Universidade de Lisboa

Faculdade de Farmácia

Characterization of HIV-2 susceptibility to protease and entry inhibitors and identification of envelope determinants of coreceptor usage, cell tropism and antibody neutralization

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Orientadores: Professor Doutor Nuno Eduardo Moura dos Santos da Costa Taveira

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Tese especialmente elaborada para a obtenção do grau de Doutor em Farmácia, especialidade Microbiologia.

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PREFACE

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Manuscripts and papers in international journals

Martins A, Calado M, Rocha C, Borrego P, Marcelino J, Azevedo-Pereira JM and Taveira N. Determinants of coreceptor usage, tropism and susceptibility to antibody neutralization in the V3 region of HIV-2. (Manuscript in preparation)

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RESUMO

O vírus da imunodeficiência humana tipo 2 (VIH-2) foi isolado e identificado pela primeira vez em 1986 como agente etiológico da Síndrome de Imunodeficiência Adquirida (SIDA). Atualmente é responsável por epidemias localizadas na África Ocidental, Continente Americano, Índia e em alguns países europeus como Portugal e França, onde a sua prevalência é ainda relativamente elevada.

O VIH-2 partilha muitas similaridades com o vírus da imunodeficiência humana tipo 1 (VIH-1), incluindo a organização estrutural e genómica, as vias de transmissão e o ciclo de replicação. No entanto, ambos os vírus exibem características distintas em termos de história evolutiva, patogénese, epidemiologia e algoritmos de tratamento.

A maior diferença clínica entre ambas as infeções reside na progressão da doença, que é mais lenta no VIH-2 comparativamente ao VIH-1. A maioria dos indivíduos infetados pelo VIH-2 apresenta cargas virais indetetáveis e contagem normal de linfócitos T CD4+. Por outro lado, ao contrário do que sucede para o VIH-1, as opções terapêuticas disponíveis para tratar a infeção pelo VIH-2 são reduzidas. Todos os fármacos antirretrovirais foram desenvolvidos especificamente para o VIH-1, sendo a sua ação menos expressiva ou inexistente para o VIH-2. Por exemplo, o VIH-2 é resistente aos inibidores não nucleósidos da transcriptase reversa e apresenta diferentes níveis de suscetibilidade aos inibidores da protease (IPs). Neste contexto, os antagonistas do coreceptor CCR5, como o maraviroc (MVC), surgem como uma nova opção terapêutica para estes doentes. Contudo, a sua utilização requer a determinação do tropismo viral e, ao contrário do HIV-1, até aqui não existia nenhuma ferramenta informática que o permitisse fazer de forma adequada a partir das sequências da região V3.

O enfuvirtide (T-20) é o único inibidor de fusão aprovado, até ao momento, para o tratamento da infeção pelo VIH-1. Porém, este péptido tem atividade muito reduzida contra o VIH-2. Recentemente foi demonstrado que com um desenho adequado se podem produzir péptidos que inibem a fusão de VIH-2 e VIH-1, o que desencadeou a pesquisa de novos fármacos peptídicos para o tratamento das infeções por estes dois vírus.

Os IPs são uma das principais classes de fármacos antirretrovirais utilizadas no tratamento da infeção pelo VIH-1. No entanto, o VIH-2 exibe suscetibilidade variável a estes fármacos e uma reduzida barreira genética de resistência.

Consequentemente, apenas três IPs estão atualmente recomendados para o tratamento dos doentes infetados por VIH-2, nomeadamente o saquinavir (SQV), lopinavir (LPV) e darunavir (DRV). Tendo em conta as opções terapêuticas limitadas e a seleção rápida de mutações de resistência no VIH-2, recomenda-se que o início do tratamento e a mudança dos esquemas terapêuticos nestes doentes devam ser realizados sob orientação de um teste de resistência. Por outro lado, desconhece-se ainda de que forma a diversidade da protease do VIH-2 afeta a resposta a longo prazo ao tratamento com IPs e de que forma a terapêutica com estes inibidores determina a evolução do VIH-2.

A entrada do VIH-2 no hospedeiro envolve a interação da glicoproteína de superfície do invólucro viral (SU) com o receptor CD4 e com os coreceptores de quimiocinas CXCR4 e CCR5 localizados nas células alvo, nomeadamente linfócitos T, macrófagos e outros tipos de células. Estirpes de VIH-2 que usam o CCR5 (variantes R5) são comuns em indivíduos assintomáticos enquanto vírus utilizadores do CXCR4 (variantes X4) são detetados apenas em indivíduos em fase avançada da doença e com contagens de linfócitos T $CD4^+$ reduzidas.
Atualmente a informação existente relativa aos determinantes genéticos e estruturais da interação do VIH-2 com os coreceptores celulares ainda é muito limitada. Tal como no VIH-1, a utilização destes coreceptores pelo VIH-2 parece estar associada a alterações específicas na região V3 do invólucro. Por outro lado, estudos recentes indicaram uma associação entre o tipo de coreceptor utilizado pelo VIH-2 e a suscetibilidade à neutralização por anticorpos. No entanto, os determinantes do invólucro envolvidos nesta associação entre tipo de coreceptor e suscetibilidade à neutralização por anticorpos estão ainda por caracterizar.

Neste sentido, o objetivo geral deste trabalho foi caracterizar a suscetibilidade do VIH-2 aos inibidores da protease e inibidores de entrada e identificar determinantes virais do uso de coreceptores, tropismo celular e neutralização por anticorpos.

Os objetivos específicos foram: 1) identificar os aminoácidos da região V3 envolvidos na utilização dos coreceptores CXCR4 e CCR5, na suscetibilidade a anticorpos neutralizantes e no tropismo celular; 2) desenvolver um método genotípico para prever o tipo de coreceptor utilizado pelo VIH-2 com base na sequência de aminoácidos da região V3; 3) determinar a potência de ação de um novo péptido inibidor de fusão sobre o VIH-2 e 4) caracterizar a evolução da protease do VIH-2 em indivíduos infetados com ou sem experiência terapêutica prévia com IPs.

No primeiro capítulo desta tese é feita uma revisão dos conhecimentos atuais sobre a infeção por VIH-2 nos temas pertinentes para este trabalho, nomeadamente os fatores genéticos e biológicos que determinam o processo de entrada do vírus na célula do hospedeiro e as opções de tratamento para doentes infetados por VIH-2. O capítulo 2 faz referência aos objetivos e plano de trabalho da presente tese. Os restantes capítulos (3-6) descrevem o trabalho científico que deu origem a esta tese. Por último, no capítulo 7, são discutidos de forma integrada os resultados obtidos e realçadas as principais conclusões deste trabalho.

Para identificar os determinantes da região V3 do VIH-2 envolvidos na interação com os coreceptores celulares CCR5 e CXCR4 (Capítulo 3) foram efetuadas mutações por substituição nas posições 18 e/ou 19 e deleções nas posições 23 e/ou 24 da V3 do pROD10, um clone molecular infecioso do VIH-2_{ROD}, o isolado X4 de referência. Os clones mutados deram origem a seis vírus mutantes após transfecção de células 293T.
Os ensaios celulares permitiram demonstrar que: 1) a conversão do fenótipo X4 em R5 no VIH-2_{ROD10} requer a substituição H18L e a deleção Δ(23,24); 2) os mutantes H18L e H23Δ + Y24Δ são mais fáceis de neutralizar do que o VIH-2_{ROD} e os outros mutantes por plasma de indivíduos infetados pelo VIH-2; por outro lado a mutação K29T parece contribuir para aumentar a resistência à neutralização; 3) os mutantes K29T adquirem tropismo macrofágico sem comprometer a capacidade de replicação em linfócitos T CD4+; 4) os mutantes Δ(23,24) e H18L + Δ(23,24) adquirem tropismo macrofágico à custa de capacidade de replicação em linfócitos T CD4+.

