT, $P=0.002$ for HIV-2 and $P=0.011$ for HIV-1; Controls, $P<0.001$ and $P=0.004$ (data not shown). Stronger selection was in general found at codons selected simultaneously at the tips and the external branches of the HIV-1 and HIV-2 trees. Importantly, however, only 2 of the 7 sites (29%) detected in terminal branches of PT HIV-2 tree were also under positive selection along the internal branches (codons 267 and 270 in C2). In contrast, for HIV-1 most positively selected sites (6/9, 67%) were present both in the internal and the terminal branches. In Control datasets these percentages were 43% for HIV-2 and 71% for HIV-1 (Table S2). These results suggest that natural selection affects less the transmission fitness of HIV-1 than HIV-2.

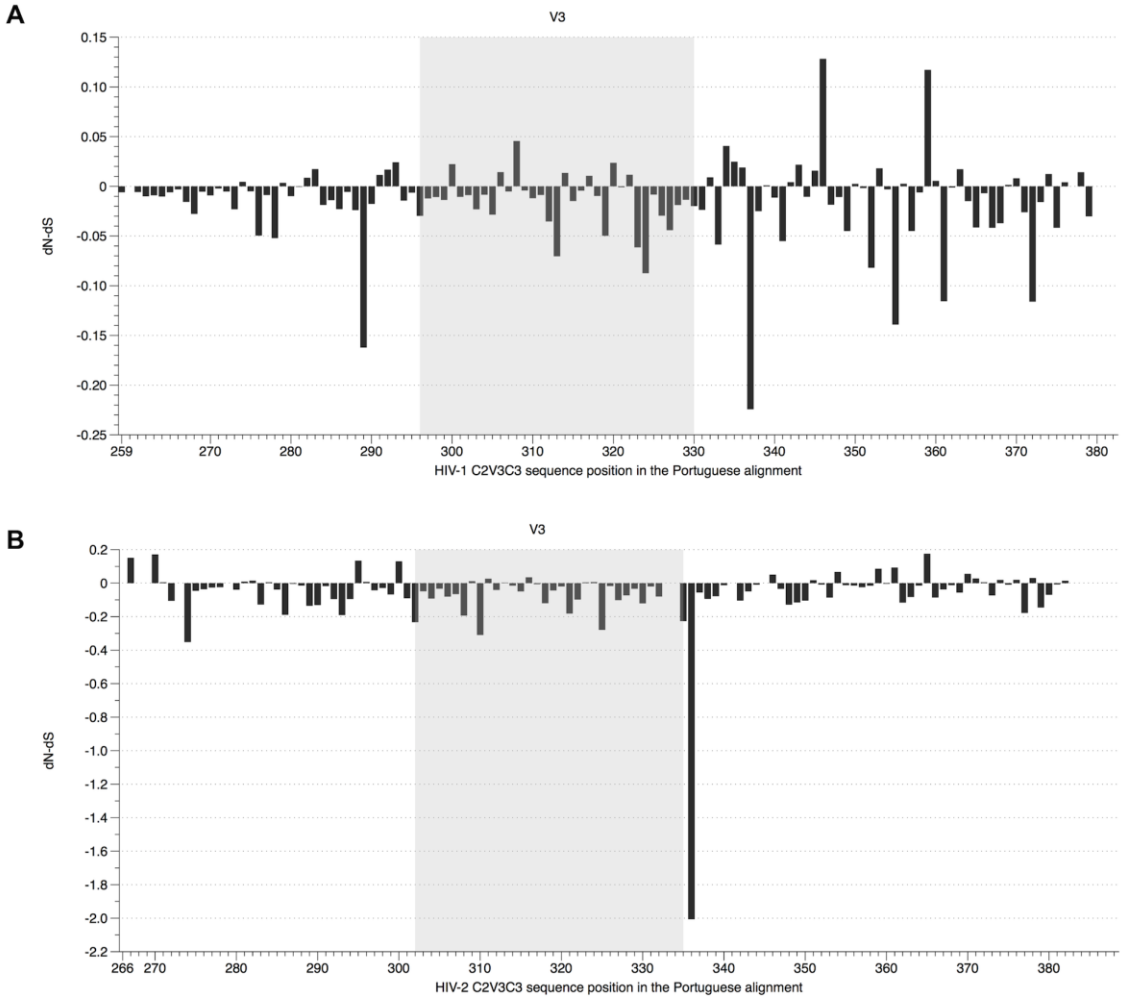

Figure 3. Positive selection in the C2, V3 and C3 envelope regions in HIV-1 and HIV-2. dN-dS values were estimated by FEL and scaled by the total codon tree length. (**A**) HIV-1 alignment (PT dataset), sites were numbered according to codon *env* position of HIV-1 HXB2 reference strain; (**B**) HIV-2 alignment (PT dataset), sites were numbered according to codon *env* position of HIV-2 ALI reference strain.

Table 1. Positively selected sites detected by SLAC, FEL, REL and/or IFEL in HIV-1 and HIV-2 env C2, V3 and C3 regions[1].

HIV-1										HIV-2									
Region	Codon	SLAC		FEL		REL		IFEL		Region	Codon	SLAC		FEL		REL		IFEL	
C2	**283**	**0.249**	(0.083)	**0.017**	(0.027)	**0.282**	(0.950)	-0.007	(0.237)	C2	**267**	**1.805**	(<0.001)	**0.151**	(<0.001)	**1.007**	(1.000)	**0.147**	(0.004)
	291	**0.252**	(0.096)	0.011	(0.334)	0.005	(0.277)	0.008	(0.648)		**270**	**1.561**	(0.003)	**0.171**	(<0.001)	**0.892**	(1.000)	**0.179**	(0.010)
	292	**0.269**	(0.063)	0.017	(0.167)	0.547	(<0.001)	0.014	(0.419)		295	**1.049**	(0.051)	**0.134**	(0.095)	0.070	(0.910)	0.109	(0.316)
	293	**0.401**	(0.066)	0.024	(0.230)	**0.924**	(0.972)	**0.063**	(0.050)		300	0.787	(0.210)	**0.130**	(0.077)	0.714	(<0.001)	0.131	(0.109)
V3	300	**0.335**	(0.079)	**0.022**	(0.022)	0.219	(0.846)	**0.016**	(0.093)	V3	331	-0.312	(0.859)	0.020	(0.591)	**0.193**	(0.987)	0.069	(0.390)
	306	0.312	(0.106)	0.014	(0.541)	**0.947**	(0.984)	-0.004	(0.867)										
	308	**0.619**	(0.008)	**0.046**	(0.065)	**0.869**	(0.973)	**0.094**	(0.012)										
	314	**0.314**	(0.052)	0.014	(0.291)	0.163	(0.178)	-0.001	(0.971)										
	317	**0.301**	(0.057)	0.011	(0.401)	0.192	(0.140)	0.005	(0.749)										
C3	332	0.124	(0.267)	0.009	(0.341)	-0.432	(<0.001)	**0.031**	(0.093)	C3	346	0.478	(0.236)	0.051	(0.173)	**1.008**	(1.000)	0.050	(0.397)
	334	**0.543**	(0.004)	**0.041**	(0.027)	**1.142**	(0.997)	**0.065**	(0.024)		351	0.207	(0.298)	**0.018**	(0.087)	0.114	(<0.001)	0.000	(1.000)
	335	**0.458**	(0.010)	0.025	(0.109)	0.893	(0.936)	0.035	(0.147)		**354**	**0.689**	(0.047)	**0.067**	(0.016)	**0.988**	(1.000)	0.000	(1.000)
	336	**0.452**	(0.058)	0.019	(0.583)	0.817	(0.907)	0.011	(0.743)		**361**	**0.887**	(0.035)	**0.093**	(0.011)	**0.988**	(1.000)	0.016	(0.693)
	343	**0.405**	(0.060)	0.022	(0.109)	**0.885**	(0.989)	0.017	(0.370)		364	-0.085	(0.704)	0.014	(0.690)	**0.130**	(0.983)	-0.069	(0.072)
	345	**0.392**	(0.024)	0.016	(0.330)	0.492	(0.657)	**0.070**	(0.018)		365	**1.074**	(0.089)	**0.175**	(0.070)	0.463	(0.561)	-0.056	(0.626)
	346	**1.080**	(<0.001)	**0.128**	(<0.001)	**0.945**	(0.982)	**0.281**	(<0.001)		378	**0.415**	(0.088)	**0.030**	(0.043)	0.116	(<0.001)	0.029	(0.119)
	348	**0.270**	(0.096)	0.011	(0.664)	1.183	(<0.001)	-0.005	(0.899)										
	353	0.319	(0.143)	0.018	(0.476)	-0.118	(0.347)	**0.120**	(0.035)										
	359	**0.558**	(0.022)	**0.117**	(<0.001)	**0.882**	(1.000)	**0.214**	(0.001)										
	363	0.169	(0.295)	0.017	(0.279)	**0.860**	(0.995)	0.003	(0.900)										
	378	**0.244**	(0.021)	**0.014**	(0.020)	0.217	(<0.001)	0.000	(1.000)										

[1]PT dataset

Codon – codons selected under 10% level of significance (SLAC, FEL and IFEL) or above a Bayes Factor of 50 (REL) and numbered according to codon *env* position of HIV-1 HXB2 for HIV-1 dataset or of HIV-2 ALI for HIV-2 dataset. Codons selected simultaneously by SLAC, FEL and REL methods are bold and underlined.

SLAC, FEL and IFEL – the first numbers are the dN-dS difference for each site scaled by the total codon tree length, the numbers in parenthesis show P-values for corresponding test of non-synonymous rate being superior to synonymous rate.

REL - the first numbers are the expected posterior dN-dS difference for each site scaled to the total codon tree length, the number in parenthesis show the posterior probability of non-synonymous rate being superior to synonymous rate.

Bold dN-dS differences correspond to significant *P*-values or posterior probabilities.

Structure and solvent accessibility of V3 differ in HIV-1 and HIV-2

A model of the structure of the C2, V3 and C3 regions was built for HIV-1 and HIV-2 based on the atomic coordinates of the HIV-1 gp120 and SIV gp120 using consensus sequences from both the PT and Control HIV-1 and HIV-2 alignments. For HIV-1, the structures of PT and Control sequences were almost identical having only a slight difference in V3, which presents less regular secondary structure in the PT sequence (Figure S5). For HIV-2, the structures of PT and Control sequences were identical. The structure of the C2, V3 and C3 regions was however markedly different between HIV-1 and HIV-2, the most striking differences being the significant retraction of the V3 loop in HIV-2 and its potential interaction both with C2 and C3 (Figure 4A). Identical results were obtained when comparing the HIV-1 and HIV-2 control sequences (Figure S6). The predicted non-covalent interaction between V3, C3 and C3 in HIV-2 involves residues Tyr296 and His301 in C2 binding, respectively, to Arg331 and Trp334 in V3, and Phe337 in C3 binding to Phe321 in V3 (Figure 4B).

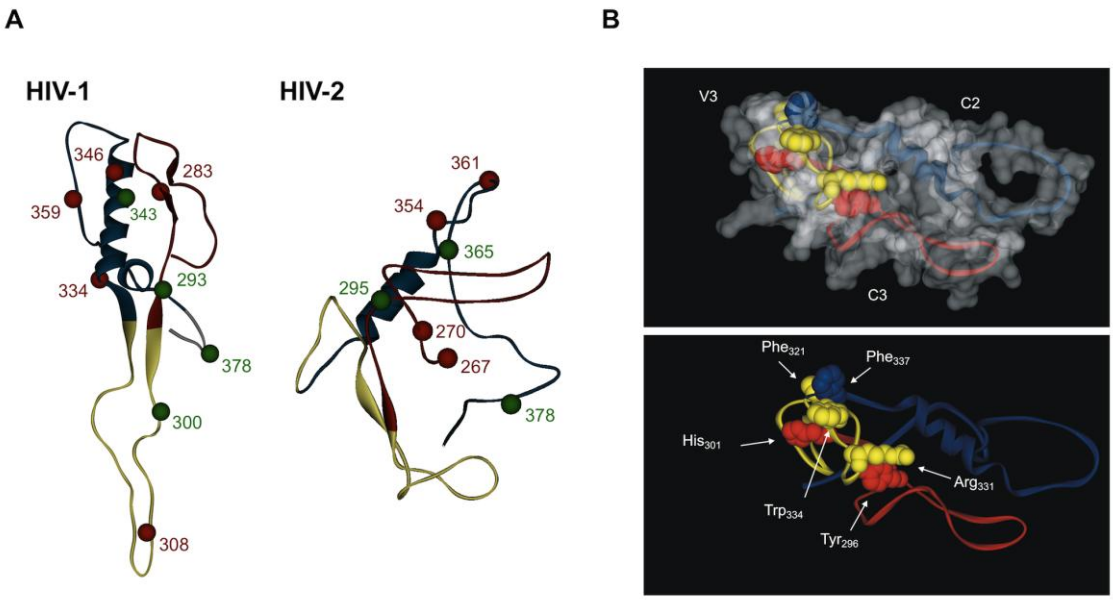

Figure 4. Conformational structure of C2, V3 and C3 envelope regions in HIV-1 and HIV-2. The conformational structure of consensus amino acid sequences derived from the PT datasets was obtained by homology modeling as indicated in Material and Methods. In the schematics, C2 is shown in red, V3 in yellow and C3 in blue. (A) Balls represent the amino acids under positive selection. The red balls represent codons selected simultaneously by SLAC, FEL and REL methods, while green balls stand for codons selected by at least two of these methods; (B) Model structure showing the predicted interactions between V3, C2 and C3 in HIV-2 gp125. The non-covalent interaction involves residues Tyr296 and His301 in C2 binding, respectively, to Arg331 and Trp334 in V3, and Phe337 in C3 binding to Phe321 in V3.

The solvent accessibilities of amino acid residues were also calculated for both models (Figure 5). As expected, both in HIV-1 and HIV-2 most PS sites and *N*-glycans had at least

50% surface exposure. In HIV-2, 8 out of 37 (22%) amino acids in C2, 8/34 (24%) in V3 and 19/53 (36%) in C3 were highly exposed (≥ 70% solvent accessibility) whereas in HIV-1 these were 9/37 (24%), 15/35 (43%) and 10/52 (19%), respectively. Consistent with the high exposure of the V3 region in HIV-1, the two amino acids at positions 306 and 320 involved in binding to co-receptors were well exposed (≥ 50% solvent accessibility). In contrast, in HIV-2, among amino acids 319/320 and 328 in V3 loop potentially involved in co-receptor binding, only 319 was relatively well exposed. Despite the potential interaction between V3 and C3 (Figure 4B), the overall exposition of C3 was higher in HIV-2 than in HIV-1. Thus, for instance, 42% (5/12) of the residues in C3 that may contribute for the formation of the CD4-binding site (positions 377-388) in HIV-2 showed high solvent accessibility. In HIV-1 only 3 out of 16 (19%) amino acids with similar function (positions 367-382) were highly exposed. Similar results were obtained when comparing the HIV-1 and HIV-2 control sequences (Figure S7).

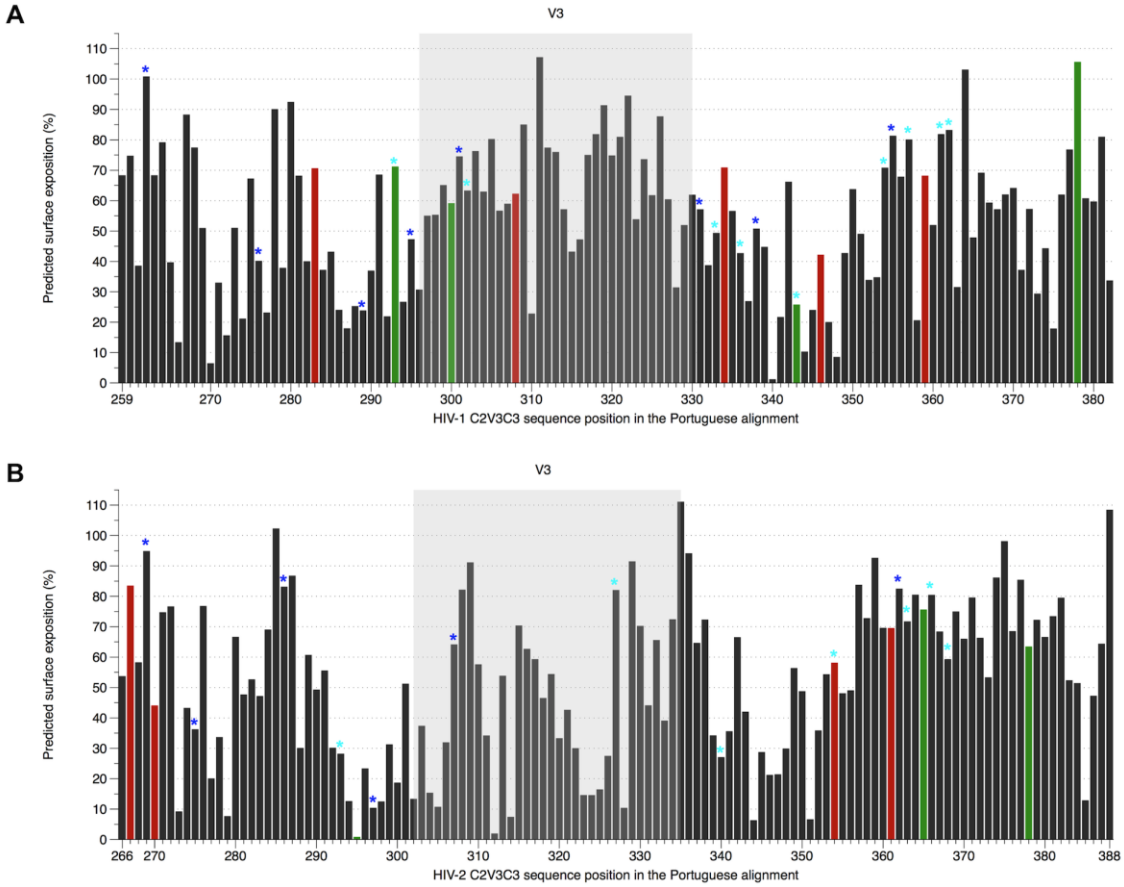

Figure 5. Solvent accessible surface area, positive selection and potential *N*-glycosylation sites in C2, V3 and C3 regions. (**A**) HIV-1 alignment (PT dataset), sites were numbered according to codon *env* position of HIV-1 HXB2 reference strain; (**B**) HIV-2 alignment (PT dataset), sites were numbered according to codon *env* position of HIV-2 ALI reference strain. Colored bars represent the amino acids under positive selection and have the same colors (red and green) as the corresponding positions (balls) highlighted in Figure 4A. The dark blue stars over the bars correspond to potential *N*-glycosylation sites conserved along the alignment (present in ≥50% of strains), whereas the light blue stars represent sites only present in less than 50% of sequences.

DISCUSSION

To investigate the molecular and structural features underlying the differences in HIV-1 and HIV-2 biology and human infection, we have analysed the C2, V3 and C3 envelope regions from a significant number of HIV-1 and HIV-2 infected patients living in Portugal and worldwide. HIV-2 sequences from Portugal belonged to group A and the majority of HIV-1 sequences belonged to subtype B (75%) followed by subtypes G, C and F, CRF02_AG and CRF14_BG. Collectively, these results are consistent with previous studies showing a highly complex HIV epidemic in Portugal caused by HIV-2 group A and different subtypes of HIV-1 group M [44,52,53,77,78,79,85].

Genetic distances and amino acid diversity between HIV-1 viruses were significantly higher compared to HIV-2. This was surprising since at the individual level HIV-2 displays a similar [52] or even faster evolutionary rate than HIV-1 in the C2, V3 and C3 regions [83]. The more pronounced evolutionary rate in synonymous sites than in non-synonymous sites in HIV-2 [83] together with the rare escape of this virus from autologous neutralizing antibodies [17], suggested that the lower amino acid diversity in HIV-2 could be related with a weaker positive selection or even with negative selection [53]. This was not the case however since most sites in C2 and C3 were under stronger positive selection in HIV-2 than in HIV-1. The C3 region of HIV-1 is antibody accessible [86] and is subject to diversifying selection because it is a major neutralizing target [87,88,89,90]. Therefore, the high level of positive selection detected in C3 together with its high solvent exposure strongly suggests that this region is also antibody accessible in HIV-2 and might be a major neutralizing domain.

Strength of selection was significantly different between internal and external branches of the HIV-1 and HIV-2 phylogenetic trees. This is expected in populations of highly variable RNA viruses and implies that non-synonymous substitutions can be highly deleterious [68,91]. In HIV-1, most of the codons selected in the tips of the tree were also under selection along the internal branches, indicating that adaptation in these sites is occurring at the host and population levels [68]. In contrast, most adaptive mutations in HIV-2 were only found in the tips of the tree indicating that they are recent maladaptive substitutions that are transitory at the population level [68,92]. Thus, in contrast to HIV-1, diversification of C2 and C3 in HIV-2 seems to have a dominant negative effect on viral fitness and transmission. This data suggests that one possible consequence of the unexpectedly high evolutionary rate of HIV-2 at the patient level can be the frequent accumulation of deleterious mutations and production of defective viruses [52,83,93]. A high frequency of defective viruses in HIV-2 infected individuals could explain the poor replication of this virus *in vivo* as well as its very low transmissibility.