Adicionalmente, a análise estrutural por *homology modelling* permitiu demonstrar que: 1) a substituição H18L compromete a ligação da histidina com a metionina em posição 15 e com a fenilalanina em posição 20; 2) a deleção da H23 e Y24 leva à eliminação das folhas beta paralelas típicas da V3 e a uma perda de conteúdo aromático muito significativo o que pode comprometer a ligação a receptores celulares ou outras moléculas (ex. anticorpos); 3) a substituição K29T reduz a carga da V3 e elimina a ligação com a isoleucina em posição 27.

Coletivamente, estes resultados demonstraram que a V3 do VIH-2 é um determinante importante da ligação do vírus aos coreceptores celulares CCR5 e CXCR4, da suscetibilidade a anticorpos neutralizantes e da capacidade replicativa em linfócitos T $CD4^+$ e macrófagos, e que estas características fenotípicas podem ser moduladas pela alteração de um único aminoácido. Estes resultados permitem atribuir à região V3 do invólucro do VIH-2 um papel crucial na patogénese da infeção por este vírus.

Até ao momento, o MVC é o único antagonista do coreceptor CCR5 aprovado para o tratamento da infeção pelo VIH-1. Estudos recentes têm demonstrado a sua eficácia também contra isolados de VIH-2. O início da terapêutica com MVC exige o conhecimento prévio do tropismo viral, dado que este fármaco pode potencialmente selecionar estirpes X4 minoritárias que estão associadas a maior capacidade replicativa, maior resistência aos anticorpos neutralizantes e a uma progressão mais rápida da doença. No entanto, ao contrário do VIH-1, ainda não existem testes genotípicos ou fenotípicos validados para a determinação do tropismo viral no VIH-2.

Nesse sentido, foi desenvolvido e validado um algoritmo para a determinação da utilização de coreceptores pelo VIH-2 com base na sequência da região V3. Este algoritmo deu origem a um serviço disponibilizado *online* semelhante ao existente para VIH-1 (geno2pheno[coreceptor-hiv2]) (Capítulo 4). O desenvolvimento e validação deste método genotípico para determinar o tropismo de VIH-2 requereu a análise de 126 sequências da região V3 obtidas a partir de indivíduos infetados pelo VIH-2, na sua maioria provenientes de Portugal, que apresentavam o perfil fenotípico definido para os coreceptores. A capacidade preditiva deste algoritmo foi ainda validada com base nas V3 mutadas produzidas e caracterizadas ao nível fenotípico no Capítulo 3. No conjunto, estes dados indicaram que o geno2pheno[coreceptor-hiv2] pode ser um instrumento útil na prática clínica, permitindo aos médicos uma melhor gestão dos doentes infetados pelo VIH-2 candidatos a terapêutica com MVC.

No Capítulo 5 determinou-se a atividade de um novo péptido inibidor de fusão designado 2P23 contra isolados de VIH-2. Tal como péptidos análogos, a sequência do péptido 2P23 é idêntica à parte da região HR2 localizada no ectodomínio da glicoproteína transmembranar. O seu mecanismo de ação envolve a ligação à região HR1, o que impede a ligação natural desta região à HR2 e a formação da estrutura hexahelicoidal (3HR1:3HR2) que é fundamental para a fusão do vírus com a célula.

O 2P23 apresenta um desenho inovador na medida em que inclui dois resíduos, uma metionina e uma treonina, adjacentes ao domínio de ligação (*pocket binding domain*, PBD) da região HR2. Estes resíduos adotam uma estrutura específica designada por gancho M-T (M-T *hook)* que é importante para a estabilização da interação entre o PBD da região HR-2 e a cavidade hidrofóbica da região HR1. Esta interação é essencial para a estabilização da estrutura hexahelicoidal e para a fusão viral constituindo assim um alvo atrativo para o desenvolvimento de novos inibidores de fusão. Para além desta estratégia, a produção do 2P23 ainda envolveu a introdução de pontes salinas e resíduos cruciais para a ligação à região HR1 da glicoproteína transmembranar do VIH.

O 2P23 demonstrou ter uma potente atividade antiviral contra isolados primários de VIH-2 e VIH-1 (IC_{50} médio, 20.17 nM e 5.57 nM, respetivamente) e SIV (IC_{50} médio, 1.8 nM para SIV_{pbj} e 2.39 nM para SIV_{239}). A sua atividade inibitória contra as seis variantes da V3 (Capítulo 3) foi igualmente muito potente (IC_{50} médio, 15.38 nM) indicando independência em relação à utilização de coreceptores. Em síntese, o 2P23 revelou ser um inibidor de fusão extraordinariamente potente contra diversos isolados primários de VIH-2, com diferentes perfis de utilização dos coreceptores, e poderá ser um fármaco promissor para desenvolvimento clínico futuro.

Os IPs são uma das principais classes de fármacos utilizadas no tratamento da infeção pelo VIH-2. Dada a prevalência significativa de VIH-2 em Portugal e a utilização frequente de IPs nestes doentes, torna-se essencial investigar a emergência de mutações de resistência nesta população e o seu impacto na resposta à terapêutica. No Capítulo 6 caracterizou-se a diversidade genética da PR e a resistência genotípica aos IPs em doentes infetados pelo VIH-2 residentes em Portugal e avaliou-se o seu impacto no resultado da terapêutica após oito anos.

Em 2007, foram colhidas amostras de sangue de 27 doentes infetados pelo VIH-2 com e sem terapêutica prévia, seguidos no Hospital de Santa Maria em Lisboa.

Procedeu-se à amplificação do gene da protease a partir de ADN proviral, que foi posteriormente clonado e sequenciado. Nesta data, 42,8% dos doentes tratados com IPs apresentavam mutações associadas a resistência (exs. I54M, I82F, L90M) ao SQV, LPV, DRV.

Após oito anos, a análise genotípica da PR permitiu constatar: 1) a perda de mutações de resistência detetadas inicialmente em dois doentes, num dos casos associada a interrupção terapêutica; 2) a persistência de mutações de resistência num doente, como resultado de falência virológica e imunológica, em contexto de troca terapêutica e 3) o desenvolvimento de novas mutações de resistência em três doentes, associado a falências terapêuticas prévias.

Relativamente à diversidade genética da PR, verificou-se um aumento da diversidade em dois doentes tratados, virologicamente suprimidos e que apresentaram um aumento da contagem de T CD4+ comparativamente ao valor basal. Por outro lado, observou-se uma diminuição da diversidade genética da PR em três indivíduos (dois tratados e um não tratado) que apresentaram em algum momento do *follow up* cargas virais detetáveis.

Estes resultados parecem evidenciar a persistência da replicação viral durante terapêutica antirretroviral a longo prazo, independentemente da supressão da carga viral plasmática. A manutenção da replicação viral poderá constituir a fonte de renovação das *quasispecies* provirais, levando a uma substituição gradual das variantes ancestrais ao longo do tempo.

Neste estudo, também identificámos dois (15.4%) casos potenciais de resistência aos IPs em doentes sem terapêutica prévia. As mutações de resistência encontradas, L90M e I84V, foram também as mais prevalentes no subgrupo de doentes tratados. No conjunto, estes dados indicam que os testes de resistência baseados em ADN proviral podem ser úteis em doentes infetados pelo VIH-2 com cargas virais reduzidas ou indetetáveis e em indivíduos sem terapêutica prévia, e que a deteção precoce de resistência adquirida ou transmitida pode predizer a resposta à terapêutica nestes doentes.

Palavras-chave: Suscetibilidade do VIH-2 aos fármacos antirretrovirais; evolução no VIH-2; determinantes do invólucro na utilização do coreceptor; determinantes do tropismo celular e dos anticorpos neutralizantes.

ABSTRACT

The main aim of this work was to characterize the susceptibility of HIV-2 to protease and entry inhibitors and to identify viral determinants of coreceptor usage, cellular tropism and antibody neutralization. The specific objectives were: 1) to determine the contribution of amino acids residues in the V3 loop involved in CCR5 and CXCR4 use, susceptibility to antibody neutralization and cellular tropism; 2) to develop a genotypic method for the prediction of HIV-2 coreceptor usage based on V3 loop; 3) to evaluate the antiviral activity of a new short-peptide fusion inhibitor in HIV-2 and 4) to characterize the evolution and diversity of protease (PR) in HIV-2 infected patients treated and untreated with protease inhibitors (PIs).

In the first study (Chapter 3), site-directed mutagenesis was used to create amino acid substitutions in residues 18 and/or 29 and/or single deletions at positions 23 and 24 in V3 loop of pROD10, an infectious molecular clone of HIV-2_{ROD}, the reference X4 isolate.

Cellular assays demonstrated that: 1) conversion from X4 to R5 phenotype in HIV-2_{ROD10} requires H18L substitution and the deletion Δ(23,24); 2) H18L and H23Δ + Y24Δ mutants are more easily neutralized than HIV-2_{ROD} and other mutated viruses by plasma from HIV-2 infected individuals; on the other hand, K29T substitution seems to contribute to increase resistance to neutralization; 3) K29T mutants acquire macrophage tropism without compromising replicative capacity in CD4+ T lymphocytes; 4) H18L + Δ(23,24) and Δ(23,24) mutants gained the ability to replicate in macrophages albeit at the cost of some capacity to replicate in CD4+ T cells.

Structural analysis by homology modelling showed that: 1) H18L substitution disrupts the interaction of histidine with methionine at position 15 and with phenylalanine at position 20; 2) deletion of H23 and Y24 leads to the elimination of the parallel β sheets presented in the V3 loop and the loss of the aromatic system which can compromise the binding of cellular coreceptors or other molecules (e.g. antibodies); 3) K29T substitution reduces the charge of V3 and leads to the loss of the interactions with isoleucine at position 27.

Collectively, these results demonstrated that V3 is an important determinant in HIV-2 coreceptor usage, susceptibility to antibody neutralization and replication capacity on CD4+ T cells and macrophages and that these phenotypic characteristics can be modulated by a single amino acid change in V3. These results support an important role for V3 in the pathogenesis of HIV-2 infection.

In the second study (Chapter 4), a genotypic method was developed for the prediction of HIV-2 coreceptor usage from the V3 loop, similar to an existing tool created for HIV-1 (geno2pheno [coreceptor-hiv2]). The development and validation of this tool was based on a data set of 126 samples from HIV-2 infected patients, most of them from Portugal, with phenotypic coreceptor usage annotations. Predictive accuracy was also validated based on the V3 mutants produced and phenotypically characterized in the previous chapter. Overall, these findings indicated that geno2pheno [coreceptor-hiv2] can be a useful tool in clinical practice, allowing better management of HIV-2 infected patients eligible for maraviroc (MVC).

In the third study (Chapter 5) a short-peptide named 2P23 was produced by combining a M-T hook structure, HIV-2 sequences and 'salt-bridge'-based strategies. This peptide showed a potent antiviral activity against HIV-2 and HIV-1 isolates (mean 50% inhibitory concentration- IC_{50}: 20.17 nM and 5.57 nM, respectively) and SIV (IC_{50}: 1.8 nM for SIV_{pbj} and 3.29 for SIV_{239}). This new fusion inhibitor also demonstrated a strong activity against the V3 variants (Chapter 3) (IC_{50}:15.38 nM), irrespectively of the coreceptor phenotype. Thus, 2P23 is an ideal candidate for further clinical development due to its broad antiviral activity against several HIV-2 isolates, with different coreceptor tropism.

The last study (Chapter 6), involved the characterization of PR diversity and genotypic resistance to PIs of HIV-2 infected individuals living in Portugal and the evaluation of the impact of resistance mutations to PIs in treatment outcome eight years post-therapy.
A high prevalence of PR mutations (e.g. I54M, I82F, L90M) associated to saquinavir (SQV), darunavir (DRV) and lopinavir (LPV) resistance, were detected in proviral DNA from these patients at baseline.

Eight years after study entry, the genotypic analysis identified: 1) loss of resistance mutations in two patients, that were initially detected at baseline, presumably as a consequence of treatment interruption; 2) long term persistence of resistance mutations in one individual as a result of virologic and immunologic failure, which might raise concern about transmission of drug resistance in the future and 3) development of new resistance mutations in three patients due to previous treatment failures.

The analysis of genetic diversity in PR showed an increase in this parameter in two treated patients, with undetectable viral loads and higher CD4+ T counts, comparing with the baseline. On the other hand, a reduction in PR genetic diversity was exhibited in three patients (two treated and one untreated), who presented detectable viral loads in at least one time point during the follow up. Due to small sample size it was not possible to investigate a potential relationship between PR genetic diversity and CD4+ T cell counts, presence of resistance mutations or/and treatment status. However, these results seem to indicate a persistent viral replication during long-term highly active antiretroviral therapy (HAART), regardless of plasma viral load. The maintenance of viral replication can act as a source of new proviral quasispecies, resulting in the gradual substitution of the ancestral variants over time.

Most importantly, we found two potential cases of transmitted drug resistance. However, due to the small sample size, additional studies with a higher number of patients are required to determine if primary drug resistance is a major problem in HIV-2 infected patients in Portugal.

Our findings suggest that proviral DNA may be useful in resistance testing in HIV-2 patients with low or suppressed viremia and in untreated patients, and that early resistance analysis of these archived viruses may predict treatment response.

Keywords: HIV-2 susceptibility to antiretroviral drugs; HIV-2 evolution; envelope determinants of coreceptor usage; determinants of cell tropism and antibody neutralization.

ABBREVIATIONS

6-HB	Six-helix bundle
3D	Three-dimensional
3TC	Lamivudine
µg	Micrograms
µl	Microliters
µM	Micromolar
ºC	Celsius degree
ABC	Abacavir
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral therapy
ASA	Accessible solvent area
ATV	Atazanavir
AUC	Area under the curve
AZT	Zidovudine
bp	Base pair
CA	Viral capsid
cART	Combined antiretroviral therapy
CD	Circular dichroism
CDC	Centers for Disease Control and Prevention
CHR	C-terminal heptad repeat
CO_2	Carbon dioxide
CRF	Circulating recombinant form
CTL	Cytotoxic T lymphocyte
d4T	Staduvine
DC	Dendritic cells
ddI	Didanosine
D/M	Dual/mixed population
DMEM	Dulbecco's minimal essential medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DRM	Drug resistance mutation

DRV	Darunavir
DTG	Dolutegravir
ECLs	Extracellular loops
ECL2	Second extracellular loop
EFV	Efavirenz
EI	Entry inhibitor
ELISA	Enzyme-Linked Immunosorbent Assay
ETV	Etravirine
EVG	Elvitegravir
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FPR	False positive rate
FPV	Fosamprenavir
FTC	Emtricitabine
GALT	Gut associated lymphoid tissue
h	Hour
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HLA	Human leukocyte antigen
HR-1	Heptad repeat 1
HR-2	Heptad repeat 2
IC50	50% inhibitory concentration
IC90	90% inhibitory concentration
IDV	Indinavir
IFN	Interferon
Ig	Immunoglobulin
IN	Integrase
INI	Integrase inhibitor
LPV	Lopinavir
LTRs	Long terminal repeats
MA	Matrix protein

mg	Miligrams
ML	Maximum likelihood method
ml	Milliliters
mm	Millimeters
nm	Nanometers
MOE	Molecular operating environment program
MPER	Membrane proximal external region
MVC	Maraviroc
Nabs	Neutralizing antibodies
NC	Nucleocapsid proteins
NFV	Nelfinavir
NHR	N-terminal heptad repeat
NIH	National Institute of Health
NK	Natural killer cells
nM	Nanomolar
nm	Nanometers
NNRTI	Nonnucloside reverse transcriptase inhibitor
NRTI	Nucleos(t)ide reverse transcriptase inhibitor
NVP	Nevirapine
PBD	Pocket binding domain
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
pDC	Plasmacytoid dendritic cell
PI	Protease inhibitor
PR	Protease
PT	Portuguese patients
RAL	Raltegravir
RNA	Ribonucleic acid
RPV	Rilpivirine
RT	Reverse transcriptase
RTV	Ritonavir

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
SQV	Saquinavir
SU	Surface glycoprotein
SVM	Support vector machine
T-20	Enfuvirtide
TAM	Thymidine analogue resistance mutation
$TCID_{50}$	50% tissue culture infectious dose
TDF	Tenofovir
TDR	Transmitted drug resistance
TLR	Toll-like receptor
TM	Transmembrane glycoprotein
TNF	Tumor necrosis factor
TPV	Tipranavir
U	Units
vs	*Versus*

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CHAPTER I

General introduction

Discovery, origins and dissemination of HIV-2

Acquired Immunodeficiency Syndrome (AIDS) is caused by two retroviruses, human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) [1, 2]. HIV-1 was isolated in 1983 and is responsible for the vast majority of HIV infections worldwide [2, 3]. HIV-2 was isolated in 1986 from two patients, one from Cape Verde interned at Claude Bernard Hospital in Paris, and another from Guinea-Bissau, interned in Hospital Egas Moniz in Lisbon with clinical symptoms similar to AIDS but negative serology for HIV-1 [1, 4].