Unlike in HIV-1, the V3 loop in HIV-2 always presented the lower amino acid diversity. This result might be a consequence of significant structural and conformational constraints due to its role in preventing chronic and disruptive immune activation [20] and in co-receptor binding [82]. On the other hand, these results imply that the V3 loop is not well exposed in the HIV-2 envelope complex *in vivo*. Indeed, by computer modelling simulations we show that in HIV-2 the V3 loop is much less exposed than C2 and C3 and likely has a retractile conformation due to non-covalent interaction both with C2 and C3. In contrast, HIV-1 had, as previously found, an extended and highly accessible V3 loop [89,90,94]. Such conformation is entirely consistent with its immunodominant and neutralizing nature and with its crucial role in HIV-1 co-receptor binding and tropism [33,34,35,95,96,97]. Conversely, the concealed nature of V3 in the HIV-2 envelope complex implies that this region may not be immunodominant in HIV-2 infection. Indeed, a significant number of HIV-2 patients do not raise antibodies against the V3 loop [43] or against a polypeptide comprising the C2, V3 and C3 regions [45]. Thus, the occlusion of V3 in the HIV-2 envelope complex may prevent it from over immune recognition and associated sequence changes thereby preserving its crucial functions in viral entry. It has been shown that removal or antigenic dampening of the HIV-1 V3 loop redirects the neutralizing immune response to other epitopes of the Env protein that otherwise would be non-neutralizing or non-antibody responsive [33,98,99,100]. In this context, the occluded nature of the V3 region in the HIV-2 envelope complex might favour a more effective production of broadly neutralizing antibodies targeting other regions in gp125 such as the C2, V1, V2, V4 and C5 regions [37,38,39,101].

In conclusion, the C2 and C3 regions are well exposed in the HIV-2 envelope complex and are under strong diversifying selection suggesting that, like in HIV-1, they may harbour neutralizing epitopes. However, extreme diversification of C2 and C3 in HIV-2 seems to be deleterious for the virus and prevent its transmission. On the other hand, V3 is highly conserved in HIV-2 and is concealed within the envelope complex, possibly due to a physical interaction with C2 and C3. In contrast, V3 is highly exposed and variable in HIV-1 which is consistent with its immunodominant and neutralizing properties. Collectively, we identify significant structural and functional constrains to the diversification and evolution of C2, V3 and C3 in the HIV-2 envelope but not in HIV-1. These studies highlight fundamental differences in the biology and infection of HIV-1 and HIV-2 and in their mode of interaction with the human immune system and may inform new vaccine and therapeutic interventions against these viruses.

ACKNOWLEDGEMENTS

This work was supported by projects PTDCSAU-FCF6767/2006 from Fundação para a Ciência e Tecnologia, Portugal and by Collaborative HIV and Anti-HIV Drug Resistance Network (CHAIN), from the European Union. PB and IB are supported by PhD grants from Fundação para a Ciência e Tecnologia. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

Alignment S1. Alignment of HIV-1 reference sequences used as a Control for the Portuguese HIV-1 dataset. Each sequence is identified by the corresponding GenBank accession number.
Found at: doi:10.1371/journal.pone.0014548.s010 (0.04 MB TXT)

Alignment S2. Alignment of HIV-2 reference sequences used as a Control for the Portuguese HIV-2 dataset. Each sequence is identified by the corresponding GenBank accession number.
Found at: doi:10.1371/journal.pone.0014548.s011 (0.02 MB TXT)

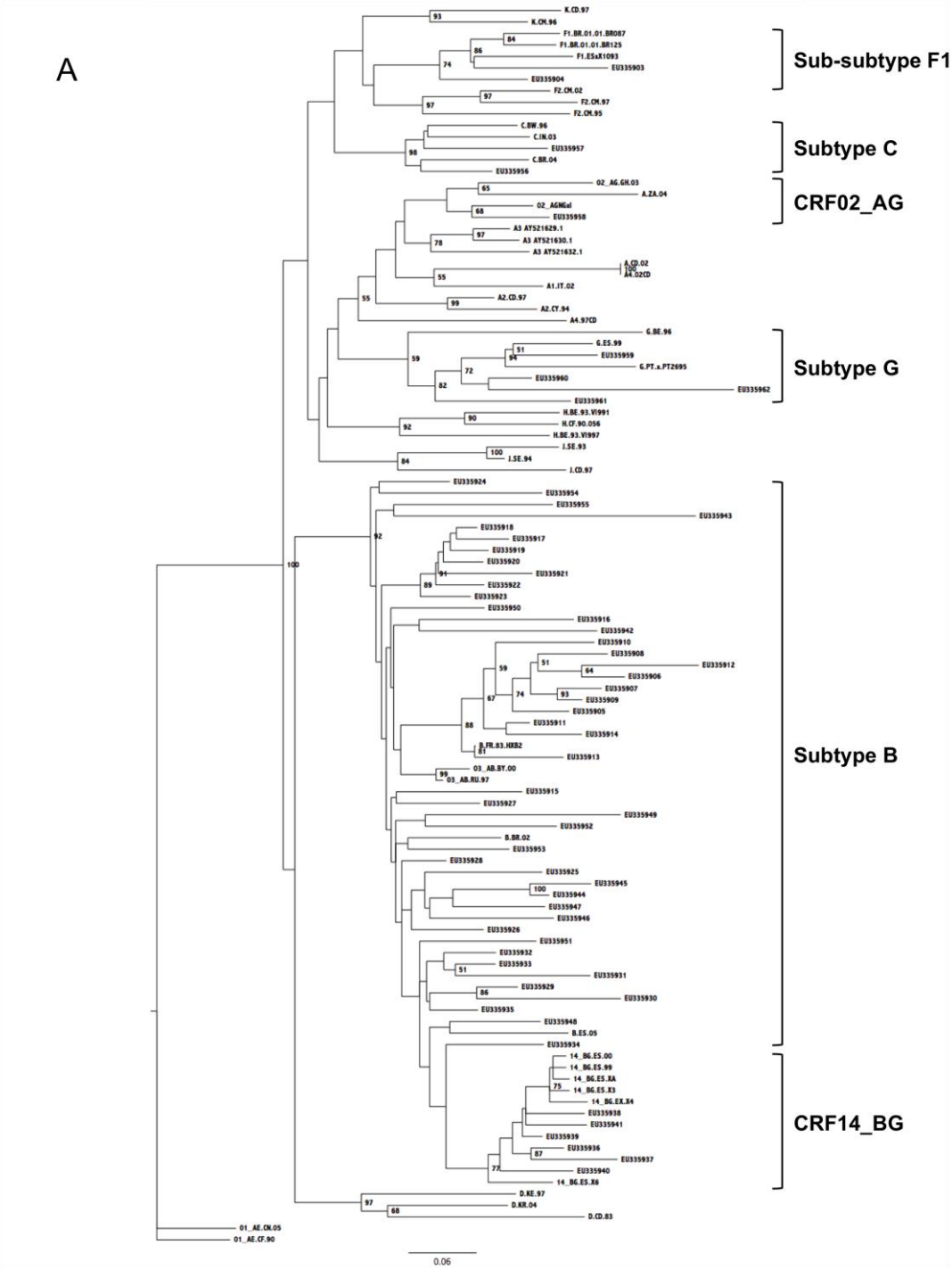

Figure S1. Genotyping HIV-1 (A) and HIV-2 (B) by maximum likelihood phylogenetic analysis. The phylogenetic trees were constructed using the SPR heuristic search strategy and 1000 bootstrap replications, with reference sequences from HIV-1, under the TVM+G+I evolutionary model (**A**) and with reference sequences from HIV-2, under the GTR+G+I evolutionary model (**B**). The bootstrap values (above 50%) supporting the internal branches are shown. The scale bar represents evolutionary distances in substitutions per site.

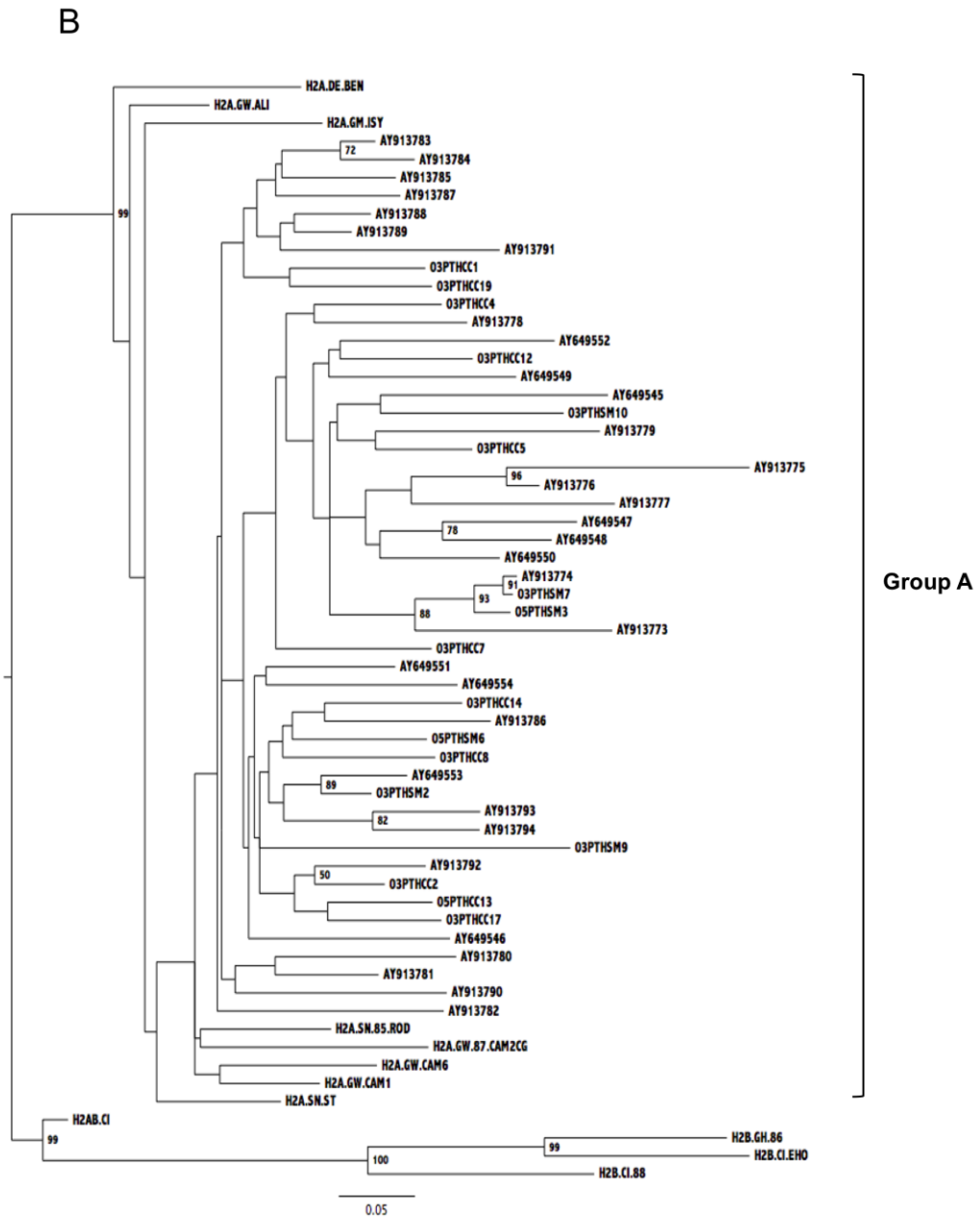

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Table S1. Summary of results for phylogenetic, codon selection and solvent accessibility analysis for C2, V3 and C3 regions of HIV-1 and HIV-2 Control datasets.

		HIV-1	HIV-2	*P* value
Nucleotide diversity, (mean, [95%CI])		0.361 [0.359; 0.364]	0.221 [0.218; 0.224]	<0.0001
Entropy, (mean, [95%CI])				
	C2-V3-C3	0.702 [0.590; 0.813]	0.353 [0.269; 0.436]	<0.0001
	C2	0.584 [0.394; 0.775]	0.244 [0.126; 0.363]	0.005
	V3	0.537 [0.382; 0.692]	0.244 [0.115; 0.373]	0.002
	C3	0.900 [0.700; 1.100]	0.496 [0.344; 0.648]	0.004
Entropy values above 1, (n. sites, (%))				
	C2-V3-C3	34 (27.6%)	16 (13%)	na
	C2	12 (32.4%)	3 (8.3%)	na
	V3	4 (11.4%)	2 (5.9%)	na
	C3	18 (35.3%)	11 (20.8%)	na
Potential glycosylation sites, (n. sites, (range)				
	C2-V3-C3	7 (5 - 9)	7 (4 - 10)	na
	C2	3 (1 - 4)	4 (3 - 5)	na
	V3	1 (0 - 1)	1 (0 - 1)	na
	C3	3 (2 - 5)	2 (0 - 5)	na
dN/dS ratio, (mean, [95%CI])		0.649 [0.621, 0.677]	0.461 [0.427, 0.497]	na
Positively selected sites, consensus from SLAC/FEL/REL (n. sites)				
	C2-V3-C3	3	3	na
	C2	1	1	na
	V3	0	1	na
	C3	2	1	na
Solvent accessibility above 70% (n. residues)				
	C2-V3-C3	27	37	na
	C2	9	8	na
	V3	8	8	na
	C3	10	21	na

[95%CI] – 95% confidence interval.

P value – P values for the non-parametric Mann-Whitney U test.

na – not applied.

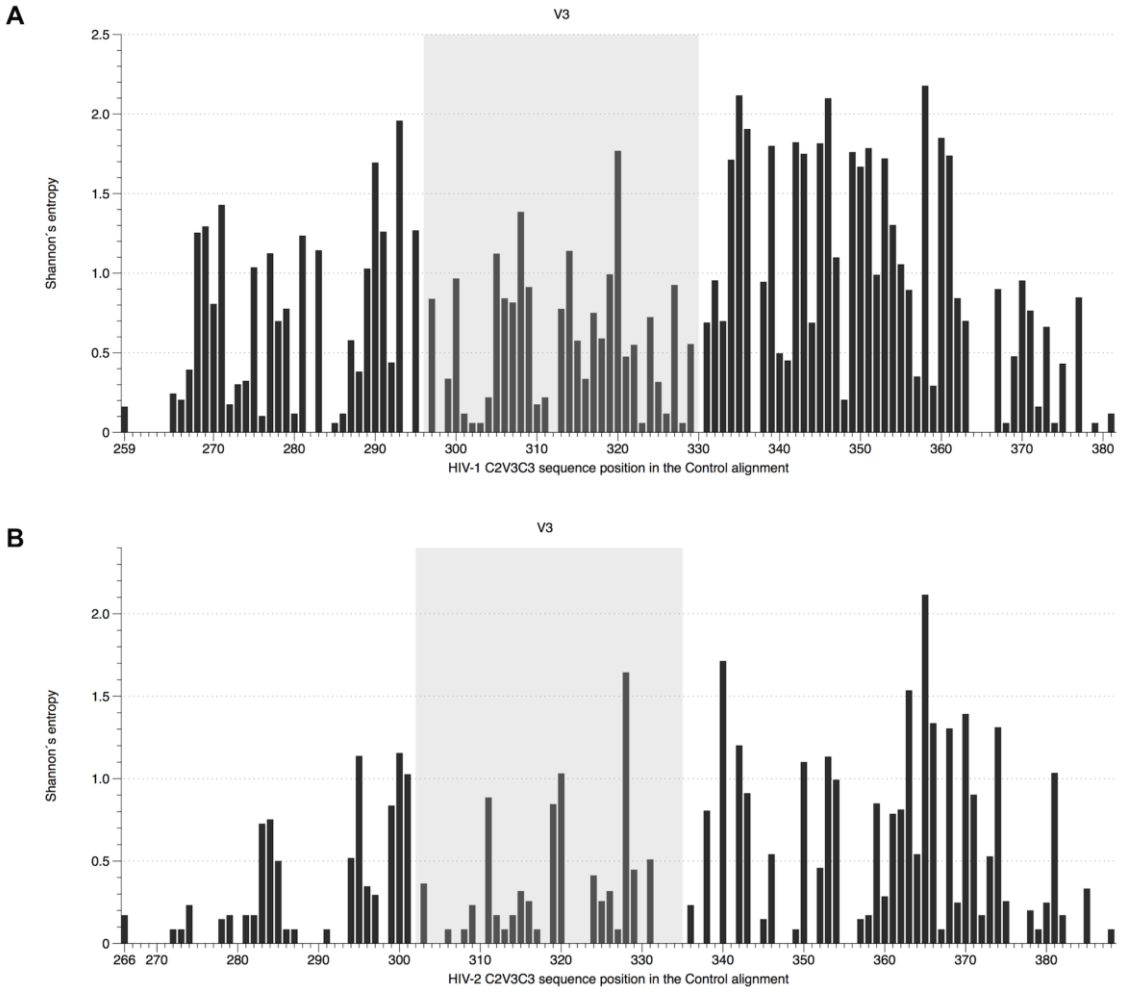

Figure S2. Shannon's entropy of individual amino acids in the C2, V3 and C3 envelope regions in HIV-1 and HIV-2. (**A**) HIV-1 alignment (Control dataset), sites were numbered according to codon *env* position of HIV-1 HXB2 reference strain; (**B**) HIV-2 alignment (Control dataset), sites were numbered according to codon *env* position of HIV-2 ALI reference strain.

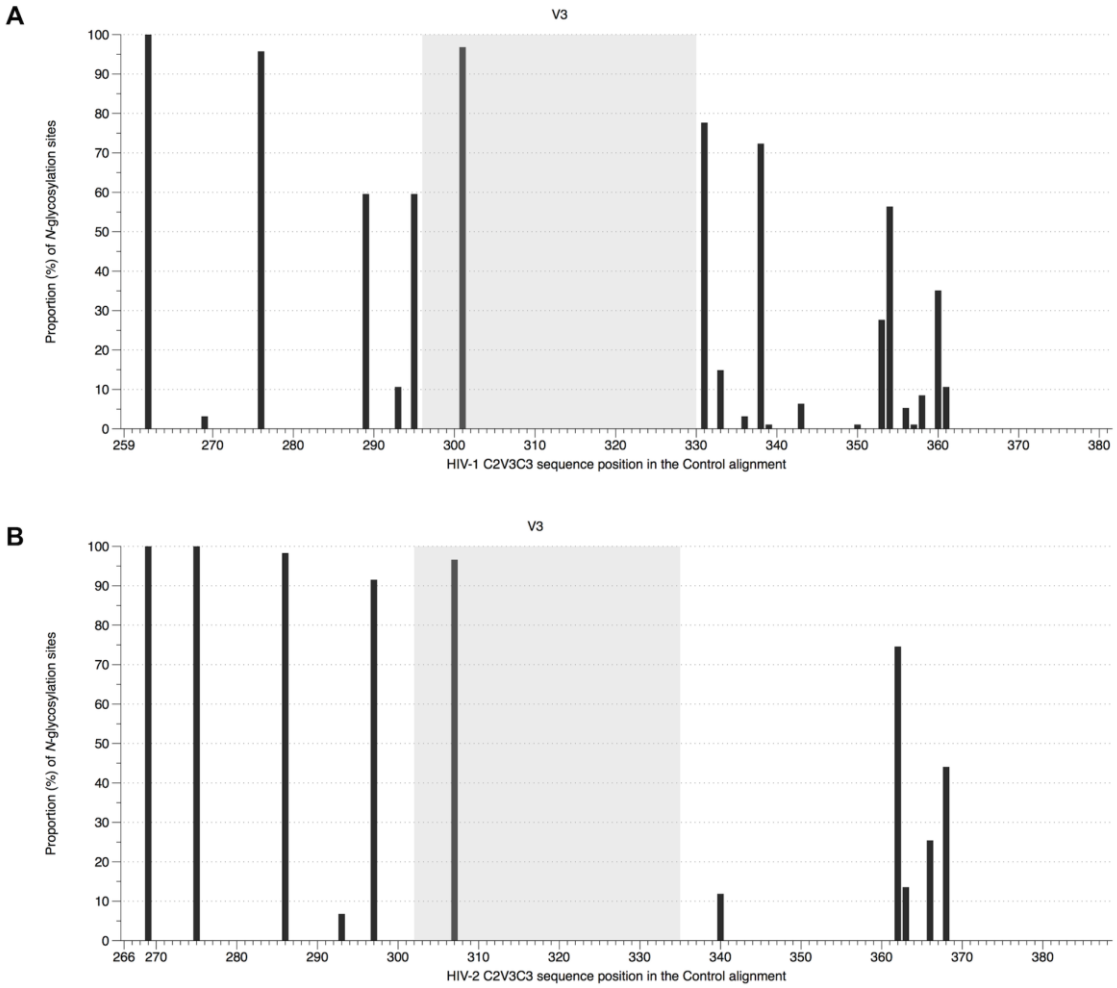

Figure S3. Frequency of N-glycosylation sites in the C2, V3 and C3 envelope regions in HIV-1 and HIV-2. (**A**) HIV-1 alignment (Control dataset). Sites were numbered according to codon *env* position of HIV-1 HXB2 reference strain. (**B**) HIV-2 alignment (Control dataset). Sites were numbered according to codon *env* position of HIV-2 ALI reference strain.