HIV-2 is a lentivirus that belongs to the *Orthoretrovirinae* subfamily and the *Retroviridae* family [5]. This virus is closely related to simian immunodeficiency virus (SIVsmm) from sooty mangabeys monkeys (*Cercocebus atys atys*) that are found in the forests of the West Coast of Africa [6-8]. In contrast, HIV-1 descends from the SIVcpz found in *Pan troglodytes troglodytes* chimpanzees and from SIVgor that infects gorillas (*Gorilla gorilla gorilla)* [9-11].

HIV-1 can be divided into groups M, N, O and P, that resulted from tree cross-species transmissions from chimpanzees and one event transmission from gorillas, respectively [11-13]. HIV-2 resulted from at least nine independent transmissions from sooty mangabeys infected with SIVsm, originating nine groups termed A through I [7, 8, 14, 15]. Among these, only groups A and B cause epidemics, with group A being responsible for most HIV-2 infections worldwide. Isolates from group A are more predominant in Guinea-Bissau but are also found in other West African countries (e.g. Gambia, Ivory Coast and Cape Verde) whereas group B is more frequent in Ivory Coast and Ghana [16-21]. Groups C to I were only detected in isolates cases from Sierra Leone, Liberia and Ivory Coast [6, 14, 15, 22-26].

The date of introduction of HIV-2 groups A and B into the human population is estimated to be approximately 1940 and 1945, respectively [27-29]. Ivory Coast is the hypothetical geographic origin of HIV-2 group B, whereas the epicentre of group A remains to be defined. Some studies suggested Guinea-Bissau, based on serologic evidences, while others found SIVsmm lineages closely related to HIV-2 group A in Ivory Coast [7, 29].

More studies are needed to clarify the geographic origin of HIV-2 group A epidemic. HIV-2 group A has spread to Portugal from Guinea-Bissau and Cape Verde and to France from Ivory Coast and Senegal [29].

Within Europe, HIV-2 group A disseminated from Portugal to Luxembourg and the United Kingdom. Transmission of group A also occurred outside Europe in countries with historical links to Portugal such as Brazil, India and Mozambique [30, 31].
A circulating recombination form (CRF) of HIV-2 comprising sequences of groups A and B (designated as HIV-2 CRF01_AB) has been described in Cameroon, Ivory Coast and Japan [32-34].

HIV-2 epidemiology

Data on HIV-2 prevalence worldwide is quite old and more limited than for HIV-1. It is estimated that only about 1 to 2 million people are infected with HIV-2 in West Africa with most of them living in countries such as Guinea-Bissau, The Gambia, Senegal, Ivory Coast and Cape Verde [35]. Furthermore, HIV-2 prevalence is decreasing everywhere and some researchers estimate that HIV-2 will be extinct by the end of the century [36-39]. For instance, in the district of Caió in Guinea-Bissau the prevalence in adults declined from 8.3 % in 1990 to 4.7 % in 2007 [39]. The prevalence remained higher in older adults (age >45) while the decrease was more dramatic in young adults (15-35). In older adults the prevalence decreased from 22 to 12 % while young adults had a decrease from 3 to 0.9 %. The pace of decline was greater from 1997 to 2007 compared to 1990 to 1997. All of this occurred without antiretroviral therapy. In Gambia, HIV-2 prevalence declined from 7.0 to 4.0 % in 2001-2003 [40]. In contrast, both countries showed a rise in HIV-1 prevalence in the same period [39, 40]. HIV-1/2 dual infections are relatively common in West African countries, representing 0.3 to 1% of all HIV infected patients [41].
Outside West Africa, HIV-2 has been reported in The Americas, India and in several European countries, with Portugal and France being the countries with the highest prevalence of HIV-2 infected individuals [42-48]. In Portugal, at the end of 2015, an overall 1791 cases were associated with HIV-2 infection, corresponding to 3.3% of all notified HIV cases; HIV-1/HIV-2 dual infections were observed in 587 (1.1%) individuals [49]. The HIV-2 cumulative cases had an overall prevalence similar in both genders, with 878 cases in men and 913 in women. The majority was found in the age of 30 to 54 years at the time of their diagnosis.
The main mode of HIV-2 transmission was associated with heterosexual transmission (82%), with more than half of the individuals (61.5%) from Sub-Saharan Africa [49].

The number of new cases of HIV-2 infection in Portugal has been decreasing in the last twelve years, with 64 new infections in 2003 compared with 30 in 2015 [49].

Biology of HIV-2

Structure

HIV-2 is an enveloped, spherical RNA virus with a diameter of around 110 nm (Figure 1) [50]. The virus is enveloped by a host cell derived phospholipid bilayer. The outer surface is covered with surface glycoproteins (SU) that are anchored to the transmembrane glycoproteins (TM) to form trimers in the mature virion [51]. The inner surface of the viral envelope is coated by the proteins (MA) and inside the matrix shell is a conical capsid core particle (CA) [52]. The capsid encapsulates two copies of single stranded RNA bound to the nucleocapsid proteins (NC) and also contains three essential viral encoded enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN) and the accessory proteins Nef, Vif, Vpr and Vpx [53].

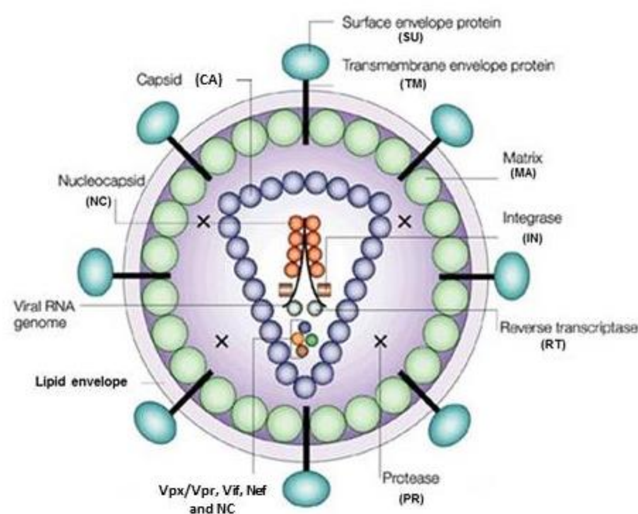

Figure 1. Schematic structure of the HIV particle. (Adapted from Robinson H. New hope for an aids vaccine. Nat Rev Immunol. 2002;2:239-50 [54]).

Genomic organization

The HIV-2 genome consists of two identical long single-stranded RNA molecules of 9800 nucleotides each, flanked by long terminal repeats (LTRs) at both ends (5' and 3') [50, 55]. It is organized into three main overlapping reading frames that comprise 9 genes; three major genes (*gag*, *pol*, *env*) encoding structural proteins or viral enzymes; two genes (rev, tat) for regulatory proteins and four (*vif*, vpx, *nef*, *vpr*) for accessory proteins (Figure 2) [50, 55].