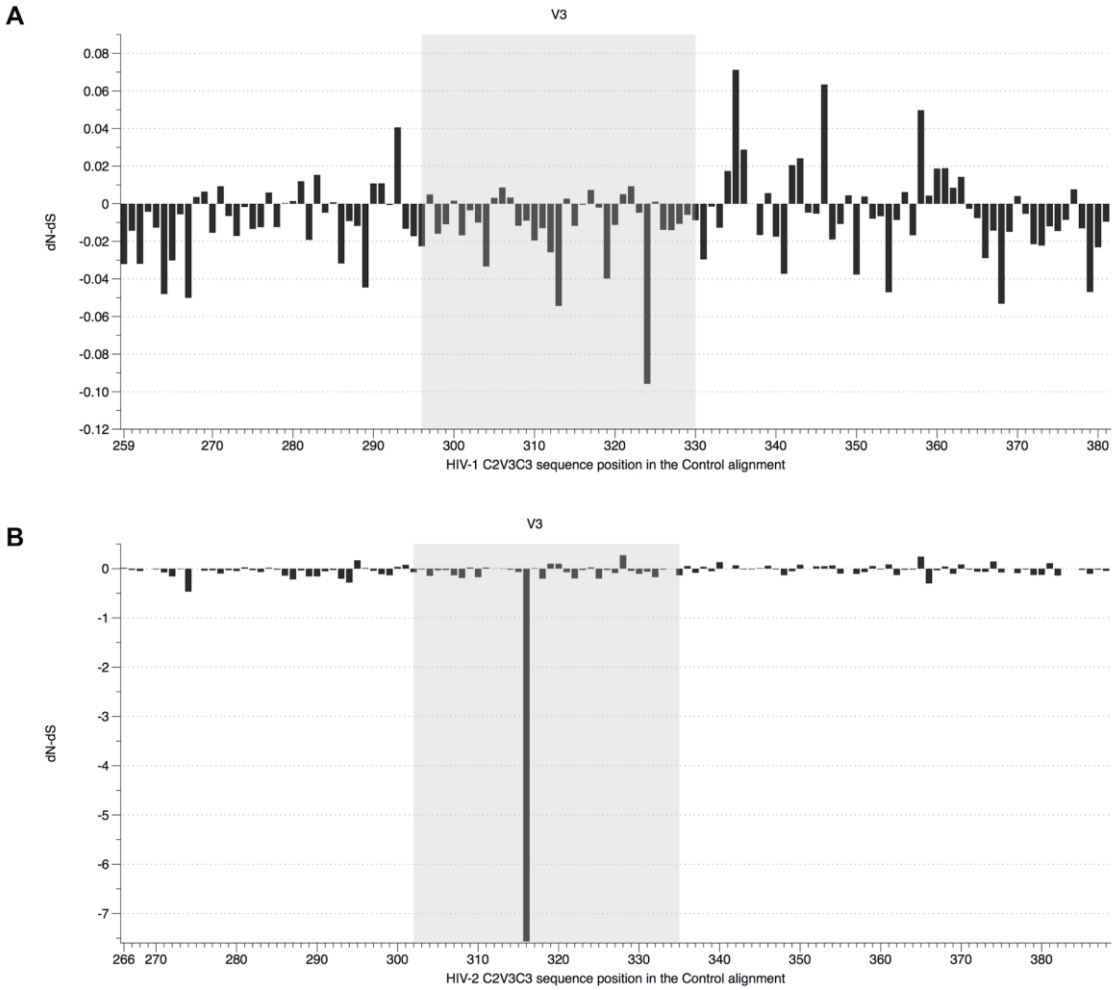

Figure S4. Positive selection in the C2, V3 and C3 envelope regions in HIV-1 and HIV-2. dN-dS values were estimated by FEL and scaled by the total codon tree length. (**A**) HIV-1 alignment (Control dataset). Sites were numbered according to codon *env* position of HIV-1 HXB2 reference strain. (**B**) HIV-2 alignment (Control dataset). Sites were numbered according to codon *env* position of HIV-2 ALI reference strain.

Table S2. Positively selected sites detected by SLAC, FEL, REL and/or IFEL in Control HIV-1 and HIV-2 env C2, V3 and C3 regions.

Control HIV-1										Control HIV-2									
Region	**Codon**	**SLAC**		**FEL**		**REL**		**IFEL**		**Region**	Codon	SLAC		**FEL**		**REL**		IFEL	
C2	268	**1.308**	(0.030)	0.003	(0.893)	0.337	(0.533)	-0.024	(0.271)	**C2**	**295**	**1.031**	(0.039)	**0.169**	(0.021)	**1.469**	(1.000)	0.114	(0.183)
	269	0.145	(0.489)	0.007	(0.513)	**0.776**	(0.949)	0.015	(0.356)		301	**0.658**	(0.092)	**0.077**	(0.097)	-0.362	(<0.001)	0.06	(0.248)
	281	0.787	(0.109)	0.012	(0.193)	**0.821**	(0.992)	0.012	(0.399)										
	283	0.775	(0.118)	**0.020**	(0.023)	**0.837**	(0.999)	0.018	(0.158)										
	290	**1.645**	(0.024)	0.013	(0.574)	-0.033	(0.185)	-0.015	(0.535)										
	291	**1.103**	(0.012)	0.013	(0.201)	**0.824**	(0.959)	0.022	(0.137)										
	293	**2.672**	(<0.001)	**0.060**	(0.003)	**0.817**	(0.948)	**0.054**	(0.021)										
V3	305	**1.034**	(0.062)	0.007	(0.712)	0.593	(0.768)	-0.008	(0.661)	**V3**	320	0.498	(0.229)	**0.099**	(0.099)	**1.197**	(0.990)	**0.28**	(0.007)
	306	**0.972**	(0.035)	0.011	(0.366)	-0.046	(0.262)	-0.010	(0.399)		**328**	**1.357**	(0.024)	**0.273**	(0.007)	**1.011**	(0.999)	0.145	(0.191)
	314	**0.943**	(0.055)	0.005	(0.651)	0.758	(0.931)	-0.009	(0.496)										
	317	**0.706**	(0.027)	0.008	(0.209)	-0.026	(0.486)	**0.018**	(0.075)										
	322	0.576	(0.146)	**0.014**	(0.041)	-0.041	(0.463)	0.005	(0.263)										
C3	332	0.184	(0.420)	0.003	(0.723)	-0.239	(0.057)	**0.031**	(0.047)	**C3**	340	0.77	(0.177)	0.132	(0.299)	**0.756**	(0.968)	0.191	(0.451)
	334	**2.069**	(0.001)	0.030	(0.106)	0.591	(0.773)	0.038	(0.123)		342	0.302	(0.378)	0.068	(0.334)	**0.851**	(0.997)	0.095	(0.397)
	335	**3.582**	(<0.001)	**0.102**	(<0.001)	0.584	(0.761)	**0.075**	(0.006)		346	0.544	(0.129)	**0.059**	(0.067)	-0.245	(<0.001)	0.02	(0.553)
	336	**1.927**	(0.002)	0.029	(0.185)	0.580	(0.761)	0.023	(0.350)		353	0.605	(0.187)	0.049	(0.470)	**0.842**	(0.999)	0.018	(0.840)
	342	**0.465**	(0.026)	0.011	(0.614)	0.307	(0.506)	-0.020	(0.250)		**361**	**0.602**	(0.091)	**0.084**	(0.021	**0.404**	(1.000)	0.042	(0.358)
	343	**0.360**	(0.045)	**0.030**	(0.004)	**0.837**	(1.000)	**0.041**	(0.012)		363	-0.22	(0.706)	-0.022	(0.821)	**0.759**	(0.969)	0.004	(0.976)
	346	**0.767**	(<0.001)	**0.052**	(0.001)	**0.961**	(0.994)	**0.053**	(0.004)		365	**1.499**	(0.043)	0.244	(0.200)	-0.338	(0.557)	0.218	(0.355)
	349	**0.273**	(0.095)	0.004	(0.781)	0.400	(0.594)	0.005	(0.791)		368	-0.17	(0.702)	0.043	(0.581)	**0.837**	(0.998)	0.005	(0.962)
	351	0.037	(0.510)	0.005	(0.657)	**0.770**	(0.943)	-0.005	(0.696)		370	0.639	(0.172)	0.085	(0.287)	**0.85**	(0.999)	0.045	(0.667)
	356	**0.183**	(0.097)	0.005	(0.531)	-0.196	(0.143)	-0.002	(0.887)		374	0.831	(0.102)	**0.145**	(0.095)	**0.926**	(0.999)	**0.245**	(0.065)
	358	0.164	(0.254)	**0.044**	(0.003)	0.708	(0.885)	**0.083**	(0.002)		381	**0.882**	(0.023)	**0.111**	(0.009)	-0.339	(<0.001)	**0.126**	(0.013)
	360	0.263	(0.122)	0.010	(0.507)	0.544	(0.730)	**0.043**	(0.087)										
	361	**0.477**	(0.005)	0.015	(0.247)	**0.796**	(0.960)	**0.041**	(0.032)										
	363	**0.262**	(0.012)	0.010	(0.101)	0.061	(0.661)	0.000	(0.980)										
	370	**0.169**	(0.077)	0.004	(0.587)	-0.103	(0.329)	0.007	(0.432)										
	377	**0.243**	(0.025)	0.007	(0.289)	-0.072	(0.392)	0.000	(0.961)										

Codon – codons selected under 10% level of significance (SLAC, FEL and IFEL), or above a Bayes Factor of 50 (REL) and numbered according to codon *env* position of HIV-1 HXB2 for HIV-1 dataset or of HIV-2 ALI for HIV-2 dataset. Codons selected simultaneously by SLAC, FEL and REL methods are bold and underlined.

SLAC, FEL and IFEL – the first numbers are the dN-dS difference for each site scaled by the total codon tree length, the numbers in parenthesis show P-values for corresponding test of non-synonymous rate being superior to synonymous rate.

REL - the first numbers are the expected posterior dN-dS difference for each site scaled to the total codon tree length, the number in parenthesis show the posterior probability of non-synonymous rate being superior to synonymous rate.

Bold dN-dS differences correspond to significant *P*-values or posterior probabilities.

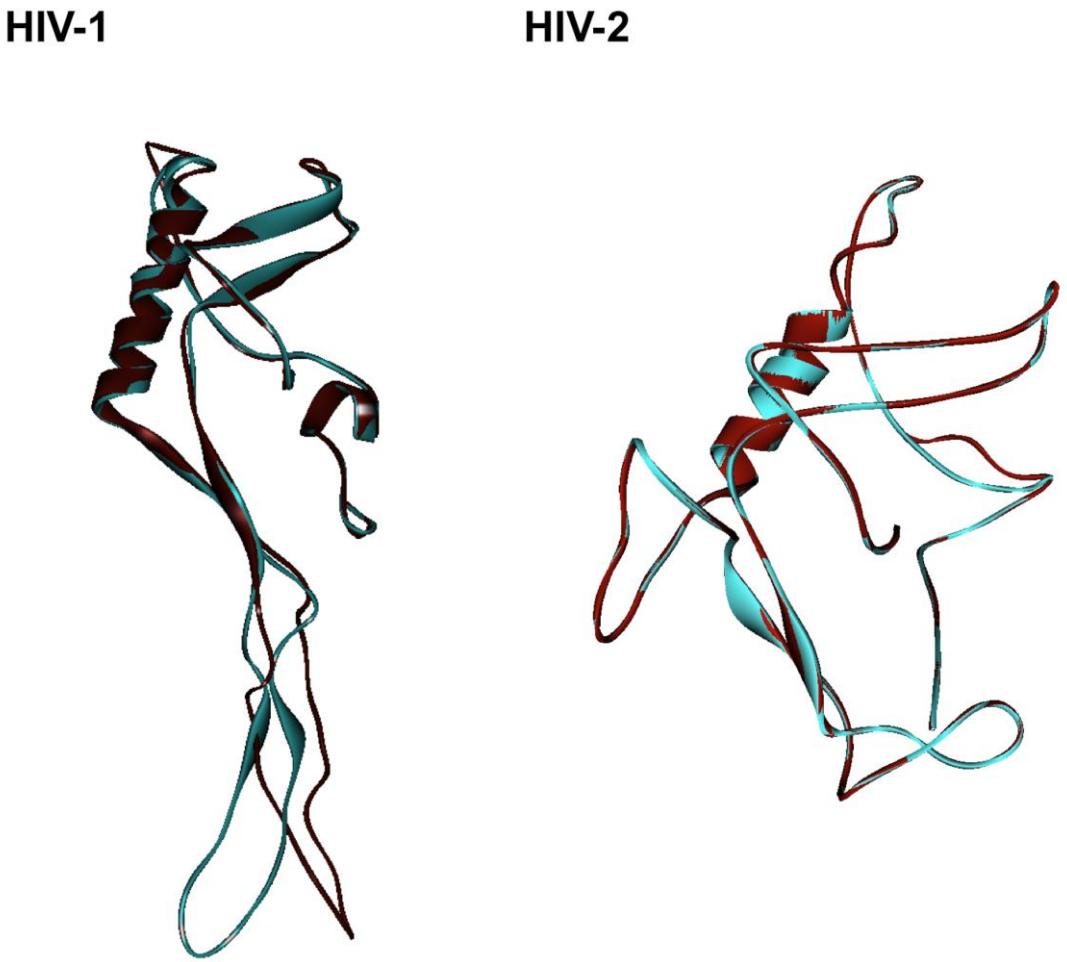

Figure S5. Superimposition of the conformational structures generated by homology modelling of Portuguese and Control C2, V3 and C3 regions of HIV-1 and HIV-2. In the schematics, Portuguese structures are represented in red, and Control structures are in blue.

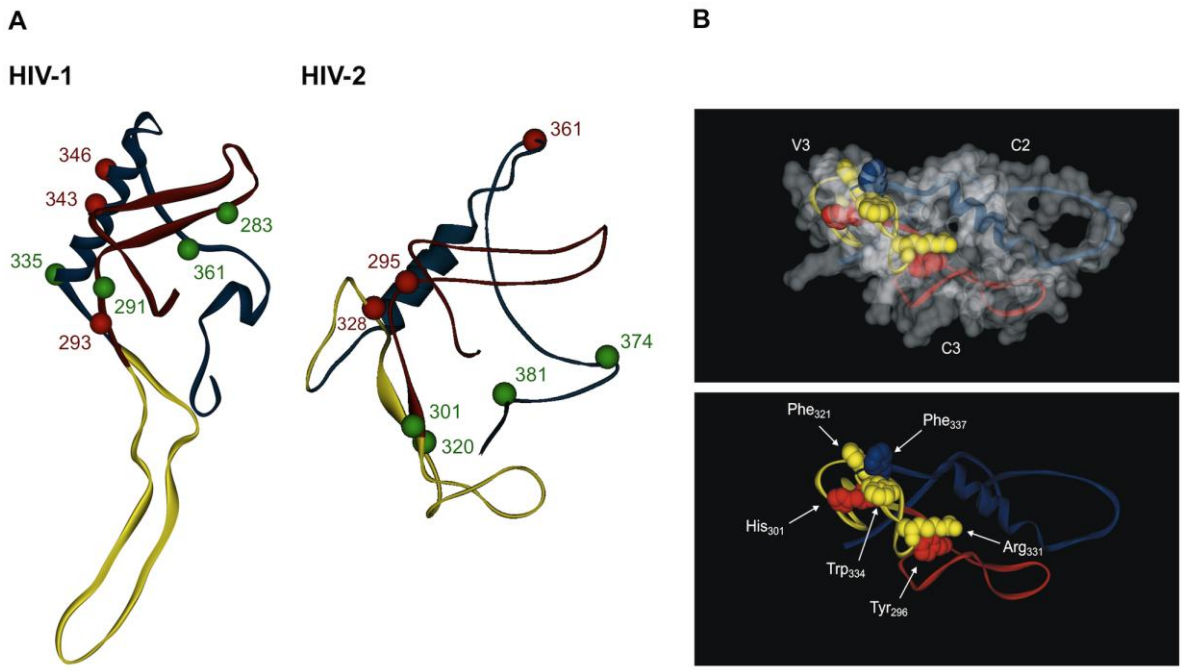

Figure S6. Conformational structure of C2, V3 and C3 envelope regions in HIV-1 and HIV-2. The conformational structure of consensus amino acid sequences derived from the Control datasets was obtained by homology modeling as indicated in Materials and Methods. In the schematics, C2 is shown in red, V3 in yellow, and C3 in blue. Balls represent the amino acids under positive selection. (**A**) The red balls represent codons selected simultaneously by SLAC, FEL and REL methods, while green balls stand for codons selected by at least two of these methods. (**B**) Model structure showing the predicted interactions between V3, C2 and C3 in HIV-2 gp125. The non-covalent interaction involves residues Tyr296 and His301 in C2 binding, respectively, to Arg331 and Trp334 in V3, and Phe337 in C3 binding to Phe321 in V3.

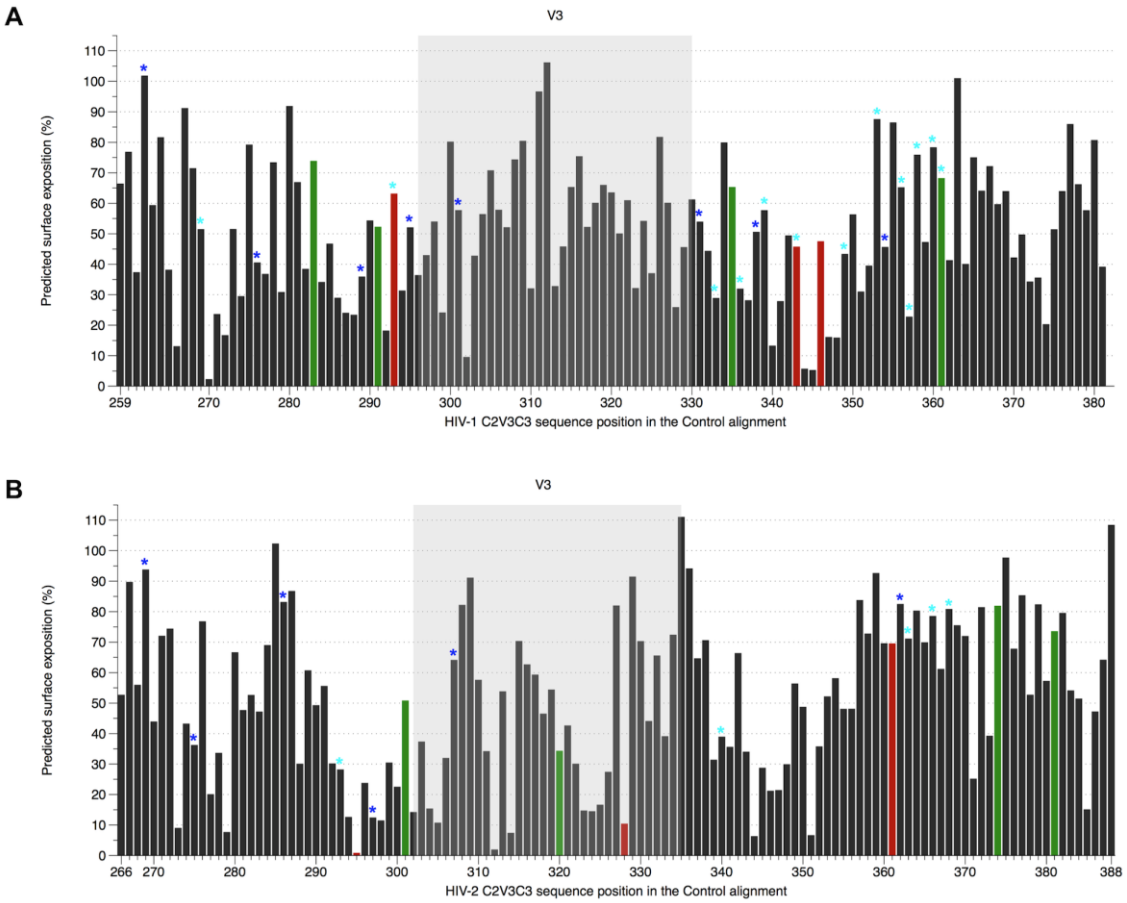

Figure S7. Solvent accessible surface area, positive selection and potential *N*-glycosylation sites in C2, V3 and C3 regions. (**A**) HIV-1 alignment (Control dataset). Sites were numbered according to codon *env* position of HIV-1 HXB2 reference strain. (**B**) HIV-2 alignment (Control dataset). Sites were numbered according to codon *env* position of HIV-2 ALI reference strain. Colored bars represent the amino acids under positive selection and have the same colors (red and green) as the corresponding positions (balls) highlighted in Figure S6. The dark blue stars over the bars correspond to potential *N*-glycosylation sites conserved along the alignment (present in ≥50% of strains), whereas the light blue stars represent sites only present in less than 50% of sequences.