The *gag* gene encodes the Gag (group specific antigen) precursor polyprotein (Pr55Gag) that is cleaved by viral protease during maturation of virion particles yielding four proteins: p6 (nucleocapsid), p6 (C-terminal protein), p16 (matrix) and p26 (capsid) [55, 56].

The Pol (polymerase) polyprotein is only expressed together with Gag as the Gag-Pol protein precursor (Pr160GagPol). GagPol precursor is cleaved by the viral protease into several enzymes that are required for virus replication: PR (p11), RT (p53) and IN (p34).

The *env* gene encodes for the polyprotein precursor Pr140Env, which is processed by a cellular protease to form the surface envelope glycoprotein SU (gp125) and transmembrane envelope glycoprotein TM (gp36), which are necessary for HIV entry into the host cell [55, 56].

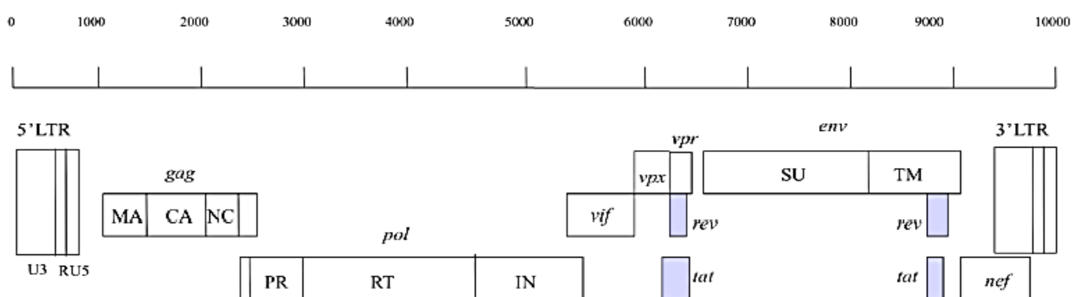

Figure 2. Genomic organization of HIV-2. (From Taveira N et al. Biologia Moelcular de VIH. In: Antunes F, editor. Manual sobre SIDA.Lisbon: Permanyer Portugal, 2008; p:27-50 [57]).

Envelope glycoproteins

The envelope glycoproteins (Env) mediate viral attachment and entry into target cells. The mature Env of HIV-1 and HIV-2 is arranged in trimeric spikes, comprising three glycoproteins SU (gp140/gp125) and three glycoproteins TM (gp40/gp36) that are non-covalently associated [58, 59]. The number and accessibility of Env spikes differ between HIV-1 and HIV-2. Compared to HIV-1, HIV-2 spikes are more stable and prominent after budding, while in HIV-1 the number of spikes decreases instantly after this process and during maturation [59-62].

The SU glycoprotein is composed of five constant (C1-C5) and five variable (V1-V5) regions. The constant domains correspond to the protein core while all variable regions, except V5, are exposed on the surface via disulphide bonds as large loops [63, 64]. The V3 loop of HIV-2, like HIV-1, is highly immunogenic, elicits antibody responses and seems to play an important role in coreceptor usage [65, 66]. On the other hand, the V4 and V5 loops are shorter and less glycosylated in HIV-2 than in HIV-1 [67].

The SU core is formed by an inner and outer domain. Both domains are linked by a bridging sheet. The inner domain is mainly formed by the C1 and C5 regions. This domain is hydrophobic and is responsible for the association between SU and TM. The outer domain is extensively glycosylated, and contains most of the antigenic determinants (neutralizing epitopes) and is implicated in the interaction between the SU and the cellular receptor (CD4) and coreceptors (mainly CCR5 and CXR4) [63, 64]. It was suggested that HIV-2 SU may sometimes adopt a CD4-induced conformation in its native state and thus may not require interaction with the receptor to induce conformational reorientation of the V1/V2 loops [68]. The TM glycoprotein consists of three major domains: an extracellular domain, a hydrophobic transmembrane domain and an intracytoplasmatic domain [69, 70]. The extracellular domain, or ectodomain, can be further divided into four segments: the hydrophobic fusion peptide at the N-terminus; two α-helices containing leucine zipper-like motifs (heptad repeat 1, HR1 and heptad repeat 2, HR2) and a membrane proximal external region (MPER). During virus entry into cells the HR1 and HR2 regions assemble into a six-helix structure consisting of a central parallel trimeric coiled-coil of the three HR1 helices, surrounded by the three HR2 helices in an anti-parallel way. The six-helix structure and the fusion peptide are essential for the fusion process between viral and host cell membranes.

The intracytoplasmatic domain of TM is required for Env incorporation into virions, during the maturation of new viral particles [64, 69, 70].

Virus entry into the target cell

Three different steps can be distinguished in the process that leads to HIV-2 entry into a target cell: binding of SU glycoprotein to the CD4 receptor, binding of SU to a coreceptor and finally the fusion of the viral envelope with host membrane [71].

The first step in viral infection is defined by the attachment of virus to the cell surface. This first interaction is mediated through the binding of the SU to a receptor CD4. After CD4 binding, SU undergoes conformational changes that rearrange its variable domains including the V1, V2, V3 and the constant region C4 and lead to the formation of the bridging sheet [63, 72]. These changes promote the reorientation and the exposure of a binding site in SU towards the target cell.

Although CD4 is the major receptor for HIV-2 as for HIV-1, some HIV-2 isolates can entry into cells independently of CD4 [73, 74]. The envelope SU glycoprotein of these isolates may have a more open structure comparing with HIV-1. It was proposed that the coreceptor binding site of CD4 independent isolates may be already formed and exposed and that this accessible conformation might facilitate the infection of target cells in the absence of CD4 [75, 76] .

Upon binding to CD4, the conformational changes mentioned before result in the interaction of V3 and eventually V1/V2 regions with a coreceptor, usually CCR5 or CXCR4. The exposure of coreceptor binding site in HIV-2 may be faster comparing to HIV-1, resulting in a more rapid fusion rate of the envelope with the membrane of the host cell [77]. It was suggested that differences in the cytoplasmic tail of the gp36/gp41 between both viruses might be the cause for the rapid rate of HIV-2 Env mediated-fusion.

Both CCR5 and CXCR4 are seven-transmembrane G-protein coupled receptors. Each has an extracellular N-terminus, three extracellular loops (ECLs), three intracellular loops and a cytoplasmic C-terminus [78]. The two coreceptor regions that are required for the interaction with the viral SU, the N-terminal region and the second extracellular loop (ECL2), are the same for HIV-2 and HIV-1 infection [78].

The binding of SU to coreceptor triggers conformational changes in the TM glycoprotein that lead to the exposure of the fusion peptide.

The fusion peptide is then inserted into the host membrane, creating a structure denominated six-helix bundle, with HR1 and HR2 packed in antiparallel orientation.

This structure brings the host cell membrane and viral envelope into close proximity and consequently a fusion pore is formed which allows the entry of viral core into the cytoplasm of the host cell [79].

Conformational alterations in gp41/gp36 required for viral entry in the host cell can be inhibited by fusion inhibitors. To date, enfuvirtide (T-20) is the only fusion inhibitor approved in treatment of HIV-1 infected patients. T-20 is based on the HR2 sequence of gp41 of HIV-1_{LAI} isolate and prevents the formation of the six-helix bundle structure by competitive binding to the HR1 region [80, 81]. However, despite its potent activity in HIV-1 isolates it presents a low genetic barrier to resistance and is not active on HIV-2 strains [82-85].
In the last years, second and third generation fusion inhibitors have been produced with increased antiviral activity against HIV-2 isolates, such as P3 and T-1249 [84, 86]. Still, there is an urgent need to produce novel fusion inhibitors with higher potency and stability than T-20, with strong antiviral activity against HIV-2 isolates and with a higher genetic barrier to resistance in order to expand treatment options for HIV-2 infection in the near future.