III

HIV-2 susceptibility to entry inhibitors

Baseline susceptibility of primary Human Immunodeficiency Virus Type 2 to entry inhibitors

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Short communication

Manuscript submitted, Antiviral Therapy, June 2011

ABSTRACT

Background: The baseline susceptibility of primary Human Immunodeficiency Virus Type 2 (HIV-2) to maraviroc (MVC) and other entry inhibitors is currently unknown.

Methods: The susceptibility of nineteen HIV-2 isolates obtained from asymptomatic and AIDS patients and seven HIV-1 clinical isolates to the fusion inhibitors enfuvirtide (T-20) and T-1249, and to the coreceptor antagonists AMD3100, TAK-779 and MVC was measured using a TZM-bl cell-based assay. 50% (IC_{50}) and 90% (IC_{90}) inhibitory concentrations and dose-response curve slopes were determined for each drug.

Results: T-20 and T-1249 were significantly less active on HIV-2 than on HIV-1 (211- and 2-fold, respectively). AMD3100 and TAK-779 inhibited HIV-2 and HIV-1 X4 and R5 variants with similar IC_{50s} and IC_{90s}. MVC, however, inhibited the replication of R5 HIV-2 variants with significantly higher IC_{90s} (42.7 vs 9.7 nM, $P < 0.0001$) and lower slope values than HIV-1 (0.7 vs 1.3, $P < 0.0001$). HIV-2 R5 variants derived from AIDS patients were significantly less sensitive to MVC than variants from asymptomatic patients, this being inversely correlated with the absolute number of $CD4^+$ T cells.

Conclusions: T-1249 is a potent inhibitor of HIV-2 replication indicating that new fusion inhibitors might be useful to treat HIV-2 infection. Coreceptor antagonists TAK-779 and AMD3100 are also potent inhibitors of HIV-2 replication. The reduced sensitivity of R5 variants to MVC, especially in severely immunodeficient patients, indicates that the treatment of HIV-2 infected patients with MVC might require higher dosages than those used in HIV-1 patients which should be adjusted to the disease stage.

Keywords: HIV-2 primary isolates; fusion inhibitors; enfuvirtide; coreceptor antagonists; maraviroc.

INTRODUCTION

HIV-2 affects an estimated 1-2 million individuals worldwide and leads to AIDS and death albeit at a slower pace when compared to HIV-1. All currently available antiretroviral drugs were specifically designed to inhibit HIV-1 entry and replication. Consequently, some drugs classes are not active on HIV-2 (non-nucleoside reverse transcriptase and fusion inhibitors) and virological and immunological responses to treatment regimens incorporating active drugs are usually poorer in HIV-2 patients [1].

The envelope glycoproteins of HIV-1 and HIV-2 are markedly different at the genetic, structural and functional levels. In contrast to HIV-1, HIV-2 may enter cells without binding to CD4 and using multiple alternative co-receptors besides CCR5 and CXCR4 [2,3]. This suggests that maraviroc (MVC), a CCR5 antagonist, might also have limited activity against

HIV-2. Currently, there is no information concerning the *in vitro* susceptibility of HIV-2 primary isolates to MVC, enfuvirtide (T-20) or any other entry inhibitor. In the absence of formal clinical trials, *in vitro* evaluation of the baseline susceptibility of HIV-2 primary isolates to MVC is crucial to assess the potential clinical value of this drug in HIV-2 therapy [4]. Here we have analysed the susceptibility of HIV-2 primary isolates obtained from asymptomatic and AIDS patients to the fusion inhibitors T-20 and T-1249 and to the coreceptor antagonists AMD3100, TAK-779 and MVC.

METHODS

Primary isolates were obtained from HIV-2-infected Portuguese patients and, for comparison, from HIV-1-infected Angolan patients, all naïve to therapy with entry inhibitors, by cocultivation with PBMCs from seronegative subjects (Table 1) [5]. Virus genotyping was performed by phylogenetic analysis using C2-V3-C3 (HIV-2) or gp41 (HIV-1) *env* sequences (HIV-1 primers described in Supplementary Table S1). GenBank accession number for newly derived HIV-2 and HIV-1 sequences are: HIV-2, HQ738345 – HQ738350; HIV-1, HQ738338 - HQ738344.

CCR5 and CXCR4 tropism was determined using a single-round viral infectivity assay performed with TZM-bl reporter cells ($CD4^+$, $CCR5^+$, $CXCR4^+$) in the presence of excessive amounts of the CCR5 antagonist TAK-779 (10 μM) and/or of the CXCR4 antagonist AMD3100 (1.2 μM), as previously described [6]. The 50% and 90% inhibitory concentrations (IC_{50s} and IC_{90s}) and dose-response curve slopes (Hill slopes) of T-20 and T-1249 (fusion inhibitors) and AMD3100, TAK-779 and MVC (coreceptor antagonists) were determined on the newly derived panel of isolates (200 $TCID_{50}$ for each virus) using also the TZM-bl reporter cell assay. IC_{50s}, IC_{90s} and Hill slopes were estimated by the sigmoidal dose-response (variable slope) equation in Prism version 4.0c for Macintosh (GrahPad Software, San Diego, California USA, www.graphpad.com). Prism was also used for statistical analyses (level of significance of 5%).

RESULTS

Genotypic and phenotypic characterization of virus isolates

Nineteen new HIV-2 primary isolates were used in this study, all belonging to group A (Table 1 and Figure S1). Ten were CCR5 tropic (R5 isolates), eight CXCR4 tropic (X4 isolates) and one used both coreceptors [dual/mixed population (D/M)]. The seven new HIV-1 primary isolates were all R5 and their genotypes were distributed as follows: subtype

G (1 isolate), J (2) and CRF02_AG (1); 3 isolates were untypable (U) (Table 1 and Figure S2).

Antiviral activity of coreceptor antagonists

AMD3100 and TAK-779 inhibited the replication of HIV-1 and HIV-2 with similar IC_{50s} and IC_{90s} and similar slope values (Tables 1 and 2). MVC also inhibited the replication of HIV-2 and HIV-1 R5 variants with similar IC_{50s} (Table 1); for HIV-1, the IC_{50s} were similar to previously reported values (range, 0.1 – 4.5 nM) (Table 1) [7]. However, MVC inhibited the replication of R5 HIV-2 variants with significantly higher IC_{90s} (42.7 vs 9.7 nM, P < 0.0001) and lower slope values (0.7 vs 1.3, P < 0.0001) than HIV-1 (Figure 1A; Table 2). R5 variants isolated from HIV-1 patients after AIDS diagnosis have reduced sensitivity to TAK-779 as compared to R5 variants isolated at the asymptomatic stage [8,9]. Strikingly, we also found a strong and significant negative correlation between HIV-2 sensitivity to MVC (as determined by the IC_{50s}) and $CD4^+$ T cell counts at the time of virus isolation (Spearman r = -0.831; *P* = 0.008) (Figure 1B). Consistent with this, isolates from AIDS patients were significantly less sensitive to MVC (required significantly higher IC_{50s} to inhibit replication) than isolates from asymptomatic patients (Figure 1C). A similar tendency was observed for TAK-779 (Figure S3). In all, these results demonstrate that HIV-2 R5 variants have lower sensitivity to MVC than HIV-1 and suggest that resistance of these variants to MVC increases as disease progresses [8,9].

Antiviral activity of fusion inhibitors

In this study, T-20 was 211-fold less active against primary isolates of HIV-2 than against HIV-1 (mean IC_{50}, 281.5 *vs* 1.2 nM, *P*<0.0001) (Figure 1D; Table 2), confirming and extending previous results based on lab-adapted isolates [10]. Interestingly, with one exception, all HIV-1 primary isolates exhibited high sensitivity to T-20 (Table 1). Sequencing analysis showed that these isolates carried the N42S polymorphism in the gp41 glycoprotein whilst the less sensitive strain did not (data not shown). These polymorphism, which is more prevalent in several non-B subtypes and recombinant forms than in subtype B, has previously been associated with higher sensitivity to T-20 both in B and non-B HIV-1 subtypes [11].

In contrast to T-20, T-1249 was active on HIV-2 although at higher concentrations than on HIV-1 (IC_{50}, 4.3 vs 2.0 nM; *P*<0.0001). Moreover, T-1249 was more active on X4 than on R5 isolates both in HIV-1 (IC_{50}, 0.6 *vs* 2.9 nM; *P*<0.0001) and HIV-2 (IC_{50}, 3.2 *vs* 6.1 nM; *P*=0.0005).

Table 1. Clinical characterization of HIV patients and primary isolates including their susceptibility to different entry inhibitors.

Isolates[a]	Clinical characterization			Genetic forms[c]	Coreceptor use[d]	Antiviral activity[e]									
						AMD3100 (nM)		TAK-775 (nM)		Maraviroc (nM)		T-20 (nM)		T-1249 (nM)	
	$CD4^+$ T cells/ml at study entry	HIV RNA copies/ml	Antiretroviral therapy[b]			IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
HIV-1															
93AOHDC249	na	na	na	U [c]	R5	-	-	6.1	173.0	1.0	6.1	78.8	1285.3	8.0	13.2
93AOHDC250	na	na	na	J	R5	-	-	8.1	7516.2	2.4	9.7	0.4	15.7	1.9	8.7
93AOHDC251	na	na	na	U	R5	-	-	5.7	23388.4	1.5	9.2	1.3	170.2	1.8	43.6
93AOHDC252	na	na	na	U	R5	-	-	153.5	24434.3	4.7	48.5	3.7	23.5	2.6	8.0
93AOHDC253	na	na	na	J	R5	-	-	15.7	423.6	1.4	4.5	0.1	2.0	1.4	8.6
01PTHDECJN	1003	< 400	na	CRF02_AG	R5	-	-	178.4	5942.9	2.7	11.9	0.7	132.1	1.6	6.7
00PTHDEEBB	409	2742788	na	G	R5	-	-	2.6	2844.5	0.8	3.8	1.5	33.6	5.5	78.5
NL4-3	-	-	-	B	X4	0.9	6.0	-	-	-	-	5.0	178.6	0.6	1.0
SG3.1	-	-	-	B	X4	5.2	29.2	-	-	-	-	0.1	0.5	0.4	6.9
HIV-2															
03PTHCC1	308	< 200	+	A	R5	-	-	0.6	1219.0	0.9	4.8	35.6	877.0	5.1	49.3
03PTHCC6	615	< 200	+	A	R5	-	-	10.1	2301.4	0.9	32.3	661.1	2192.8	8.4	74.1
03PTHCC7	144	< 200	+	A	R5	-	-	16.2	3581.0	2.9	28.9	549.1	2138.0	7.3	12.6
03PTHCC12	66	< 200	-	A	R5	-	-	45.1	7030.7	3.8	78.7	2857.0	32062.7	6.3	36.6
03PTHCC17	367	< 200	+	A	R5	-	-	3.0	55080.8	0.9	27.9	138.4	2162.7	2.5	40.3
03PTHCC19	175	na	-	A	R5	-	-	128.3	167880.4	4.3	81.5	250.0	1729.8	7.2	24.6
00PTHDECT	2919	1355	-	A	R5	-	-	24.8	3741.1	1.6	61.1	109.3	881.0	2.3	24.4
10PTHSJIG	164	4257	+	A	R5	-	-	121.8	8128.3	5.5	108.6	586.3	14092.9	21.9	412.1
03PTHSM2	275	< 200	+	A	R5	-	-	8.8	15922.1	2.4	53.1	114.0	4375.2	3.4	61.9
10PTHSMNC	231	< 200	+	A	R5	-	-	57.4	3396.3	2.2	40.1	265.4	3507.5	8.9	71.3
10PTHSMAK	40	1793	+	A	D/M	3.2	17.9	0.7	29922.6	116.0	30903.0	125.2	1458.8	1.5	45.6
ROD	-	-	-	A	X4	1.0	16.1	-	-	-	-	76.1	3380.6	9.1	174.6
03PTHCC10	48	< 200	+	A	X4	3.6	78.3	-	-	-	-	293.6	3047.9	2.4	12.4
00PTHCC20	1033	< 200	-	A	X4	1.9	17.5	-	-	-	-	151.3	1422.3	0.9	8.4
03PTHCC20	78	< 200	+	A	X4	2.0	18.6	-	-	-	-	362.7	3548.1	1.9	10.9
03PTHDECT	209	20968	na	A	X4	1.6	20.7	-	-	-	-	373.4	5520.8	2.1	32.1
01PTHDESC	44	1250	+	A	X4	4.0	32.7	-	-	-	-	241.5	3672.8	4.9	49.1
03PTHSM9	15	< 200	+	A	X4	4.2	27.7	-	-	-	-	1281.0	6729.8	7.0	12.6
04PTHSM10	265	4792	+	A	X4	3.6	47.1	-	-	-	-	293.6	3047.9	6.7	24.3
10PTHSMAUC	177	< 200	-	A	X4	3.0	20.9	-	-	-	-	167.3	952.8	1.7	12.0

(please find the annotations on the next page)

[a] Lab-adapted reference strains NL4-3 (HIV-1), SG3.1 (HIV-1) and ROD (HIV-2) were obtained by transfection of 293T cells with pNL4-3 (HIV-1), pSG3.1 (HIV-1) or pROD10 (HIV-2) plasmids.

[b] +, yes; -, no.

[c] U, untypable HIV-1: 93AOHDC249 and 93AOHDC252, sequences are basal to subtypes 19_cpx and 37_cpx; 93AOHDC251 sequence is basal to subtype H (Figure S2).

[d] R5, CCR5 coreceptor usage; X4, CXCR4 coreceptor usage; D/M, Dual/Mixed viral population using CCR5 and CXCR4 coreceptors.

[e] IC_{50} and IC_{90} best-fit values were inferred from sigmoidal dose-response (variable slope) curves and represent geometric mean values of two independent experiments performed in duplicate wells; AMD3100 was only tested against X4 isolates, while TAK-779 and maraviroc were tested against R5 isolates.

na, not available.

Table 2. Comparison of antiviral activities of the different entry inhibitors on HIV-1 and HIV-2 primary isolates

Antiviral	Parameter[a]	HIV-1 Mean (95% confidence interval)	HIV-2[b] Mean (95% confidence interval)	*P* value[c]
AMD3100 (nM)				
(HIV-1 n=2; HIV-2 n=9)	IC_{50}	2.1 (1.1 - 3.8)	2.6 (2.2 - 3.0)	0.288
	IC_{90}	16.7 (4.4 - 62.8)	29.0 (20.8 - 40.5)	0.213
	Hill slope	1.0 (0.5 – 1.6)	0.9 (0.8 - 1.0)	0.391
TAK-779 (nM)				
(HIV-1 n=7; HIV-2 n=10)	IC_{50}	23.3 (12.0 - 45.4)	18.9 (11.8 - 30.3)	0.595
	IC_{90}	5200.0 (1161.4 - 23334.6)	11587.8 (3899.4 - 34514.4)	0.379
	Hill slope	0.4 (0.3 – 0.5)	0.3 (0.3 – 0.4)	0.237
Maraviroc (nM)				
(HIV-1 n=7; HIV-2 n=10)	IC_{50}	1.7 (1.4 - 2.2)	2.1 (1.7 - 2.6)	0.201
	IC_{90}	9.7 (6.6 - 14.4)	42.7 (26.6 - 68.4)	< 0.0001
	Hill slope	1.3 (1 – 1.6)	0.7 (0.6 – 0.8)	< 0.0001
T-20 (nM)				
(HIV-1 n=9; HIV-2 n=20)	IC_{50}	1.2 (0.7 - 2.2)	281.5 (223.2 - 354.9)	< 0.0001
	IC_{90}	95.9 (26.3 - 350.8)	3881.5 (2393.3 - 6280.6)	< 0.0001
	Hill slope	0.5 (0.4 – 0.6)	0.8 (0.7 – 1)	0.001
T-1249 (nM)				
(HIV-1 n=9; HIV-2 n=20)	IC_{50}	2.0 (1.4 - 2.8)	4.3 (3.6 - 5.2)	< 0.0001
	IC_{90}	14.3 (6.9 - 29.5)	40.6 (28.1 - 58.5)	0.006
	Hill slope	1.1 (0.8 – 1.4)	1 (0.8 – 1.1)	0.426

[a] IC_{50}, IC_{90} and Slope best-fit values were inferred from sigmoidal dose-response (variable slope) curves adjusted to combined results of HIV-1 and HIV-2 isolates.

[b] Estimates for AMD3100, TAK-779 and maraviroc didn't include the HIV-2 10PTHSMAK isolate, a virus with Dual/Mixed tropism.

[c] *P* value for comparison of best-fit values between HIV-1 and HIV-2, using the F test.

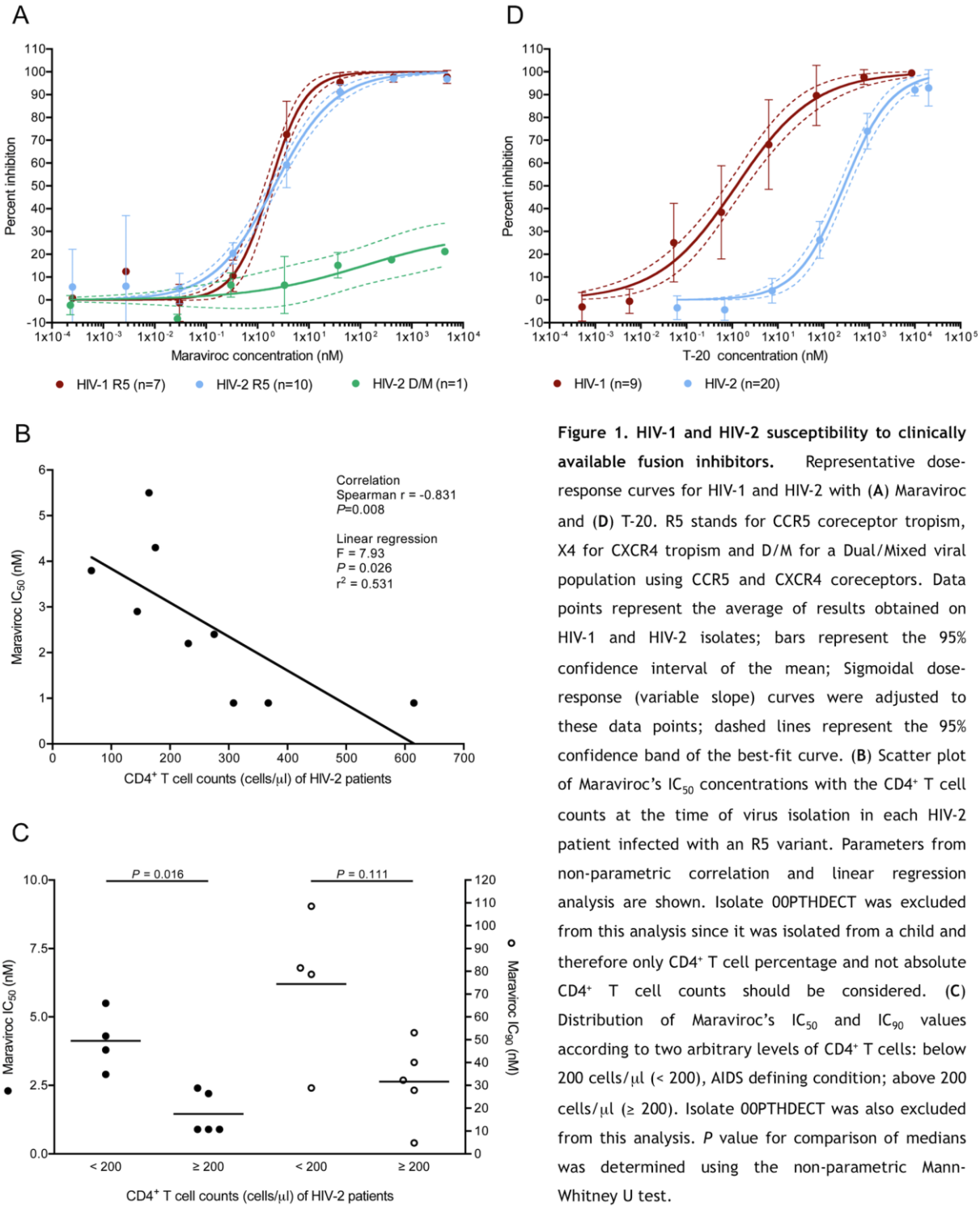

Figure 1. HIV-1 and HIV-2 susceptibility to clinically available fusion inhibitors. Representative dose-response curves for HIV-1 and HIV-2 with (**A**) Maraviroc and (**D**) T-20. R5 stands for CCR5 coreceptor tropism, X4 for CXCR4 tropism and D/M for a Dual/Mixed viral population using CCR5 and CXCR4 coreceptors. Data points represent the average of results obtained on HIV-1 and HIV-2 isolates; bars represent the 95% confidence interval of the mean; Sigmoidal dose-response (variable slope) curves were adjusted to these data points; dashed lines represent the 95% confidence band of the best-fit curve. (**B**) Scatter plot of Maraviroc's IC_{50} concentrations with the $CD4^+$ T cell counts at the time of virus isolation in each HIV-2 patient infected with an R5 variant. Parameters from non-parametric correlation and linear regression analysis are shown. Isolate 00PTHDECT was excluded from this analysis since it was isolated from a child and therefore only $CD4^+$ T cell percentage and not absolute $CD4^+$ T cell counts should be considered. (**C**) Distribution of Maraviroc's IC_{50} and IC_{90} values according to two arbitrary levels of $CD4^+$ T cells: below 200 cells/μl (< 200), AIDS defining condition; above 200 cells/μl (≥ 200). Isolate 00PTHDECT was also excluded from this analysis. *P* value for comparison of medians was determined using the non-parametric Mann-Whitney U test.