HIV-2 coreceptor usage and tropism

As for HIV-1, CCR5 and CXCR4 are the major coreceptors *in vivo* for HIV-2 [87, 88]. Variants that use the CCR5 coreceptor are termed R5, those that use CXCR4 are named X4, and those that use both are designated R5X4 (or dual tropic). Furthermore, a population of R5 and X4 variants is designated as dual/mixed (D/M) [89, 90].
Usually, CCR5 usage corresponds to a slow/low (viruses that replicate slowly and poorly), non-syncytium inducing phenotype while CXCR4 usage is associated with a rapid/high, syncytium inducing phenotype [89, 91]. Although HIV infects CD4+ T cells and peripheral blood mononuclear cells (PBMCs), CCR5 tropic strains tend to infect cells of the monocyte/macrophage lineage whereas X4 variants preferentially infect lymphocytic cell lines, according to the expression levels of CCR5 and CXCR4 in these target cells [89, 92].
Of note, *in vitro* some HIV-2 isolates obtained from patients in advanced disease stages have the ability to use a broad range of chemokine receptors: CCR1, CCR2b, CCR3, CCR6, CCR8, GPR15 (BOB), and CXCR6 (BONZO [87, 93-95].
The role of those alternative coreceptors in the pathogenicity of HIV-2 remains to be clarified [87, 88, 90].

R5 HIV-2 strains are common in asymptomatic patients or in acute stage of infection, while X4-tropic HIV-2 isolates have been found only in patients with advanced disease, low levels of CD4+ T cells and higher viral loads [66, 96-100].
In HIV-1 infection, switch from CCR5 to CXCR4 occurs in 50% of the infected individuals and has been associated with accelerated depletion of CD4+ T cell counts and progression to AIDS [91, 101]. However, in contrast to HIV-2, X4 HIV-1 variants are more sensitive to plasma antibody neutralization comparing to R5 strains [102].
The transition from R5 to X4 phenotype in HIV-2 seems to implicate specific alterations in the V3 region and perhaps also in V1/V2 region of SU glycoprotein. Particularly in V3, a global net charge superior to 6 and the presence of mutations in positions 18, 19 and amino acid insertions at position 24 were associated to CXCR4 usage [65, 66, 75, 97, 100, 103-106]. These alterations have an impact on the structural conformation of V3. In fact, it was shown that R5 viruses are characterized by the absence of a secondary structure in the V3 region while transition to X4 tropism is characterized by the acquisition of a secondary structure (β-hairpin structure) in the V3 loop [65, 66, 97]. These alterations on the structure and conformation of the V3 may prevent the efficient binding of neutralizing antibodies (Nabs) to this region thus leading to escape and resistance to Nabs [65, 66, 97].

In HIV-1, V3 glycosylation has also been reported to influence coreceptor usage [107, 108]. Although HIV-2 has been reported to have lower number of glycosylation sites in V3 than HIV-1, the impact of these on coreceptor usage is still unknown [66, 75, 109].

Pathogenesis and immune response in HIV-2 infection: differences for HIV-1

HIV-2 infection is characterized by a longer asymptomatic phase and slower progression towards AIDS, when compared with HIV-1 [110, 111].
Clinically, a significant proportion of HIV-2 infected individuals (~80%) have higher CD4+ T cell counts and lower or undetectable plasma viral loads, in the absence of therapy, than that seen in HIV-1 infection [111-115].
HIV transmission occurs mainly across mucosal tissues. In this context, the dendritic cells (DC) may play a role, capturing the virus and spreading the infection to lymph nodes and secondary lymphoid tissue to present the virus to CD4+ T cells [116-118].

Therefore, the acute phase of HIV-1 infection is associated to a massive depletion of CD4+ T cells, especially in gut associated lymphoid tissue (GALT), as a consequence of several mechanisms, including direct viral infection, apoptosis, activation-induced cell death and host cytotoxic responses [119, 120]. In contrast, HIV-2 infection does not affect gut mucosal integrity and CD4+ T cells number despite local viral replication [121].

The viral set point establishes the beginning of the chronic phase and is associated with the rate of disease progression in untreated patients [122]. Viral set point in HIV-2 is 30 fold lower than in HIV-1 (median, 2500 *versus* 70000 RNA copies/ml, respectively) [123].The chronic phase (clinical latency) is the asymptomatic stage of HIV infection, with a median of duration 8-10 years in HIV-1, and 20 years or more in HIV-2 in the absence of treatment [124]. This stage is characterized by a persistent immune activation, which is manifested by high levels of CD4+ and CD8+ T cell apoptosis, polyclonal B cell activation with hypergammaglobulinemia, increased cell turnover of T cells, monocytes and natural killers (NK) cells [110, 122, 125]. This results in immunological abnormalities including, poor cell renewal, senescence and cellular exhaustion [122]. Several mechanisms have been proposed to be involved in the establishment and maintenance of HIV-associated chronic immune activation [126-128]. The innate and adaptive responses of the immune system against HIV replication and viral antigens may play an important role in this context. Moreover, the effect induced by specific viral proteins, including Tat, Env and Nef and the presence of opportunistic infections that are reactivated more frequently in these individuals (e.g. cytomegalovirus and Epstein-Barr virus) have also been suggested to be involved in HIV induced immune activation [126, 128].

Additionally, HIV response to the increased levels of type I interferons and pro-inflammatory mediators, the loss of the integrity of the gastrointestinal tract, with the consequent microbial products translocation from the GALT and the massive depletion of memory CD4+T cells are other potential players associated to chronic immune activation in HIV infection [126-128].

Immune activation is strongly linked to disease progression in HIV infection. The levels of immune activation are similar between HIV-1 and HIV-2 infected patients, when matched for the same degree of CD4+ T cell depletion [129, 130].

As mentioned before, in HIV-2, progression to AIDS is less frequent than in HIV-1, however, the clinical manifestations are very similar [131, 132]. One prospective study demonstrated that the probability of AIDS-free survival at five years after seroconversion, was near 100% in HIV-2 *versus* 67% in HIV-1 infected patients [133]. In the same way, mortality rates in HIV-2 infected individuals are lower compared with HIV-1, being only about two thirds of that for HIV-1 infected patients [134, 135].
A detectable viral load at baseline significantly predicts the rates of disease progression as determined by a decline in CD4+ T cell count or death [113, 136]. However, a substantial proportion of untreated HIV-2 infected patients (13%-46.5%) displays undetectable viral loads [137-141]. Therefore, as with HIV-1 infected patients, HIV-2 individuals with high viral loads undergo rapid CD4+ T cell count declines and death, while those who present low or undetectable HIV-2 RNA viral loads have decreased or no disease progression [136].
In HIV-2 infected patients, CD4+ T cell count rises as a response to an effective antiretroviral therapy [142-144]; however this response appears to be lower than in HIV-1 infected patients [114, 145, 146].

Innate and intrinsic immune responses against HIV-2

The innate immune response comprises several cellular and humoral components, such as cytokines, complement proteins, DCs, macrophages and NK that interact and cooperate to induce a robust immune response against pathogens until the adaptive response is established [147, 148].
HIV infection triggers innate immune receptors, including toll-like receptors (TLR), like TLR-7, TLR-8, and TLR-9, inducing the activation of DCs and the secretion of type 1 interferons (IFN) and tumor necrosis factor α (TNF- α) [147-149]. IFN and TNF-α play a role in the inhibition of viral replication, enhancing antiviral activity of immune cells (NK, T and B cells and macrophages) and recruiting other cells of the immune system to the sites of infection [147, 150].
Plasmacytoid DC (pDC) are one subset population of human DCs that specializes in the production of IFN- α upon TLR9 stimulation [151, 152]. The responsiveness of HIV-1 and HIV-2 infections to TLR-9 is defective in the advanced disease stage, along with CD4+ T cell loss [153].

On the other hand, the levels of circulating pDCs are diminished in HIV-2 infection, despite the absence of viremia, and are associated with CD4+ T cell depletion and immune activation [154].