DISCUSSION

We have demonstrated that MVC inhibits the replication of R5 HIV-2 variants with significantly higher IC_{90} and lower slope values than HIV-1 indicating that higher dosages of MVC might be required for the treatment of HIV-2 infected patients [12,13]. So far, MVC use in HIV-2 infection was reported on only two occasions with uncertain results [14,15]. Clinical trials are therefore needed to determine if the MVC dosages recommended in HIV-1 infection are also effective for HIV-2 infection. This may prevent the administration of sub-therapeutic dosages that favour the selection of X4 variants which, in HIV-2, have been associated not only with CD4 depletion and disease progression [2] but also with resistance to neutralization [16].

Similarly to previous results obtained with RANTES for HIV-2 [17] and with TAK-779 and C-C chemokines for HIV-1 [8,9], MVC inhibits the replication of R5 HIV-2 variants isolated from AIDS patients with significantly higher IC_{50s} than R5 variants isolated from asymptomatic patients this being inversely associated with the number of $CD4^+$ T cells. In HIV-2 infected patients, CD4 depletion and higher immune activation are also closely associated with a increased frequency of memory $CD4^+$ T cells expressing CCR5, the preferential target cells of this virus [18]. Hence, these results suggest that in HIV-2 infected patients MVC dosage may need to be adjusted according to the number of $CD4^+$ T cells (higher dosage in severely immunodeficient patients and lower dosage in asymptomatic patients). Increased MVC resistance of late stage disease R5 variants might be explained by increased affinity for CCR5 [19] and/or an enhanced viral infectivity and replicative capacity [8,19]. Alternatively, these R5 variants may be evolutionary intermediates toward X4 use [8,17].

The reduced activity of T-20 on primary HIV-2 isolates provides definitive evidence that T-20 is not useful for HIV-2 therapy. The low activity of T-20 in HIV-2 is likely related with the high genetic variability between HIV-1 and HIV-2 in the HR1 and HR2 domains in the gp41 glycoprotein [10,20]. On the other hand, T-1249 a second-generation fusion inhibitor available only for research use was highly active on both HIV-1 and HIV-2 indicating that new fusion inhibitors (peptides or small-molecules) might be useful to treat HIV-2 infection.

In summary, primary isolates of HIV-1 and HIV-2 with X4 or R5 tropism have similar sensitivities to AMD3100 and TAK-779, respectively. However, significantly higher 90% inhibitory concentrations of MVC are required to inhibit replication of HIV-2 R5 variants than HIV-1 variants. Additionally, the sensitivity of HIV-2 R5 variants to this drug is inversely related with CD4+ T cell counts at time of virus isolation. If MVC is to be used in HIV-2 patients, clinical trials should be performed to fully evaluate the clinical efficacy of this drug in HIV-2 infection and determine the best therapeutic dosage in early and late

stage disease. Because X4 HIV-2 variants and dual/mixed HIV-2 populations are totally or partially resistant to MVC, coreceptor tropism should be determined before initiation of MVC therapy in HIV-2 infected patients. To this end, genotypic tropism assays, possibly based on the sequence of the V3 loop [2], should be developed to facilitate tropism assignment. Once used regularly in HIV-2 patients, the impact of MVC in the phenotypic evolution of this virus *in vivo* should be fully investigated as MVC has the potential to select for HIV-2 X4 variants that are associated with bad disease prognosis.

ACKNOWLEDGEMENTS

This work was supported by Fundação para a Ciência e Tecnologia [grant number PTDCSAU-FCF6767/2006], Portugal, and by Collaborative HIV and Anti-HIV Drug Resistance Network (CHAIN), from the European Union. PB, RC, IB and CR are supported by PhD grants from Fundação para a Ciência e Tecnologia. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: bicyclam JM-2987 (hydrobromide salt of AMD-3100); pNL4-3 from Dr. Malcolm Martin; pSG3.1 from Drs. Sajal Ghosh; T-20 (enfuvirtide, Fusion Inhibitor from Roche; TAK-779; TZM-bl from John C. Kappes, Xiaoyun Wu, and Tranzyme, Inc. pROD10 plasmid was a kind gift from Keith Peden. Trimeris Inc (USA) and Pfizer Inc (USA) provided T-1249 and maraviroc, respectively.

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SUPPLEMENTARY MATERIAL

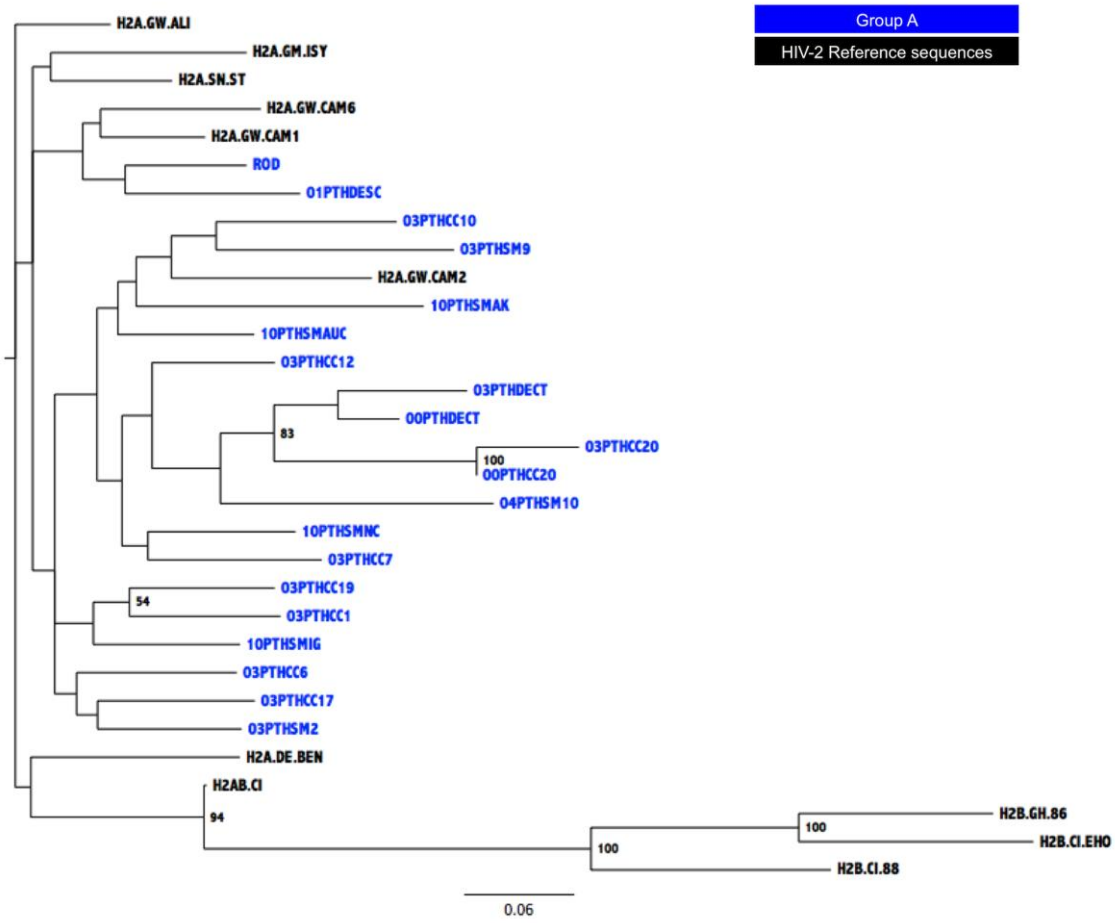

Figure S1. Genotyping HIV-2 by maximum-likelihood phylogenetic analysis. The phylogenetic tree was constructed in PAUP* using the NNI heuristic search strategy and 1000 bootstrap replications, with reference sequences from HIV-2, under the TVM+I+G evolutionary model. The bootstrap values (above 50%) supporting the internal branches are shown. The scale bar represents evolutionary distances in substitutions per site.

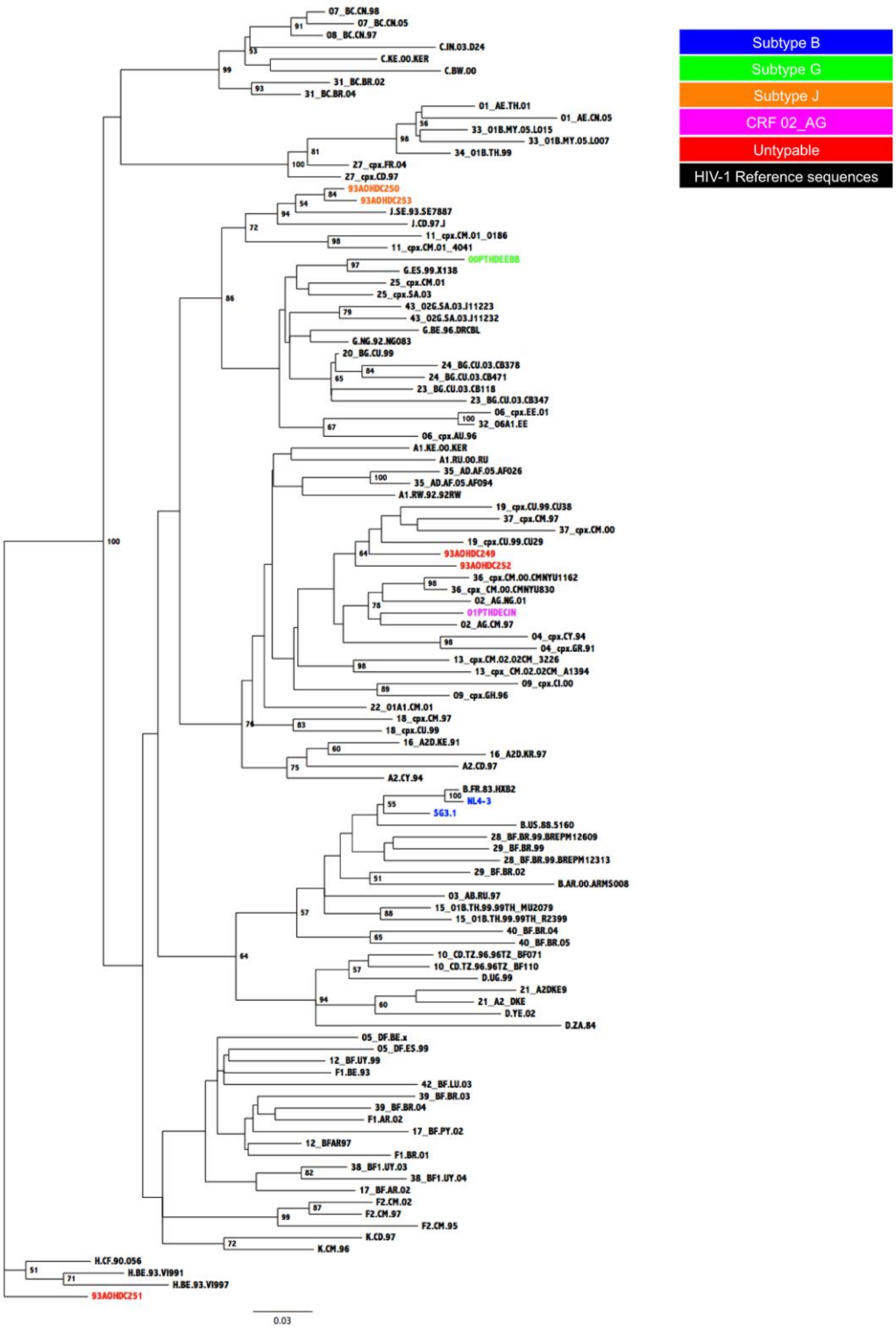

Figure S2. Genotyping HIV-1 by maximum-likelihood phylogenetic analysis. The phylogenetic tree was constructed in PAUP* using the NNI heuristic search strategy and 1000 bootstrap replications, with reference sequences from HIV-1, under the GTR+I+G evolutionary model. The bootstrap values (above 50%) supporting the internal branches are shown. The scale bar represents evolutionary distances in substitutions per site.

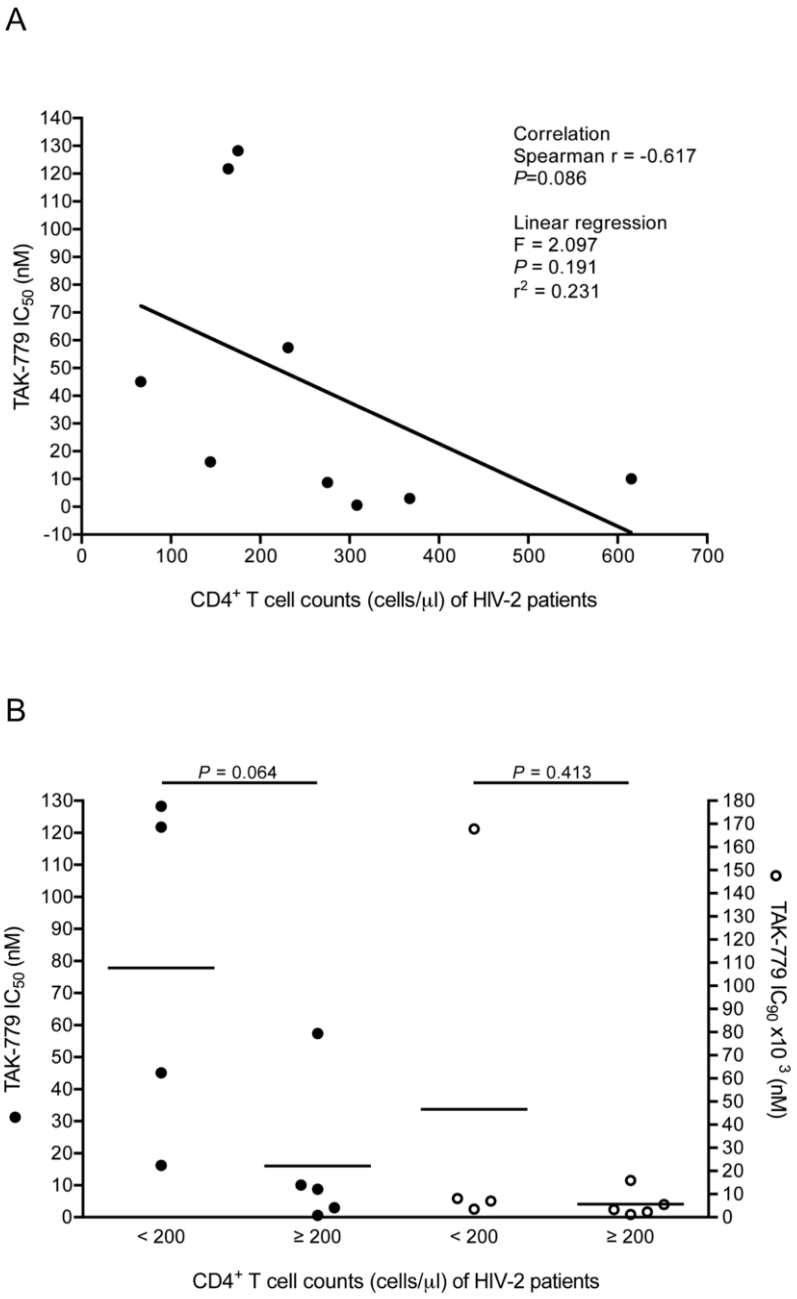

Figure S3. Association between the HIV-2 susceptibility to TAK-779 and the immuno-deficiency degree of HIV-2 infected patients. (**A**) Scatter plot of IC_{50} concentrations with $CD4^+$ T cell counts at the time of virus isolation in each HIV-2 patient infected with an R5 variant. Parameters from non-parametric correlation and linear regression analysis are shown. Isolate 00PTHDECT was excluded from this analysis since it was isolated from a child and therefore only $CD4^+$ T cell percentage, and not absolute $CD4^+$ T cell counts, should be considered. (**B**) Distribution of IC_{50} and IC_{90} values according to two arbitrary levels of $CD4^+$ T cells: below 200 cells/μl (< 200), AIDS defining condition; above 200 cells/μl (≥ 200). Isolate 00PTHDECT was also excluded from this analysis. *P* value for comparison of medians, using the non-parametric Mann-Whitney U test.

IV

Design and evaluation of an ancestral HIV fusion inhibitor peptide

Design and evaluation of an ancestral peptide with potent and broad HIV fusion inhibitor activity

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Research article

Manuscript submitted, July 2011

ABSTRACT

Background: Enfuvirtide (T-20) is the only inhibitor of HIV-1 fusion in clinical use. It has no activity against HIV-2 and its genetic barrier to resistance is low. The aim of this work was to use evolutionary biology methods to produce new peptides with broad and potent HIV-1 and HIV-2 fusion inhibitor activity.

Methods: We reconstructed ancestral transmembrane protein sequences at several nodes of the envelope gene phylogenies that represent ancestors to diverse HIV and SIV virus clades. Several peptides were derived from the helical region 2 (HR2) of these ancestral protein sequences. The antiviral activity [50% (IC_{50}) and 90% (IC_{90}) inhibitory concentrations] of one selected peptide (named P3) was examined on 20 HIV-2 isolates and nine highly divergent HIV-1 isolates from untreated patients and on four T-20- resistant strains using a TZM-bl cell-based assay. Circular dichroism was used to determine the secondary structure of P3. ELISA assays were developed to measure binding of P3 to a recombinant transmembrane envelope protein and to determine the antigenic reactivity of P3 with plasma from HIV-1 and HIV-2-infected patients. Selection of P3 resistance mutations was attempted using increasing concentrations of the peptide in HIV infected PBMCs.

Results: P3 has 34 residues and overlaps the N-terminal pocket-binding region and heptad repeat (HR) core of the HR2 region. It differs by 21 aa from the consensus HIV-1 sequence, 14 aa from T-20 and 6 aa from consensus HIV-2. In contrast to T-20, P3 forms a typical α-helix structure in solution and potently inhibits both HIV-1 and HIV-2 replication (mean IC_{50} HIV-1, 11 nM vs IC_{50} HIV-2, 63.8 nM; $P < 0.0001$). P3 also potently inhibits the replication of T-20 resistant HIV-1 isolates harbouring the V38A, V38A/N42D and V38A/N42T mutations (IC_{50} range, 0.15 – 11.8 nM). In a primary isolate of HIV-1, the N43K resistance mutation (HR1 region) was selected in the presence of P3 and led to a moderate (120-fold) decrease in susceptibility to this peptide, when compared to HIV-1 NL4-3. Under the same conditions we were not able to select HIV-2 resistant strains. Finally, P3 bound strongly to a recombinant HIV transmembrane envelope protein and was significantly less antigenic than T-20 in drug naive HIV-1 infected patients.

Conclusions: P3 is the first ancestral peptide to exhibit a broad and potent activity against both HIV-1 (including variants resistant to T-20) and HIV-2. The results indicate that the HR1 region in the TM glycoprotein is the target of P3 and suggest that the pathway of HIV-1 resistance to P3 differs from that of T-20 and that the genetic barrier to P3 resistance is significantly higher in HIV-2 than in HIV-1. Our findings provide proof of principle that viable antiviral peptides can be constructed using evolutionary biology strategies. Such strategies should be explored to enhance the production of peptide drugs and vaccines.

Keywords: Ancestral P3 peptide: inhibition of HIV-1 and HIV-2 cell fusion and entry; P3 resistance; P3 mechanism of action; P3 antigenicity.

INTRODUCTION

Over the last decade, the inhibition of viral entry has become one of the most attractive fields in the research for new anti-HIV molecules. Entry inhibitors are a recent class of antiretroviral drugs, which can be classified in four groups according to the step of viral entry targeted: inhibitors of the SU-CD4 interaction, CCR5 antagonists, CXCR4 antagonists and fusion inhibitors [1,2]. Despite numerous efforts, currently there are only two entry inhibitors approved for HIV type 1 (HIV-1) antiretroviral therapy, the fusion inhibitor peptide enfuvirtide (or T-20; FUZEON, Roche) [3] and the CCR5 coreceptor antagonist maraviroc (SELZENTRY, Pfizer) [4].

Like all other available antiretroviral drugs, entry inhibitors were specifically designed to inhibit HIV-1 replication. HIV type 2 (HIV-2), the second causative agent of AIDS, is responsible for localized epidemics manly in West Africa and a in few other countries (e.g. Portugal and France), affecting an estimated 1-2 million patients worldwide [5]. HIV-1 and HIV-2 have different evolutionary histories [6], share only 50% of genetic similarity [7] and their envelope glycoproteins are markedly different at the structural and functional levels [8]. Consequently, some drugs have limited or no activity on HIV-2, namely non-nucleoside reverse transcriptase inhibitors, some protease inhibitors and T-20 [9,10,11].

T-20 is a linear peptide composed of 36 amino acids that mimics the gp41 HR2 sequence of the HIV-1 LAI isolate [9,12]. T-20 inhibits HIV-1 entry by competitive binding to the complementary HR1 region, thereby blocking the formation of the six-helix bundle structure and preventing viral fusion [13,14,15]. Despite strong anti-HIV-1 activity, there is considerable variability (up to 500-fold) in the T-20 sensitivity of HIV-1 primary isolates within subtypes B and non-B [16,17,18]. In addition, the genetic barrier for T-20-resistance is low [15,19]. Resistance mutations are usually found within the 36-45 positions of HR1 region, especially in the GIV motif (codons 36-38) [15,20]. T-20 has poor bioavailability and has to be injected subcutaneously twice daily, complicating patient adherence to treatment. Currently, T-20 is only used as a salvage therapy in HIV-1 infection [21,22,23]. T-20 has no activity on HIV-2 possibly because its sequence divergence prevents it from binding to the HR1 target in gp36 envelope glycoprotein [10,24,25].

Second and third generations fusion inhibitor peptides have been developed in an attempt to improve antiviral potency, increase *in vivo* stability, and overcome T-20 resistance

[15,26]. T-1249 is a representative second generation 39-mer peptide derived from HR2 consensus sequences of HIV-1, HIV-2 and SIV strains [27,28]. It is a potent inhibitor of HIV-1 replication, including isolates resistant to T-20, and it also inhibits HIV-2 [27,28,29]. However, the elevated production costs and drug formulation difficulties associated with its long size, have hampered its' clinical development beyond phase I/II trials [30]. Sifuvirtide is a third generation fusion inhibitor peptide, with increased α-helical content (93%), which have showed promising results in phase II clinical studies being active against a broad range of HIV-1 isolates, including T-20- resistant strains [15,26,31]. Despite better pharmacokinetic profile than T-20, it is still administered as a subcutaneous injection. In addition, since its sequence is solely based on HIV-1, one can expect that it also won't be active against HIV-2 [24].

The initial aim of this work was to produce new HR2-based peptides that inhibit HIV-2 fusion and entry. HIV-2 is a highly variable virus composed of 8 groups termed A to H of which only groups A and B have generated human epidemics [32,33,34]. To enhance the likelihood of inhibiting replication of all HIV-2 strains, the candidate peptides were derived from ancestral HIV-2 and SIV gp36 sequences. We found that one selected peptide, named P3, potently inhibited the replication of highly divergent HIV-2 and HIV-1 primary isolates.

MATERIALS AND METHODS

Cells, plasmids, virus and fusion inhibitors

293T cells were purchased from American Type Culture Collection (Rockville, MD). The following reagents were provided by the AIDS Research and Reference Reagent Program, National Institutes of Health: TZM-bl [35,36,37,38] and CEM-SS cells [39,40,41]: pNL4-3 [42], pSG3.1 [43], pHEF-VSVG [44] and pSG3Δenv [36,45] plasmids; T-20-resistant pNL4-3 gp41 (36G) variants bearing the V38A, V38A/N42D, V38A/N42T, or N42T/N43K mutations [42,46]; T-20 (Enfuvirtide) fusion inhibitor. pROD10 plasmid was a gift from Keith Peden [47]. Trimeris Inc (USA) provided T-1249. 293T and TZM-bl cells were cultured in complete growth medium (GM) that consists of Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS and 100 U/ml of penicillin-streptomycin (Gibco/Invitrogen, USA). CEM-SS cells were propagated in RPMI-1640 medium with 10% FBS and 100U/ml of penicillin-streptomycin (Gibco/Invitrogen, USA). Healthy peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque PLUS (GE Healthcare, Waukesha, WI, USA) density gradient centrifugation and stimulated for 3 days with 5 μg/ml of phytohemagglutinin (PHA; Sigma-Aldrich, St. Louis, MO, USA). PBMCs cultures were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml of Penicillin-

Strepotmycin, 0.3 mg/ml of Gentamicin (Gibco/Invitrogen, Carlsbad, CA, USA), 5 μg/ml of Polybrene (Sigma-Aldrich, St. Louis, MO, USA) and 20 U/ml units of recombinant interleukin-2 (Roche, Basel, Switzerland). All cell cultures were maintained at 37ºC in 5% CO_2.

Virus stocks and titration

A total of 26 primary isolates were included in this study (seven HIV-1 and 19 HIV-2 viruses), which were previously isolated, titrated and characterized for coreceptor usage [25]. HIV laboratory-adapted reference strains were obtained by transfection of 5×10^6 HEK293T cells with 6 μg of pNL4-3 (HIV-1), pSG3.1 (HIV-1) or pROD10 (HIV-2) plasmids using Fugene 6 reagent (Roche, Switzerland) according to manufacturer's instructions. A pseudovirus carrying the vesicular stomatitis virus (VSV) envelope was produced by co-transfection of 5×10^6 HEK293T cells with 2 μg of pHEF-VSVG plasmid and 4 μg of pSG3Δenv plasmid, using also Fugene 6 reagent. Transfections and co-transfection were performed in 100 mm culture dish plates in a total volume of 10 ml of GM, and the supernatants were collected after 48h and cleared by filtration. Variants resistant to T-20 were propagated in CEM-SS cells according to protocol available at www.aidsreagent.org. The 50% tissue culture infectious dose ($TCID_{50}$) of all viruses was determined in a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells [25,48] and calculated using the statistical method of Reed and Muench [49].

Peptide design

Custom peptides were derived from ancestral gp36 HR2 sequences reconstructed from a phylogenetic tree of HIV-2 and SIV reference sequences (see Supplementary Table S1 for a list of reference sequences used). Reconstruction of ancestral character states was performed by maximum likelihood in PAUP version 4 software [50]. MODELTEST [51] estimated best-fit models of molecular evolution for maximum likelihood analyses. The chosen model was GTR+G+I [52]. Tree searches were also performed in PAUP version 4.0 using the nearest-neighbor interchange (NNI) and tree bisection and reconnection (TBR) heuristic search strategies, and bootstrap resampling.

Peptides were produced commercially by Genemed Synthesis (San Antonio, Texas, USA). They were modified with the N-terminus acetylated and the C-terminus as a carboxamide, the salt form being acetate. Reverse-phase high-pressure liquid chromatography (HPLC) was used for purification (>95%) and mass spectrometry for confirmation analysis.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded for P3 and T-20 at a concentration of 100 and 50 μM, respectively, in 10 mM phosphate buffer + 100 mM NaF (pH 7.4) using a Jasco® 810 spectropolarimeter. Spectra were recorded in the far UV region (185-240 nm), using a 0.1 cm pathlength cell, with a 50 nm/s scan speed, an 8 s response time, 2 nm bandwidth and accumulation of 4 scans.

Binding assay

An enzyme-linked immunosorbent assay (ELISA) was developed to study the binding specificity of peptide P3 to its predicted target, HIV-2 *env* gp36. Polystyrene immune module microwells (Maxisorp; Nunc, Denmark) were independently coated (100 μl/well) with each peptide at a concentration of 50 μg/ml in phosphate buffered saline (PBS) solution and incubated overnight at 4°C. After two washes with PBS, microwells were blocked with 5% of bovine serum albumin (BSA; Sigma-Aldrich, USA) in PBS for 2h at 37ºC and washed twice with PBS. A recombinant gp36 protein with a polyhistidine tag (rgp36) previously produced in our lab [53] was diluted in PBS containing 0.05% of Tween-20 (Bio-Rad, USA) (PBS-T) and added (100 μl) at a concentration of 2.5 μg/ml and incubated for 1 h at 37ºC. After five washes with PBS-T, a 1:2000 dilution of mouse monoclonal anti-polyhistidine antibody conjugated to alkaline phosphatase (Sigma-Aldrich) in PBS-T was added (100 μl) and incubated for 1 h at 37ºC. After another five washes with PBS-T, p-Nitrophenyl Phosphate tablets (Sigma-Aldrich, USA) were added as a chromogenic substrate, and the optical density (OD) was measured in a Tecan MP-500 plate reader (Tecan, Switzerland) at 405 nm against a reference wavelength of 620 nm. The cut-off value of the assay, calculated as the mean OD value of negative controls + 2 times the standard deviation [SD], was determined for each peptide using wells in which the peptide was incubated with PBS instead of rgp36. The results of the assay are expressed quantitatively as $OD_{peptide}$ / $OD_{cut\text{-}off}$ ratios (OD/cut-off ratio).

Phenotypic sensitivity assays

The antiviral activity of entry inhibitors was determined in a single-round viral infectivity assay using the TZM-bl reporter cells as previously described [25]. Briefly, cells were infected with 200 $TCID_{50}$ of each virus. Infections were performed in the presence of serial-fold dilutions of fusion inhibitors in GM, supplemented with DEAE-dextran (19.7 μg/ml). After 48h of infection, luciferase expression was quantified with the One-Glow luciferase assay substrate reagent (Promega, USA) according to manufacturer's instructions.

Background luminescence was measured by using control wells that contained only target cells and GM. At least two independent experiments were performed for each analysis and each assay was set up in duplicate wells. The cytotoxicity of the compounds was evaluated using control wells in the absence of the virus.

The 50% (IC_{50}) and 90% (IC_{90}) inhibitory concentrations, as well as the dose-response curve slopes (Hill slope), were estimated by plotting the percent inhibition of infection (y axis) against the log_{10} concentration of each fusion inhibitor (x axis) and using the sigmoidal dose-response (variable slope) equation in Prism version 4.0c for Macintosh (GrahPad Software, San Diego, California USA, www.graphpad.com).

Cellular viability assay

The potential *in vitro* cytotoxicity of all peptides was also evaluated in PBMCs. PBMCs (25,000 cells/well in 96-well plates) were incubated in absence or presence of serial-fold dilutions of peptides, with starting concentrations of 20 μM. After 48h, cell viability was examined with alamarBlue reagent (Invitrogen, USA) according to manufacturer's instructions.

Antigenic reactivity assay

A new ELISA assay was developed to measure antigenic reactivity of peptides in HIV-infected patients using an ELISA protocol similar to the one described for the binding assay. Briefly, microwells were independently coated with each peptide at a concentration of 10 μl/ml in PBS solution and incubated overnight at 4°C. After blocking with BSA, 100 μl of a 1:300 dilution of plasma samples collected from 30 HIV-1 and 29 HIV-2 infected patients (all naive to T-20) in PBS-T was added and incubated for 1h at 37ºC. Wells were then washed six times with PBS containing 0.1% of Tween 20 and a 1:2000 dilution of goat anti-human immunoglobulin G (Fc specific) conjugated to alkaline phosphatase (Sigma-Aldrich) in PBS-T was added. Following incubation, the colour was developed and ODs were measured as described above. The clinical cut-off value of the assay, calculated as the mean OD value of HIV-seronegative samples + 2 times the SD, was determined using samples from healthy HIV-seronegative subjects (n = 10). The results of the assay are expressed quantitatively as $OD_{clinical\ sample}$ / $OD_{cut\text{-}off}$ ratios (OD/cut-off ratio).

In vitro selection of resistance mutations to P3

Primary HIV-1 (subtype G) and HIV-2 (group A) strains were used for selection of resistance mutations to P3 in PBMCs, using a standardized procedure as previously described [54].

Briefly, PBMCs were infected at a multiplicity of infection of 0.01 for two hours at 37ºC, washed, and then seeded in 24-well plates at a density of 250,000 cells/well. Selections were performed by a standard procedure, starting with concentrations below the IC_{50} level. Control wells were maintained with infected cells and without peptide. Viral replication was monitored weekly by p24 antigen assay (Innotest from Innogenetics, Belgium). At each passage, one aliquot of culture supernatant was used to infect fresh PBMCs and peptide concentrations were maintained or increased by two-fold based on p24 values. The remaining culture fluids were harvested and kept at -80°C for a subsequent genotypic analysis by sequencing. To this end, RNA was extracted using QIAmp viral RNA Mini Kit (Qiagen, Germany), according to manufacturer's instructions, and reverse transcribed using Titan One Tube RT-PCR System (Roche, Switzerland). The *env* gene, (positions 6203 – 8817 in HIV-1 HXB2 and positions 6673 - 9268 in HIV-2 BEN) was amplified by nested PCR, using the Expand Long Template PCR System kit (Roche, Switzerland), and sequenced. Primers used for amplification and sequencing are described in Supplementary Tables S2 and S3.

Statistical analysis

Statistical analyses were performed using Prism version 4.0c for Macintosh (GrahPad Software, San Diego, California USA, www.graphpad.com), with a level of significance of 5%.

RESULTS

Design of ancestral peptides

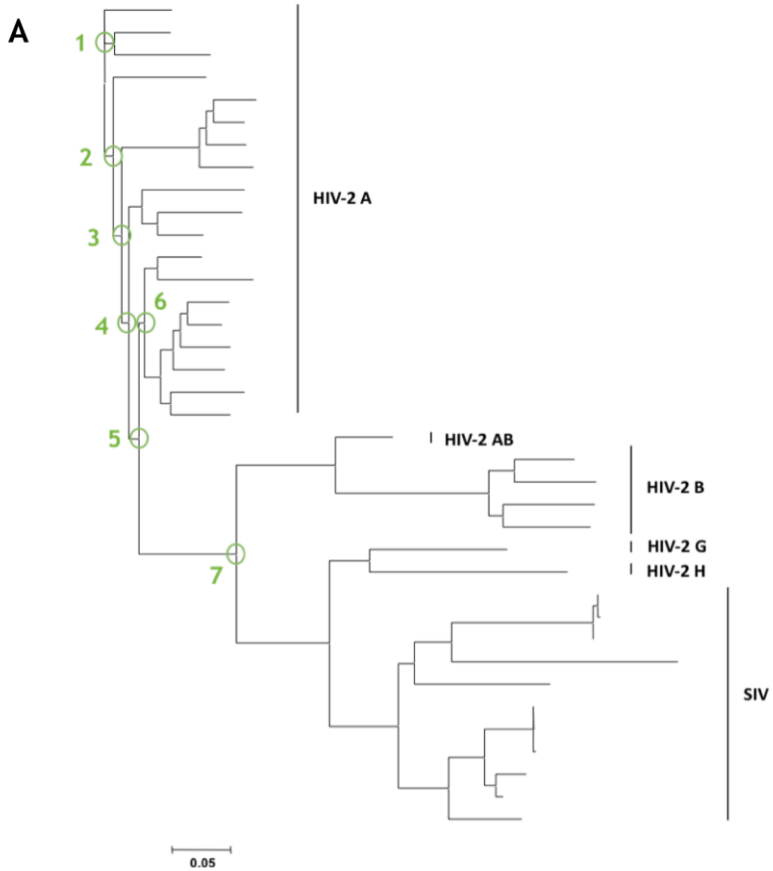
Maximum likelihood methods were used to reconstruct ancestral transmembrane protein sequences at several nodes of the envelope gene phylogenies that represent ancestors to diverse HIV and SIV virus clades (Figure 1A). These sequences were aligned and several peptides were derived from the helical region 2 (HR2) (Figures 1B and 1C). Three peptides were produced: P1 (36 amino acids) and P2 (42 amino acids) included the C-terminal lipid-binding domain whereas P3 (34 amino acids) covered the N-terminal pocket-binding sequence (Figure 1C). In contrast to P1 and P2, P3 doesn't comprise the epitopes of the 2F5 and 4E12 HIV-1 neutralizing antibodies [55,56]. Due to high hydrophobicity peptides P1 and P2 were very hard to synthesise and reconstitute in an appropriate buffer suitable for cell culture assays. Therefore, only peptide P3 was further analysed for structure and antiviral activity. P3 has 34 residues and overlapps the N-terminal pocket-binding region and heptad repeat (HR) core of the HR2 region (positions 628 – 661 of HIV-1 HXB2 Env). It

differs by 21 aa from the consensus HIV-1 sequence, 14 aa from T-20 and 6 aa from consensus HIV-2. However, the positions *a* and *d* of the heptad repeat, critical for HIV-1 HR1/HR2 binding [57], were quite conserved. There were only four changes at these positions: I635V, involving aliphatic amino acids from the same chemical group; Y638L, L645R, S649A, from different chemical groups. Notably, the S649A substitution (or S138A in gp41) is a common secondary mutation selected during therapy with T-20 [58,59] and, when introduced in the context of a fusion inhibitor like the modified T-20_{S138A}, it increases its binding affinity to the HR1-target region and enhances the antiviral activity [60].

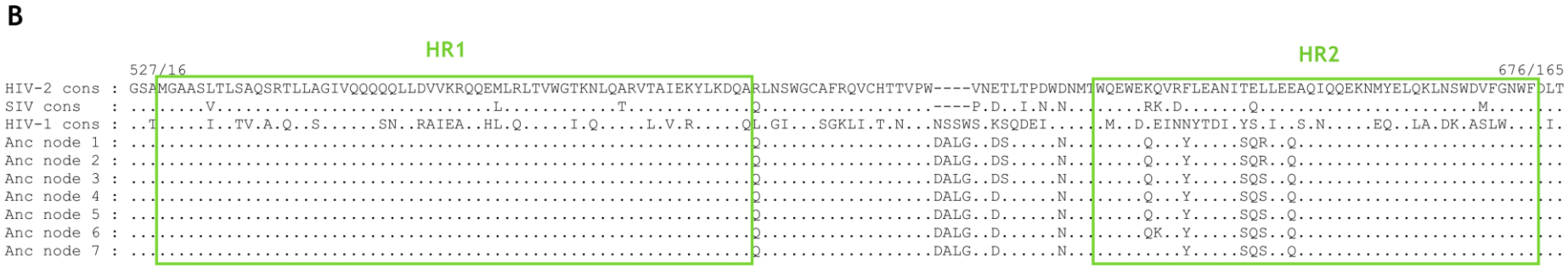
The sequences of T-20 and P3 overlap in 24-amino acids of the HR core and between them there are 14 residue changes, while the differences between the 27 overlapping positions of T-1249 and P3 (over the pocket-binding region and HR core), are of only 5 residues (19%). The percentage of hydrophilic residues is of 62% in P3 which compares with 56% in T-20 and 54% in T-1249 [61].

(on the following page)

Figure 1. Design of ancestral peptides. (**A**) Ancestral reconstruction of HIV-2 gp36 sequences; the interior nodes highlighted by numbered circles correspond to the ancestral used for peptide design. The scale bar represents evolutionary distances in substitutions per site. (**B**) Alignment of the gp41/gp36 HR1-HR2 segment, containing HIV-2, SIV and HIV-1 consensus sequences, as well as the ancestral sequences derived in each of the interior nodes highlighted above in the phylogenetic tree. Points represent similarity relative to HIV-2 consensus and dashes represent gaps in the alignment. Sequences are numbered according to HIV-1 HXB2, Env position / gp41 position. (**C**) Comparison of the HR2 amino acidic sequences between HIV-2, SIV and HIV-1 consensus sequences and the HR2-based peptides (T-20, T-1249, P1, P2 and P3). Sequences are numbered according to HIV-1 HXB2, Env position / gp41 position. Positions *a* and *d* of HR2 represent the residues involved in HIV-1 HR1/HR2 interaction. The HR2 region contains tree functional domains: 1, pocket-binding domain (PBD, in blue); 2, HR core in the center (3HR, black); 3, lipid-binding domain (LBD, in pink). Asterisks indicate complete (2F5) or partial (4E10) neutralizing epitopes in HIV-1.


A
1
2
3
4
5
6
7
HIV-2 A
HIV-2 AB
HIV-2 B
HIV-2 G
HIV-2 H
SIV
0.05


C
628/117
673/162
2F5
4E10
a d a d a d a d a d a d
HIV-2 cons : WQEWEKQVRFLEANITELLEEAQIQQEKNMYELQKLNSWDVFGNWF
SIV cons : .....RK.D.....Q.....M.....
HIV-1 cons : .M..D.EINNYTDI.YS.I..S.N.....EQ..LA.DK.ASLW...
T-20 : YTSL.HS.I..S.N.....EQ..LE.DK.ASLW...
T-1249 : .....QK-------..A...Q.....E.....DK.ASLWE..
P1 : .....SQR..Q.....
P2 : .Q...Y.....SQR..Q.....
P3 : .....Q...Y.....SQR..Q.....