Consequently, the production of IFN-α is decreased in HIV-2 infection [154]. The depletion of pDC in HIV-2 infected individuals with undetectable viremia might result from other mechanisms besides direct viral infection [154, 155]. Moreover, monocyte-derived dendritic cells and myeloid DCs were also shown to be less sensitive to HIV-2 infection, suggesting a preservation of these cells' function through infection [155-157]. However, despite this less sensitivity, a progressive loss in circulating levels of myeloid DCs was observed in advanced disease stages in association with increase in viral load, CD4+ T depletion and immune activation [125].

NK cells are another important component of innate response. These cells secrete inflammatory chemokines such as IFN-γ and TNF-α and can recognize HIV infected targets leading to cytolysis of the infected cells [158]. Unlike HIV-1, HIV-2 asymptomatic patients show higher levels of NK cytotoxicity, similar to that found in uninfected controls [159]. However, the cytolytic and chemokine response of NK cells deteriorates with CD4+ counts decline, reaching similar levels to those seen in HIV-1 infection [159].

Important differences exist between the responses elicited by HIV-1 and HIV-2 against the four major host restriction factors, TRIM5-α, APOBEC3G, SAMHD1 and tetherin [160]. These proteins integrate the so called intrinsic branch of immunity that constitute the first line response to HIV infection and other viruses and are often blocked by specific viral proteins, such as Gag, Vif, Env and Vpx [160].

TRIM5-α is a member of the tripartite motif family of proteins that destabilizes the viral capsid core leading to premature uncoating, perturbing subsequently reverse transcription [161]. HIV-2 is more susceptible to restriction by TRIM5-α than is HIV-1, although there are strain specific variations, depending on motifs in the viral capsid [162, 163].

APOBEC3G belongs to the cytidine deaminases family [164]. In the absence of Vif, this protein, is packaged into virions and induces G to A hypermutation in the viral DNA, leading to degradation of the nascent proviral DNA [165, 166]. Compared with HIV-1, HIV-2 seems to be more resistant to APOBEC3G [167].

SAMHD1 contains nuclease and deoxyribonucleoside triphosphate phosphohydrolase (dNTPase) activity [168].

This enzyme lowers the concentration of deoxyribonucleotide triphosphate (dNTPs) in nondividing cells such as DCs, monocytes, macrophages and resting CD4+ lymphocytes thereby blocking HIV reverse transcription [169].
This effect could be more pronounced on HIV-2 replication in these cells because the RT of HIV-2 is less processive than HIV-1 and requires higher concentration of dNTPs to work properly [170-173].
However, the accessory protein Vpx, which is present in HIV-2 and related SIV lineages but not in HIV-1, degrades SAMHD1 [174]. It was suggested that HIV-2 would trigger a more efficient immune response relative to HIV-1 by productively infecting DC [110, 175]. However, a recent study showed that, HIV-2, like HIV-1, does not efficiently infect monocytes derived dendritic cells *in vitro*, suggesting that other factors not linked to SAMHD1 blockade may disturb HIV-2 infection in this cell population [156].
Tetherin, also known as bone marrow stromal antigen 2 (BST-2) or CD317 is a type II transmembrane protein that prevents virus release by inserting its N-terminal transmembrane domain in the plasma membrane and its GPI-linked C terminus in the virus envelope lipid bilayer. The tethered virus is then endocytosed [160]. HIV-1 and HIV-2 counteract tetherin by two very different mechanisms. In HIV-2, the envelope gp36 glycoprotein blocks its activity by interacting with the tetherin cytoplasmatic ectodomain, whereas in HIV-1, the anti-tetherin activity is mediated by the protein Vpu. Sequestration of tetherin by HIV-1 Vpu and HIV-2 Env in the endoplasmic reticulum prevents its transit to the plasma membrane. Tetherin proteasomal degradation is then induced by its interaction with the SCF-β-TRCP complex [176-178].

Adaptive immunity

Cellular immune response

HIV infects several types of immune cells, such as CD4+ T lymphocytes, DCs, monocytes/macrophages [122, 147]. CD4+ and CD8+ T cells are the most important players in HIV infection, with the former being responsible for the activation of diverse innate and adaptive immune cells and the latter mediating cell killing and secreting antiviral factors in order to control viral infection [179].

CD8+ T cells (cytotoxic T lymphocytes-CTL) play a critical role in the cellular immune response to HIV infection, since the initial decline in viral load, in acute infection, is assumed to be attributable to the activity of these cells [180, 181].

CTL are able to recognise viral determinants, at the surface of the infected cells, in the context of antigen presentation by human leukocyte antigen (HLA) class I and, subsequently lyse these cells [179, 182]. Additionally, CD8+ T cells secrete chemokines to control infection, particularly MIP-1a (CCL3), MIP-1b (CCL4) and RANTES (CCL5), that bind to HIV coreceptors on the surface of CD4+ T cells, inhibiting viral entry [179, 182, 183].

The degree of polyfunctionality of CD8+ T cells seems to be higher in HIV-2 infected patients, with production of higher levels of IFN-γ, TNF-α and other cytokines relative to HIV-1 infected patients [184, 185]. The heterogeneity and promiscuity of TCR usage is higher in HIV-2 infected patients, leading to a more efficient response in these patients comparing with HIV-1 infected individuals [186].

CD4+ T cells are preferentially infected by HIV, and depleted from the host as disease progresses [120, 187, 188]. Naïve CD4+T cells are activated after recognition of viral determinants through HLA class II, differentiating into specific subtypes and releasing cytokines (like IL-2). These cells have multiple functions, playing an important role in induction and maintenance of CTL and macrophages and in maturation of B cells [122, 188, 189].

In the majority of HIV-2 infected patients, qualitative and quantitative features of CD4+ T cells function seem to be preserved [190]. In HIV-2 infection, these cells appear to be more polyfunctional, with improved proliferative capacity, secreting a wider range of cytokines (namely IL-2 and IFN-γ), compared with HIV-1 [110, 184, 190, 191].

Antibody responses to HIV-2 infection

The humoral immune response is mediated by antibodies, which belongs to a family of globular proteins named immunoglobulins (Ig) that are secreted by B cells [192, 193]. Antibodies consist of two heavy and two lights chains linked by disulphide bonds and noncovalent interactions. Each chain is composed by two variable and two constant regions at the amino- and carboxyl-terminal end, respectively. The antigen-binding sites are located in the variable regions of both light and heavy chains [192, 194].

The activation of B cells into antibody producing cells is antigen-dependent. The mature naïve B cell contains IgM and IgD on the surface. Once antigen activates the B cell, upon interaction with a T cell, the B cell proliferates and differentiates into an antibody-secreting effector cell, switching the isotype (class) of immunoglobulin that is produced [194, 195].

Alterations in human B-cell populations have been reported in HIV-1 infection, particularly, hyperactivation of B cells, hypergammaglobulinaemia, loss of resting memory B cells and increased levels of: - polyclonal B-cell activation, - differentiation of B cells to plasmablasts, - cell turnover and expression of activation markers [196-198].

In HIV-2, B cell activation and hypergammaglobulinemia are also present and associated with decrease in CD4+ T cell count [199, 200]. Moreover, Tendeiro *et al*. reported a depletion in memory B cells levels (unswitched and switched) in association with CD4+ T cell decrease, despite the absence of detectable viremia [201]. Similar to HIV-1, these alterations in B cells were not recovered by antiretroviral therapy (ART) [201].

After HIV infection, B cell responses are initially detected as anti-gp41 IgM antibodies, followed by IgG and IgA antibodies [202, 203]. The antibodies against gp41 form immune complexes with the virions and generally have no detectable effect on viremia [202, 203]. Autologous Nabs targeting mainly the variable loops of gp120 appear weeks later after infection and are strain-specific Nabs [203, 204]. The appearance of these Nabs is associated with the emergence of mutations in the Env region that lead to viral escape from neutralization [205, 206].

During the course of the infection, viruses continuously mutate and escape from neutralizing antibody leading to a limited effect of Nabs on the control of virus replication and disease course.

It is estimated that only about 20% of HIV-1 infected individuals generate antibodies in two to four years after infection, capable of neutralizing a wide range of strains from different subtypes (named broadly neutralizing antibodies or bNAbs) [207-209].