B
HR1
HR2
527/16
676/165
HIV-2 cons : GSAMGAASLTLSAQSRTLLAGIVQQQQQLLDVVKRQQEMLRLTVWGTKNLQARVTAIEKYLKDQARLNSWGCAFRQVCHTTVPW----VNETLTPDWDNMTWQEWEKQVRFLEANITELLEEAQIQQEKNMYELQKLNSWDVFGNWFDLT
SIV cons : ..V.....L.....T.....Q.....----P.D..I.N.N.....RK.D.....Q.....M.....
HIV-1 cons : ..T.....I..TV.A.Q..S.....SN..RAIEA..HL.Q.....I.Q.....L.V.R.....QL.GI...SGKLI.T.N...NSSWS.KSQDEI.....M..D.EINNYTDI.YS.I..S.N.....EQ..LA.DK.ASLW....I.
Anc node 1 : .....Q.....DALG..DS.....N.....Q...Y.....SQR..Q.....
Anc node 2 : .....Q.....DALG..DS.....N.....Q...Y.....SQR..Q.....
Anc node 3 : .....Q.....DALG..DS.....N.....Q...Y.....SQS..Q.....
Anc node 4 : .....Q.....DALG..D.....N.....Q...Y.....SQS..Q.....
Anc node 5 : .....Q.....DALG..D.....N.....Q...Y.....SQS..Q.....
Anc node 6 : .....Q.....DALG..D.....N.....QK..Y.....SQS..Q.....
Anc node 7 : .....Q.....DALG..D.....N.....Y.....SQS..Q.....

P3 forms a typical α-helical structure in solution

The secondary structure of P3 was determined by CD spectroscopy and compared to that of T-20 (Figure 2). The predominant conformation of P3 in solution is an α-helix (42%), whereas the T-20 spectrum is indicative of a less defined secondary structure in solution with only 19% of helical content, as previously reported [62].

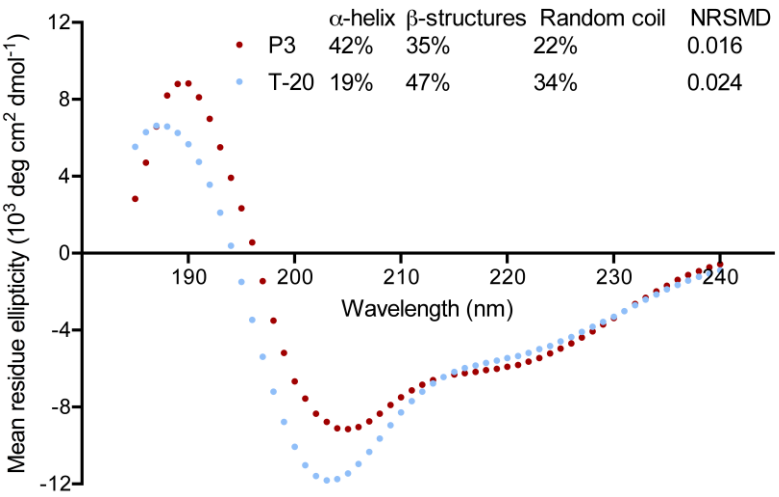


Figure 2. Circular dichroism spectra for P3 and T-20 peptides. β-structures include sheets and turns; NRSMD, Normalised Root Mean Square Deviation.

P3 is a potent inhibitor of HIV-1 and HIV-2 infection

The antiviral activity of peptide P3 was evaluated against a set of 20 group A HIV-2 isolates, of which 19 were primary isolates, and a group of nine HIV-1 viruses, including seven highly diverse primary isolates (Table S4). Overall, P3 proved to be a potent inhibitor of both HIV-2 and HIV-1 infection (Table 1 and Figure 3). P3 inhibited HIV-2 infection at an IC_{50} of 63.8 nM and an IC_{90} of 709.6 nM. Interestingly, however, an even stronger activity was displayed against HIV-1, with an IC_{50} of 11 nM (P<0.0001) and an IC_{90} of 366.4 nM (P=0.239). In addition, coreceptor tropism of virus isolates determined susceptibility to P3, as it was more effective on X4 than on R5 isolates both in HIV-1 (IC_{50}, 0.9 *vs* 20.4 nM; P<0.0001) and HIV-2 (IC_{50}, 50.4 *vs* 80.5 nM; P<0.035). The sensitivity of R5 HIV-2 variants to P3 was not correlated with the level of $CD4^+$ T cells at the time of virus isolation (P=0.493). No cytotoxicity was observed either in TZM-bl culture cells or primary PBMCs at all concentrations tested (up to 20 μM) (data not shown).

Compared with T-20, P3 was significantly more active against HIV-2 and significantly less active against HIV-1 (P<0.0001 for both cases) (Table 1 and Figure 2). Nonetheless, the P3

and T-20 had similar IC_{90} ranges (P3, 6.2 – 1785.5 nM; T-20, 0.5 – 1285.3 nM) and similar dose-response curve slopes when tested against HIV-1 of predicting similar antiviral activity in vivo [25,63]. Interestingly, T-1249 had better HIV-1 and HIV-2 inhibitory profiles than T-20 or P3.

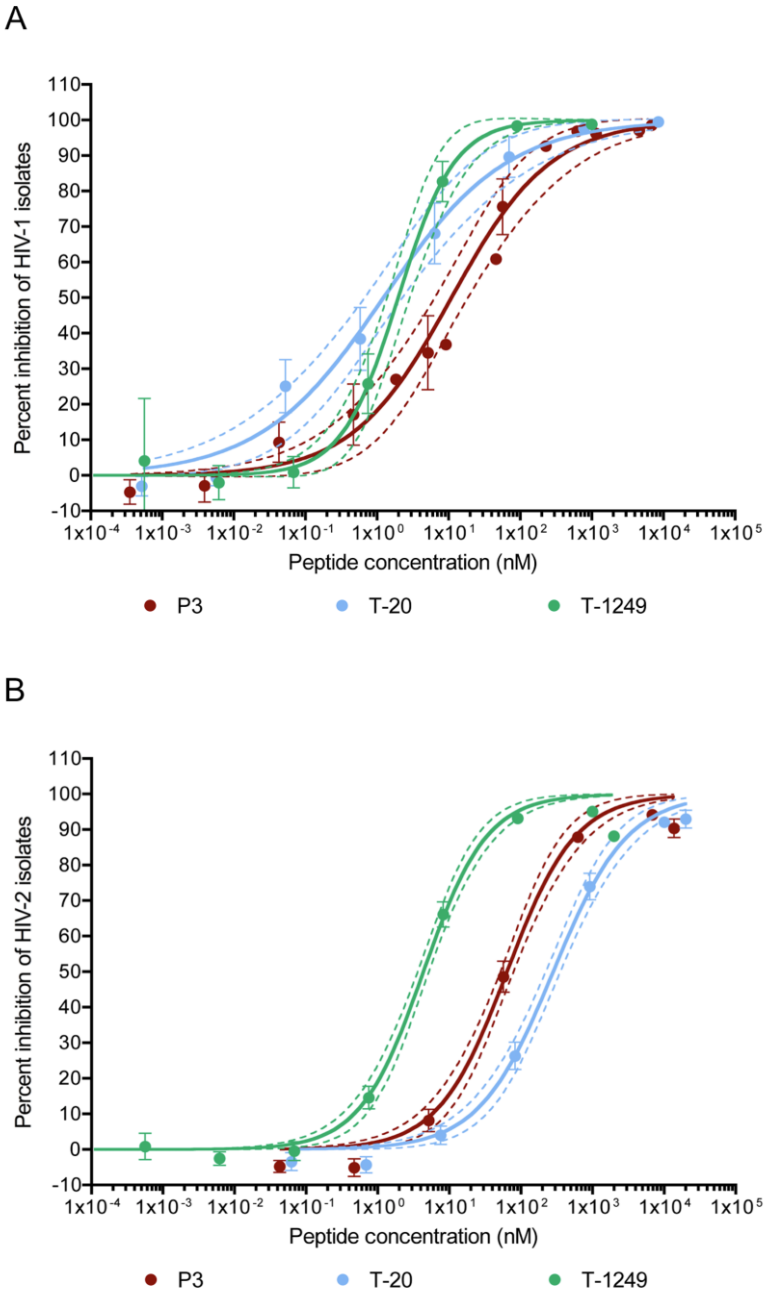


Figure 3. Representative dose-response curves for (A) HIV-1 and (B) HIV-2 with peptide fusion inhibitors: comparison of P3 with T-20 and T-1249 [25]. Data points represent the average of results obtained from HIV-1 and HIV-2 isolates; bars represent standard error of the mean. Sigmoidal dose-response (variable slope) curves were adjusted to these data points; dashed lines represent the 95% confidence band of the best-fit curve.

Table 1. Antiviral activity of P3, T-20 and T-1249 against HIV-1 and HIV-2 isolates.

Parameter[a]	**P3** (nM)	**T-20** (nM)[b]	***P* value**[c]	**T-1249** (nM)[b]	***P* value**[c]
HIV-1 (n=9)					
IC_{50} (95% CI)	11 (6.5; 18.4)	1.2 (0.7; 2.2)	< 0.0001	2.0 (1.4; 2.8)	<0.0001
IC_{90} (95% CI)	366.4 (117.5; 1145.5)	95.9 (26.3; 350.8)	0.107	14.3 (6.9; 29.5)	<0.0001
Hill slope (95% CI)	0.6 (0.4; 0.82)	0.5 (0.4; 0.6)	0.263	1.1 (0.8; 1.4)	0.010
HIV-2 (n=20)					
IC_{50} (95% CI)	63.8 (51.9; 78.5)	281.5 (223.2; 354.9)	< 0.0001	4.3 (3.6; 5.2)	<0.0001
IC_{90} (95% CI)	709.6 (435.5; 1158.8)	3881.5 (2393.3; 6280.6)	< 0.0001	40.6 (28.1; 58.5)	<0.0001
Hill slope (95% CI)	0.9 (0.7; 1.1)	0.8 (0.7; 1)	0.492	1 (0.8; 1.1)	0.540

[a] IC_{50}, IC_{90} and Hill slope best-fit values were inferred from sigmoidal dose-response (variable slope) curves adjusted to combined results of HIV-1 and HIV-2 isolates, and represent geometric mean values; 95% CI – 95% confidence interval.

[b] T-20 and T-1249 susceptibilities were obtained for the same HIV-1 and HIV-2 viral panel and previously published elsewhere [25].

[c] *P* value for comparison of best-fit values between P3 and T-20 or P3 and T-1249, using the F test.

P3 mechanism of action is envelope specific

P3 and T-20 bound strongly to a recombinant transmembrane protein (rgp36, [53]) in an ELISA assay (30-fold above cut-off for P3) (Figure 4A). Moreover, P3 did not inhibit the entry of a pseudovirus carrying the vesicular stomatitis virus envelope glycoprotein (VSV-G) (Figure 4B). These results indicate that the antiviral activity of P3 is HIV-envelope specific.

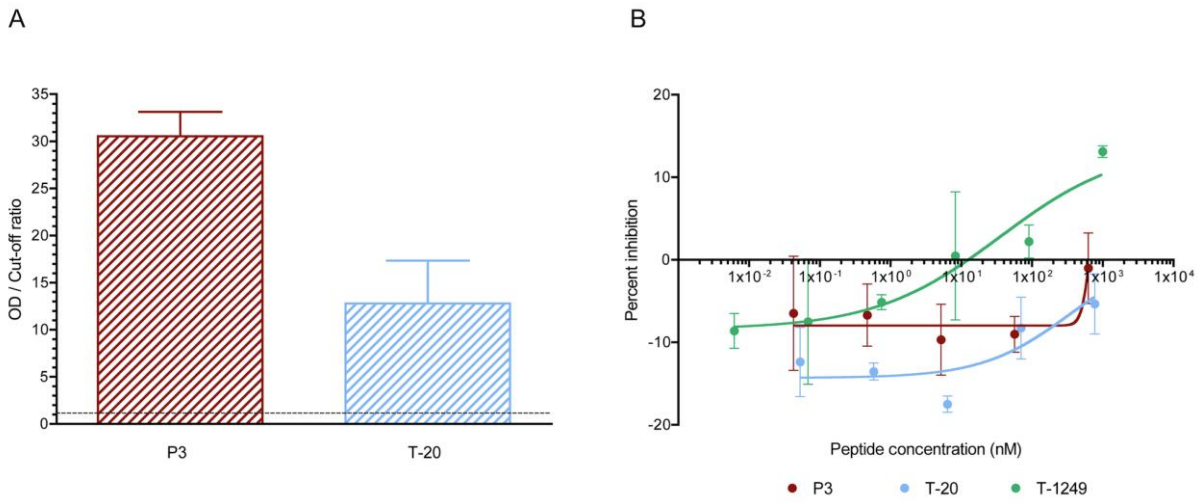


Figure 4. HIV-envelope specificity of P3. (**A**) Binding activity of peptides P3 and T-20 to HIV-2 gp36 in an ELISA assay. (**B**) Representative dose-response curves for VSV-G pseudovirus with peptide fusion inhibitors. Data points represent the average of results and bars represent standard error of the mean. Sigmoidal dose-response (variable slope) curves were adjusted to these data points.

P3 inhibits the replication of T-20- resistant HIV-1 variants

To determine if P3 is able to inhibit the infection of HIV-1 strains resistant to T-20, we measured the susceptibility of HIV-1 variants carrying well-defined T-20 resistance mutations to P3 [42,46]. Notably, P3 exhibited potent activity against T-20 resistant variants harbouring the V38A, V38A/N42D and V38A/N42T mutations (IC_{50} range, 0.15 – 11.8 nM) (Table 2). In fact, the V38A/N42D mutations seem to confer increased susceptibility to P3 (7-fold lower IC_{50}). However, P3 did not inhibit the replication of a resistant strain harbouring the N43K mutation. These results indicate that P3 potently inhibits the replication of most T-20 resistant strains and suggest that P3 could be useful as an alternative fusion inhibitor for treatment of patients infected with HIV-1 strains resistant to T-20.

Selection of P3 resistant variants

To investigate the mechanism of action and the pathways of resistance to P3, i*n vitro* selection of resistance mutations to P3 was performed by repeated passage of HIV-1 and HIV-2 primary isolates in PBMCs in the presence of either constant or increasing concentrations of P3, according to the viral replication capability [54]. An HIV-1 subtype G variant containing the N43K mutation in the HR1 region of gp41 was selected after 59 days in culture (8 passages) in the presence of 212 nM of P3. Inhibition of replication of this mutant virus with P3 occurred at an IC_{50} of 1.9 μM and IC_{90} of 13.1μM, which represent a 120-fold and 56.4-fold decrease in susceptibility, respectively. Of note, a T-20 resistant HIV-1 subtype B isolate that also harboured the N43K mutation was 2140-fold more resistant to P3 than the wild-type virus (Table 2). On the other hand, under the same experimental conditions and despite repeated attempts, we were not able to select an HIV-2 strain resistant to P3. Collectively, these results indicate that the HR1 region in the TM glycoprotein is the target of P3 and suggest that the pathway of HIV-1 resistance to P3 differs from that of T-20 and that the genetic barrier to P3 resistance is significantly higher in HIV-2 than in HIV-1.

Table 2. Comparison of antiviral activity of P3 and T-20 on T-20- resistant HIV-1 variants.

		P3		T-20		
HIV-1 variant	**Phenotype[a]**	**IC_{50} nM (95% CI)**	***Fold-increase*[b]**	**IC_{50} nM (95% CI)**	***Fold-increase*[b]**	***P* value[c]**
NL4-3 D36G (parental)	S	0.4 (0.2; 1.2)	*1*	0.03 (0.01; 0.06)	*1*	0.0002
NL4-3 (D36G) V38A	R	1.5 (0.5; 5.1)	*3.8*	43.8 (21.8; 87.8)	*1460*	<0.0001
NL4-3 (D36G) V38A/N42D	R	0.06 (0.01; 0.3)	*0.15*	118.2 (63.0; 221.7)	*3940*	<0.0001
NL4-3 (D36G) V38A/N42T	R	4.7 (1.9; 11.5)	*11.8*	482.0 (324.1; 716.8)	*16066.7*	<0.0001
NL4-3 (D36G) N42T/N43K	R	855.9 (628.0; 1167.0)	*2139.8*	80.3 (61.1; 105.6)	*2676.7*	<0.0001

[a] Sensitive (S) or Resistant (R) to T-20.

[b] Fold-increase of IC_{50} concentration relative to NL4-3 D36G (parental).

[c] *P* value for comparison of best-fit values between P3 and T-20, using the F test.

P3 is significantly less antigenic than T-20 in HIV-1 infected patients

Virtually any foreign protein introduced into the body has the potential to trigger the production of antibodies [64,65]. Therapeutic proteins are no different, and one of the major concerns regarding the development of anti-drug antibodies is the possible reduction of therapeutic efficacy of such drugs [64]. The HR1 region is under strong immunological pressure [66,67] implying that fusion inhibitor peptides derived from this region may be highly antigenic. The antigenicity of P3 was examined with plasmas from HIV-1 and HIV-2 infected patients, all naïve to T-20. A significantly higher number of HIV-2 patients had antibodies reacting with P3 then with T-20 (93% and 45%, respectively) and the mean binding affinity of the P3-specific antibodies was significantly higher in HIV-2 patients then in HIV-1 patients (P<0.0001) (Figure 5). In contrast, a significantly higher number of HIV-1 patients had antibodies reacting with T-20 (used as a control in this experiment) then with P3 (90% and 67%, respectively) and the mean binding affinity of the T-20- specific antibodies was significantly higher in HIV-1 patients then in HIV-2 patients (P<0.0001). In all, these results demonstrate that, in contrast to T-20, P3 is weakly antigenic in HIV-1 patients and is highly antigenic in HIV-2 patients.

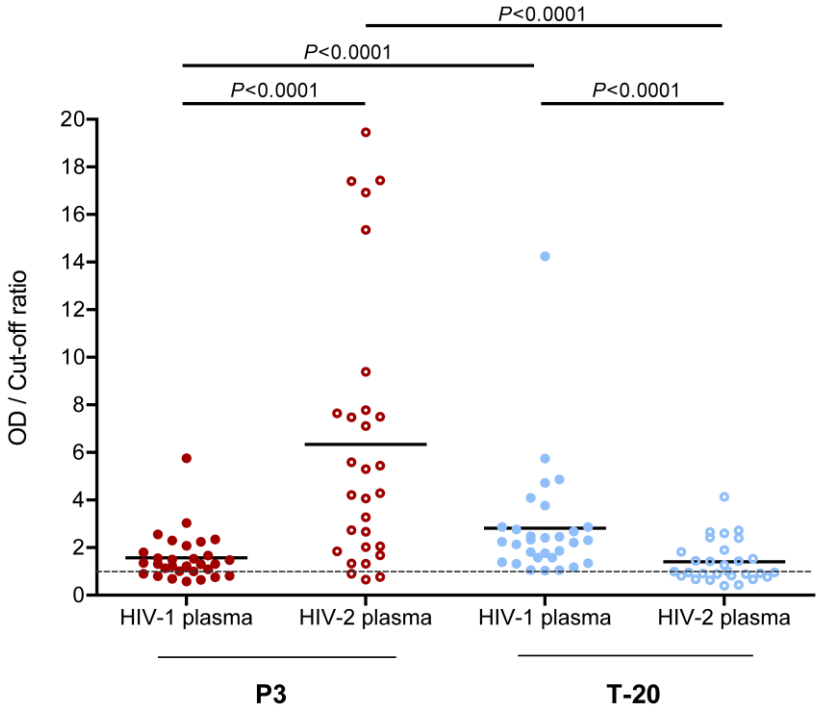


Figure 5. Antigenic reactivity of P3 and T-20 in HIV-infected patients. Results from P3 are in red and from T-20 are in blue. Closed circles represent the antigenic reactivities of HIV-1 plasmas, while open circles represent the ones from HIV-2 plasmas. Statistical significance is shown for comparisons using non-parametric tests: Mann-Whitney U test (independent samples) and Wilcoxon Signed Rank test (paired samples).

DISCUSSION

We show here that an ancestral peptide (named P3) derived from the helical region 2 (HR2) of HIV-2 and SIV potently inhibits HIV-1 and HIV-2 entry and replication. The rational for using ancestral sequences of the transmembrane envelope glycoprotein as a source for the new antiviral peptide was to minimize HIV sequence divergence by tracing the most likely evolutionary path along the phylogeny and capture more conserved structural features of the HR2 sequences [68,69].

In contrast to T-20, P3 displayed a potent activity against highly divergent HIV-1 and HIV-2 primary isolates demonstrating that our strategy was highly successful. The activity on HIV-1 might be partly explained by the conservation in P3 of the residues located in critical positions involved in the HR1/HR2 interaction (*a* and *d* residues) [57]. In addition, in contrast to T-20, P3 has an Alanine at position 22 that corresponds to residue 138 of HIV-1 transmembrane gp41 glycoprotein. S138A is a secondary mutation that arises in HR2 to compensate for the reduction of viral fitness of HIV-1 variants carrying T-20- primary resistant mutations in HR1 [58,59]. It increases the binding affinity of this region to HR1 and can therefore contribute to the potent inhibitory activity of P3 against HIV-1 [60]. P3 potently inhibited the replication of most T-20 resistant HIV-1NL4-3 clones, which suggest that it could be useful as an alternative for treatment of patients infected with HIV-1 strains resistant to T-20. However, a clone containing the N43K mutation (together with N42T) was highly resistant to P3 (2140-fold resistance). Moreover, the same N43K mutation was selected in presence of P3 and led to 120-fold resistance to this peptide. This is a common mutation selected in T-20 and T-1249 treated patients [19,59,70,71] but, by itself, it is responsible for only a modest resistance to T-20 [19] and T-1249 [72]. Collectively, these results suggest that (1) the HR1 region in the TM glycoprotein is the target of P3 and (2) the pathway of HIV-1 resistance to P3 differs from that of T-20 and T-1249.

We were not able to generate HIV-2 isolates resistant to P3, even after 60 days in culture, which prevented identification of the drug target in this virus. However, P3 bond strongly to a recombinant HIV-2 gp36 suggesting that its mechanism of action in HIV-2 might also involve binding to the HR-1 region. The strong binding of P3 to its target protein may also have prevented the emergence of resistance mutations in our experimental conditions [19,59].

As previously observed for other fusion inhibitors (T-20, T-1249 and T-649) [16,25,37], P3 was more active on X4 variants than on R5 variants both in HIV-1 and HIV-2. As there were no significant differences in the target HR-1 region in R5 and X4 viruses, these results can be explained by the more rapid fusion kinetics in R5 viruses due to a higher affinity of

gp120/gp125 for CCR5. This accelerated fusion kinetics can reduce significantly the window of opportunity for peptide-gp41 interactions in R5 variants (reviewed in [73]).

It has been demonstrated that the helical content of peptide fusion inhibitors correlates with higher anti-HIV-1 potency by increasing their binding affinity for HR1 [74,75]. Moreover, unstructured peptides like T-20 are less stable and more susceptible to proteolytic degradation in the blood [15,26]. In this sense, the strong α-helix structure of P3 might increase its stability in physiological conditions and decrease the likelihood of adopting non-helical conformations thereby favouring the binding of the peptide to its target site [72].

We showed that when compared to T-20, P3 has a significantly lower antigenicity in HIV-1 infected patients. Drug-specific antibodies can compromise their clinical efficacy of either by preventing their exposure to the active site or by decreasing their half-life [64]. Hence, the weaker antigenicity of P3 might translate into a better bioavailability profile and durable clinical efficacy in HIV-1 infected patients.

In summary, we successfully derived an ancestral peptide (P3) with broad antiviral activity against HIV-1 and HIV-2 strains, including HIV-1 variants resistant to T-20. P3 is a peptide with predominant α-helix conformation that binds strongly to the transmembrane glycoprotein and is weakly antigenic in HIV-1 patients. The N43K mutation in the HR-1 region leads to moderate HIV-1 resistance to P3 in primary isolates, when compared to HIV-1 NL4-3. No HIV-2 resistant strains could be selected in the presence of P3. Collectively, the results indicate that the HR1 region in the TM glycoprotein is the target of P3 and suggest that the pathway of HIV-1 resistance to P3 differs from that of T-20 and that the genetic barrier to P3 resistance is significantly higher in HIV-2 than in HIV-1. Our findings provide proof of principle that viable antiviral peptides can be constructed using evolutionary biology strategies. Such strategies should be explored to enhance the production of peptide drugs and vaccines.

ACKNOWLEDGEMENTS

This work was supported by Fundação para a Ciência e Tecnologia [grant number PTDCSAU-FCF6767/2006], Portugal, and by Collaborative HIV and Anti-HIV Drug Resistance Network (CHAIN) from the European Union. PB and RC are supported by PhD grants from Fundação para a Ciência e Tecnologia, Portugal. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

Table S1. GenBank accession numbers of HIV-2 and SIV reference sequences used for the reconstruction of ancestral character states.

HIV-2	SIV
AB100245	AF077017
AF082339	AF334679
AF208027	AY033146
AY530889	AY033233
D00835	L03295
J03654	L09212
J04498	M31325
J04542	M33262
L07625	M83293
L36874	U72748
M15390	X14307
M30502	
M30895	
M31113	
U05352	
U05353	
U05355	
U05356	
U05357	
U05358	
U05359	
U22047	
U27200	
U38293	
X61240	
Z48731	

Table S2. Primers used for PCR amplification and sequencing of HIV-1 *env* gene segments.

Name	Position [a]	Sequence (5' – 3')
PBENV1 (fwd) [b]	5968 – 5986	CTATGGCAGGAAGAAGCGG
PBENV2 (fwd) [c]	6203 – 6223	GAAAGAGCAGAAGAYAGTGGC
PBENV3 (rev) [b]	9016 – 9036	AGTCATTGGTCTTARAGGTAC
PBENV4 (rev) [c]	8797 – 8817	TTTTGACCACTTGCCHCCCAT
PBSEQ1 (fwd) [d]	6567 - 6583	AGCYTAAAGCCATGTGT
PBSEQ2 (rev) [d]	6567 – 6583	ACACATGGCTTTARGCT
PBSEQ3 (fwd) [d]	6955 – 6970	CAGTACAATGTACACA
PBSEQ4 (rev) [d]	6955 – 6970	TGTGTACATTGTACTG
PBSEQ5 (fwd) [d]	7344 - 7363	CATAGTTTTAATTGTRGAGG
PBSEQ6 (rev) [d]	7344 – 7363	CCTCYACAATTAAAACTATG
PBSEQ7 (fwd) [d]	7745 – 7762	GAGAGAAAAAAGAGCAGT
PBSEQ8 (rev) [d]	7745 – 7762	ACTGCTCTTTTTTCTCTC
PBSEQ9 (fwd) [d]	8031 – 8047	ATCTGCACCACTAATGT
PBSEQ10 (rev) [d]	8031 – 8047	ACATTAGTGGTGCAGAT
PBSEQ11 (fwd) [d]	8510 – 8529	CCTGTGCCTCTTCAGCTACC
PBSEQ12 (rev) [d]	8510 – 8529	GGTAGCTGAAGAGGCACAGG

[a] HIV-1 HXB2.

[b] Outer primer for PCR.

[c] Inner primer for PCR.

[d] Sequencing primer.

Table S3. Primers used for PCR amplification and sequencing of HIV-2 *env* gene segments.

Name	**Position** [a]	**Sequence (5' – 3')**
CR1 (fwd) [b]	5927 – 5946	AGGAAACAGYGGMGAAGAGA
CR2 (rev) [b]	9391 – 9369	TCTACATCATCCATATTTTGYTG
CR3 (fwd) [c]	6673 – 6692	CTCATYCGTCTTCTGCATCA
CR4 (rev) [c]	9286 – 9268	TCACAGGAGGGCGATTTCT
HB5 (fwd) [d]	7321 – 7344	CACATCAGTCATCACAGAGTCA
CRSEQ2 (rev) [d]	7363 – 7345	ATCCCAATAGTGCTTRTCA
CRSEQ3 (fwd) [d]	7313 – 7334	CATTGCAACACATCAGTCATCA
HIV2SEQ2 (rev) [d]	7873 – 7859	GCAGTTAGTCCACAT
CRSEQ4 (rev) [d]	7918 - 7898	CCAATTGAGGAACCAAGTCAT
CRSEQ5 (fwd) [d]	7859 – 7879	ATGTGGACTAACTGCAGAGGA
CRSEQ6 (rev) [d]	8360 – 8344	GCTGTTGCTGTTGCTGC
CRSEQ7 (fwd) [d]	8344 – 8360	GCAGCAACAGCAACAGC
CRSEQ8 (rev) [d]	8835 – 8817	GAGAAAACAGGCCTATAGC
CRSEQ9 (fwd) [d]	8817 – 8835	GCTATAGGCCTGTTTTCTC
CRSEQ10 (fwd) [d]	7159 – 7173	AGACAATTGCACAGG
CRSEQ11 (rev) [d]	7424 - 7410	TGGTATCATTGCATC

[a] HIV-2 BEN.

[b] Outer primer for PCR.

[c] Inner primer for PCR.

[d] Sequencing primer.

Table S4. Characterization of HIV-1 and HIV-2 isolates included in this study, and the antiviral activities of peptide P3 against these isolates in TZM-bl cells.

HIV-1			P3 (nM) [c]		HIV-2			P3 (nM) [c]	
Isolates	**Genetic forms**	**Tropism[b]**	IC_{50}	IC_{90}	**Isolates**	**Genetic forms**	**Tropism[b]**	IC_{50}	IC_{90}
NL4-3	B	X4	0.3	6.2	ROD	A	X4	95.2	438.5
SG3.1	B	X4	2.5	64.4	03PTHCC1	A	R5	18.7	296.5
93AOHDC249	U[a]	R5	62.2	312.6	03PTHCC6	A	R5	114.3	1020.9
93AOHDC250	J	R5	38.6	191.0	03PTHCC7	A	R5	78.4	450.8
93AOHDC251	U	R5	53.8	1786.5	03PTHCC10	A	X4	108.8	822.2
93AOHDC252	U	R5	22.9	140.3	03PTHCC12	A	R5	70.8	411.1
93AOHDC253	J	R5	12.5	194.1	03PTHCC17	A	R5	142.4	1336.6
01PTHDECJN	CRF02_AG	R5	0.5	130.9	03PTHCC19	A	R5	120.6	659.2
00PTHDEEBB	G	R5	15.9	231.7	00PTHCC20	A	X4	13.3	145.9
					03PTHCC20	A	X4	16.3	212.8
					00PTHDECT	A	R5	48.9	792.5
					03PTHDECT	A	X4	51.7	584.8
					01PTHDESC	A	X4	14.89	116.7
					10PTHSJIG	A	R5	184.1	5445.0
					03PTHSM2	A	R5	44.2	1013.9
					03PTHSM9	A	X4	62.1	246.6
					04PTHSM10	A	X4	369.4	2074.9
					10PTHSMAK	A	D/M	51.65	1124.6
					10PTHSMAUC	A	X4	17.4	242.1
					10PTHSMNC	A	R5	88.51	1142.9

[a] U, untypable HIV-1 subtype: 93AOHDC249 and 93AOHDC252, sequences are basal to subtypes 19_cpx and 37_cpx; 93AOHDC251 sequence is basal to subtype H.

[b] R5, CCR5 coreceptor usage; X4, CXCR4 coreceptor usage; D/M, Dual/Mixed viral population using CCR5 and CXCR4 coreceptors.

[c] IC_{50} and IC_{90} best-fit values were inferred from sigmoidal dose-response (variable slope) curves and represent geometric mean values.

V

General Discussion and Conclusions

GENERAL DISCUSSION AND CONCLUSIONS

In a context of limited tools and studies specifically addressing HIV-2 infection, the clinical management of patients infected with HIV-2 is often dependent on the growing knowledge and evidence produced for its counterpart, HIV-1. However, HIV-1 and HIV-2 have different genetic characteristics and evolutionary histories. Hence, it is not surprising that the therapeutic armamentarium for HIV-2 infection is more restricted than that for HIV-1 and that response to treatment with available options is usually poorer in HIV-2 patients [1,2,3,4]. In this context, MVC and other entry inhibitors could be useful to treat HIV-2 patients. However, up to now, despite the recent use of MVC in salvage therapy of two HIV-2 infected patients [5,6], there was no information on the *in vitro* activity of MVC on HIV-2 primary isolates. Hence, the main objectives of the work presented here were to determine HIV-2 susceptibility to MVC and other available entry inhibitors and produce a new fusion inhibitor for HIV-2.

It is now well known that the sensitivity of HIV to entry inhibitors is modulated by the interactions of the surface envelope glycoprotein with the coreceptors. The envelope structure and function of HIV-1 and HIV-2 are subtly different as judged by the different tropism profiles of these viruses [7,8,9] and susceptibility to neutralizing antibodies [10,11]. Changing the sequence and structure of the HIV-1 or HIV-2 V3 loop often results in different levels of susceptibility to selected coreceptor antagonists [12,13,14] and fusion inhibitors [15,16,17]. Interestingly, such modifications can modulate in opposite ways the susceptibility of HIV-1 and HIV-2 to neutralizing antibodies. Indeed, whereas in HIV-2 viruses with charged V3 loops and X4 tropism are resistant to neutralization [18] in HIV-1 this type of viruses are usually more sensitive to neutralization [19]. These data suggests that co-receptor antagonist might act differently in HIV-1 and HIV-2 and that resistance to these drugs might also develop in different ways [12,20,21]. To better understand and explain the perceived differences in HIV-1 and HIV-2 susceptibility to co-receptor antagonists we first performed a detailed side-by-side molecular, evolutionary and structural comparison of the target of all co-receptor antagonists, the V3 loop and surrounding C2 and C3 envelope regions (Chapter 2). Overall, the C2, V3 and C3 regions were more conserved and occluded in HIV-2 than in HIV-1. This was particularly evident in the V3 loop that was highly conserved in HIV-2 and concealed within the envelope complex, possibly due to a physical interaction with C2 and C3. The strong conservation of these envelope regions in HIV-2 can be related with its multiple functional roles (e.g. immunosuppressive activity, interaction with multiple co-receptors and Vpu-like function [22,23,24]). On the other hand, the extended and highly accessible HIV-1 V3 loop is

consistent with its immunodominant and neutralizing nature [25,26,27,28,29,30] and with its major role in CXCR4 and CCR5 binding [31,32]. Remarkably, we found that diversification of C2, V3 and C3 in HIV-2, but not in HIV-1, has a dominant negative effect on viral fitness and transmission. Hence, selection against change in the C2, V3 and C3 envelope regions seems to be a distinctive evolutionary feature of HIV-2 [33,34]. Whether the different molecular, functional and structural features of HIV-1 and HIV-2 in these envelope regions had any impact on the susceptibility to entry inhibitors was evaluated on Chapter 3.

In Chapter 3 we determined the *in vitro* baseline susceptibility of HIV-1 and HIV-2 primary isolates to fusion inhibitors (T-20 and T-1249) and coreceptor antagonists (AMD3100, TAK-779 and MVC) and related this susceptibility with the time of virus isolation as well as with the genetic and phenotypic characteristics of the isolates. We found that primary isolates of HIV-1 and HIV-2 have similar sensitivities to AMD3100 and TAK-779. However, different dose-response profiles were obtained for MVC. Indeed, MVC inhibits the replication of R5 HIV-2 variants with significantly higher IC_{90s} and lower slope values than HIV-1. This suggests that higher dosages of MVC may be required for the treatment of HIV-2 infected patients [35,36]; otherwise, the administration of sub-therapeutic dosages might favour the selection of X4 variants, which in HIV-2 have been associated in with CD4 depletion, disease progression [37] and resistance to neutralization [38]. Additionally, we found an inverse correlation between HIV-2 sensitivity to MVC (IC_{50}) and CD4+ T cell counts at time of virus isolation. Indeed, R5 HIV-2 variants isolated from AIDS patients were significantly less susceptible to MVC than R5 variants isolated from asymptomatic patients. In HIV-1, R5 variants with lower susceptibility to MVC and other entry inhibitors had V3 loops with higher charges as compared with variants with higher sensitivity to MVC. We could not find such a relationship in our study possibly due to the reduced number of HIV-2 isolates that were studied. In addition, there were no obvious relationship between MVC susceptibility of R5 variants and conformational structure of the C2, V3 and C3 envelope regions (data not shown). Increased MVC resistance of late stage disease R5 variants might be explained by increased affinity for CCR5 [39] and/or an enhanced viral infectivity and replicative capacity [39,40]. Alternatively, these R5 variants may be evolutionary intermediates toward X4 use [40,41]. Future studies of a longitudinal nature should address these issues.

Collectively, our results argue in favour of further clinical studies to fully evaluate the clinical efficacy of MVC in HIV-2 infection and determine the best therapeutic dosage in early and late stage disease. Equally important will be the development of tropism assays for HIV-2 (currently unavailable) to determine coreceptor tropism before initiation of MVC

therapy [42,43]. Failure to do so might favour the selection for HIV-2 X4 variants that are associated with bad disease prognosis.

In what concerns to fusion inhibitors, our results confirm the reduced activity of ENF on HIV-2. Conversely, the high susceptibility of this virus to T-1249 indicates that fusion inhibitors are potentially useful against HIV-2; hence, it could be of interest to develop new drugs of this class (peptides or small-molecules) that are active against both HIV-1 and HIV-2.

Finally, we evaluated the activity of a newly designed peptide (P3) on both HIV-1 and HIV-2 isolates (Chapter 4). The sequence of this peptide was selected from an alignment of HIV-2 and SIV gp36 ancestral sequences. The rational for this approach was to minimize the amount of sequence divergence between contemporary strains and capture the more conserved features of the HR1/HR2 sequences [44,45]. Additionally, we expected to improve the tolerance of these molecules for natural polymorphisms on their target region or genotypic substitutions selected under drug pressure, without significant loss of sensitivity to their antiviral activity.

P3 has 34 residues and overlapps the N-terminal pocket-binding region and heptad repeat (HR) core of the HR2 region. It differs by 21 aa from the consensus HIV-1 sequence, 14 aa from T-20 and 6 aa from consensus HIV-2. In contrast to the unstructured nature of T-20, P3 has a typical α-helix conformation, which might increase its stability in physiological conditions and enhance its binding affinity to the target site [46,47,48,49,50]. In contrast to T-20, P3 potently inhibited both HIV-1 and HIV-2 replication (mean IC_{50} HIV-1, 11 nM vs IC_{50} HIV-2, 63.8 nM; $P < 0.0001$). P3 also potently inhibited the replication of T-20 resistant HIV-1 isolates (IC_{50} range, 0.15 – 11.8 nM). The N43K resistance mutation in HR1 region was selected in the presence of P3 and led to a 120-fold decrease in HIV-1 susceptibility to this peptide. Under the same conditions were were not able to select HIV-2 resistant strains. P3 did not inhibit the replication of a HIV pseudovirus containing the VSV-G envelope and bound strongly to a recombinant HIV transmembrane envelope protein (30-fold above cut-off). Finally, P3 was significantly less antigenic than T-20 in drug naive HIV-1 infected patients. Overall, these results indicate that P3 is a strong antiviral molecule that inhibits HIV fusion by binding to the HR1 region in the TM glycoprotein (like other HR2-based peptides), and suggest that the pathway of HIV-1 resistance to P3 differs from that of T-20. Moreover it seems that the genetic barrier to P3 resistance is significantly higher in HIV-2 than in HIV-1. Our findings provide proof of principle that viable antiviral peptides can be constructed using evolutionary biology strategies. Such strategies should be explored to enhance the production of peptide drugs and vaccines.

FUTURE PERSPECTIVES

The work described in this thesis suggests several lines of future investigation. Firstly, clinical trials are required to accurately determine the therapeutic dosages of MVC in HIV-2 infection (with different support regimens). Secondly, longitudinal studies with HIV-2 infected patients treated with MVC will be required to: (1) study the mode and tempo of resistance development to this drug *in vivo,* and (2) better characterize and understand the relationship between disease progression and susceptibility of R5 variants to MVC. The results obtained with these studies will be crucial to manage HIV-2 therapy with MVC and to determine whether MVC should be preferentially offered in the beginning of the HIV-2 infection (in a first-line regimen) as our results would suggest. Our studies demonstrated that dual tropic HIV-1 isolates are not responsive to MVC. Therefore, development of a genotypic and/or a phenotypic tropism assay for HIV-2 is urgently required so that MVC is not used in patients harbouring dual tropic viruses or mixed infections with X4 and R5 strains.

We have found that R5 variants isolated from AIDS patients are more resistant to MVC than R5 variants from asymptomatic patients (Chapter 3). Could this R5 isolates be transition isolates toward X4 usage? Are the envelope glycoproteins of this late R5 isolates conformational different from early R5 isolates? It will be important to characterize the molecular, structural and functional determinants of this different susceptibility to MVC. Interestingly, we found recently that while most R5 variants are sensitive to neutralizing antibodies, some HIV-2 R5 variants are resistant to neutralizing antibodies [18]. Is there any association between evolution of MVC resistance in R5 isolates and neutralization? Could this evolution be driven by the neutralizing antibody response in HIV-2 patients? Responses to these questions will lead to a more informed use of MVC in HIV-2 patients and to a better knowledge of the HIV-2 evolution in a highly selective environment.

Concerning the new fusion inhibitor peptide (P3), biophysical studies on the specific interaction between P3 and the HR1-target are underway and will help to clarify the mechanism of action of P3. Resistance to P3 should be further investigated in HIV-2; in HIV-1, site directed mutagenesis should be used to insert the N43K mutation alone into the backbone of an infectious molecular clone of HIV-1 in order to see if this mutation confers high-level resistance to P3. We think that P3 may be useful as a microbicide. These are substances designed to reduce or prevent the sexual transmission of HIV and other sexually transmitted infections when applied topically inside of the vagina or rectum. Thus, we plan to formulate P3 with an appropriate carrier [e.g. HydroxyEthyl Cellulose (HEC) gel] [51], test its stability under different circumstances (e.g. in the presence of seminal

plasma and in the presence of the microflora of the vagina, especially *Lactobacillus acidophilus*) and test its activity on the vagina microflora [52]. The preclinical trial of this peptide as a microbicide or as a drug will be tested in the RAG-Hu mice [53]. Finally, if all this trials succeed we plan to push P3 into clinical trials with monkeys, which will require, in first instance, the confirmation that P3 is active in SIV isolates.

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