Unlike HIV-1, most HIV-2 infected individuals produce potent and broad neutralizing antibodies [66, 97, 109, 210-212]. The V3 region has been reported as a potent neutralizing domain in several studies; however, other epitopes were also recognized in V1, V2, V4, C5 regions in gp125 and the C-terminal region of gp36 ectodomain, although with weakly neutralizing activity [66, 97, 109, 212-215].

The association between susceptibility to antibody neutralization and HIV-2 coreceptor usage was first reported by Marcelino and colleagues in vaccinated mice [65].

They demonstrated that C2V3C3 region of gp125 was a potent broad neutralizing domain and that X4 tropic viruses, which showed specific alterations in the V3 loop, were more resistant to neutralization, than R5 tropic variants [65].
Subsequently, the authors confirmed the association between antibody neutralization and coreceptor usage in HIV-2 infected adults [97]. They found that X4-tropic viruses isolated from adult patients with late stage infection were resistant to antibody neutralization and had major changes in V3 loop sequence and conformation relative to R5 isolates isolated from asymptomatic infection [65, 97]. Similar findings were reported by Rocha and colleagues [66], who characterized for the first time the evolution of the Nab response in two HIV-2 infected children from acute to late stage infection. This study demonstrated that Nabs can be elicited very early after HIV-2 infection and that escape from antibody neutralization was associated with R5-to-X4 tropism switch, increased diversity in V1 and V3 regions and specific changes in V3 conformation [66]. Overall, these findings revealed that acquisition of CXCR4 tropism in HIV-2 primary isolates was associated with escape from neutralizing antibody response, involving significant changes in V3 loop charge, size and conformation that might inhibit the proper binding of the neutralizing antibodies that target this region [65, 66, 97].
Currently there is no information on the targets and dynamics of the neutralizing antibody response during the acute phase of infection in adult HIV-2 infected patients.

Antiretroviral therapy and drug resistance in HIV-2 infection

Highly active antiretroviral therapy (HAART), also designated as cART (combination antiretroviral therapy), which involves the combination of antiretroviral drugs from different drug classes, has dramatically improved the outcome of treatment of HIV infection [216]. HAART regimens generally consist of two nucleos(t)ide analogues and a third agent which may be an integrase, protease or a non-nucleoside reverse-transcriptase inhibitor, depending on the type of virus (HIV-1 or HIV-2), clinical status of the patient or the available resources. Entry inhibitors (fusion inhibitors or CCR5 antagonists) are in general reserved for salvage therapy (i.e. treatment of patients with resistance to the three most commonly used and widely available classes of antiretroviral drugs) [216, 217].

Currently there are 24 antiretroviral drugs that have been approved by Food and Drug Administration (FDA) for use in the treatment of HIV-1 infection (Table 1) [217]. So far, no drug has been specifically designed against HIV-2.

As mentioned above, these drugs can be divided into several groups according to the steps in the viral replication cycle which they aim to interfere: nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), nonnucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), integrase inhibitors (INI) and entry inhibitors (EI) (CCR5 antagonist and fusion inhibitor) [217].

Principles of antiretroviral therapy

The primary goals of ART are to reduce HIV-associated morbidity, prolong the duration and quality of survival, restore and preserve immunologic function and prevent HIV transmission [218-221]. These goals can be achieved by a maximal and durable suppression of viral replication (e.g. <50 RNA copies/mL, depending on the assay used), in order to maximize immunological recovery and to prevent the emergence of drug resistant variants. Despite the high success in suppressing viral replication to almost undetectable levels, ART does not cure HIV infection because it does not eradicate the virus from the cellular reservoirs such as resting memory $CD4^+$ T cells and anatomic reservoirs like central nervous system [222, 223].

When to start ART

Currently, several international guidelines (e.g. EACS, DHHS,) recommend ART in all HIV-1 infected individuals, regardless of CD4+ T cell count [224, 225].

This recommendation is based on data from two randomized trials, START and TEMPRANO, that evaluated the optimal time to initiate antiretroviral therapy [227, 228]. These studies demonstrated a 50% reduction in morbidity and mortality in HIV-1 infected individuals who started ART with a CD4+ T cell count superior to 500 cells/mm^3 *versus* HIV-1 infected individuals who ART initiation was delayed [227, 228].

However, this principle is not applied in HIV-2 setting, due to lack of scientific evidence [229]. In addition, many HIV-2 untreated patients who are eligible for ART based on CD4+ T cell counts have low or undetectable viral loads [112, 230]. Therefore, the available data suggest that the initiation of antiretroviral therapy should be based on CD4 cell count together with the clinical status of the patient. Clinical guidelines recommend starting HIV-2 therapy when one or more of the following situations are present: - symptomatic patients (presence of specific opportunist infections or neoplasic diseases - AIDS-defining conditions- according to Centers for Disease Control and Prevention-CDC list); - CD4 cell count below 500 cells/mm^3; - CD4 cell decrease equal to or higher than 30 cells/year; - repeatedly detectable viral load [226, 229, 231, 232].

Table 1. Approved antiretroviral drugs in use for the treatment of HIV-1 infection.

Drug (acronym)	Approval Date
Nucleos(t)ide reverse transcriptase inhibitors (NRTIs)	
Zidovudine (AZT)	1987
Didanosine (ddI)	1991
Stavudine, d4T	1994
Lamivudine (3TC)	1995
Abacavir (ABC)	1998
Tenofovir (TDF)	2001
Emtricitabine (FTC)	2003
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	
Nevirapine (NVP)	1996
Efavirenz (EFV)	1998
Etravirine (ETV)	2008
Rilpivirine (RPV)	2011
Protease inhibitors (PIs)	
Saquinavir (SQV)	1995
Ritonavir (RTV)	1996
Indinavir (IDV)	1996
Nelfinavir (NFV)	1997
Lopinavir (LPV)	2000
Atazanavir (ATV)	2003
Fosamprenavir (FPV)	2003
Tipranavir (TPV)	2005
Darunavir (DRV)	2006
Entry inhibitors (EIs) - Fusion inhibitors	
Enfuvirtide (T-20)	2003
Entry inhibitors (EIs) - CCR5 Co-receptor antagonist	
Maraviroc (MVC)	2007
Integrase inhibitors (INIs)	
Raltegravir (RAL)	2007
Dolutegravir (DTG)	2013
Elvitegravir (EVG)	2012

Note: All PIs except NFV are always boosted with RTV which is not used per se.

What to start

To date no randomized trials addressing the question of the choice of initial or second-line therapy for HIV-2 infection have been made; thus, the optimal treatment scheme has not been defined. Therefore, decisions of what constitutes the best choices of therapy in HIV-2 infection rely in general on both *in vitro* as well as *in vivo* data from small cohort studies and case series [142, 146, 233-238].

In addition, as mentioned above antiretroviral drugs have been developed for HIV-1 and may not provide optimal suppression of HIV-2 replication. One thing is clear: HIV-2 is intrinsically resistant to NNRTIs [239, 240] and to T-20 [83, 241] and these should not be used to treat HIV-2 infected patients.

In contrast, HIV-2 is sensitive to the currently available NRTIs, although with a lower barrier to resistance when compared to HIV-1 [172, 242, 243].

Given the high toxicity and the faster emergence of resistance mutations, didanosine (ddI) and stavudine (d4T) should be avoided [229, 231].

Regarding PI drug class, darunavir (DRV), lopinavir (LPV) and saquinavir (SQV) are the most active inhibitors of HIV-2 [225, 244, 245], whereas others PIs (fosamprenavir-FPV, atazanavir-ATV, tipranavir-TPV, and indinavir-IDV) should be avoided because of their lack of activity and/or high failure rates [229, 245, 246].

All three INIs, raltegravir (RAL), elvitegravir (EVG) (boosted with cobicistat) and dolutegravir (DTG), have potent activity against HIV-2 and are therefore recommended in these patients [225, 232, 247-250].

Finally, MVC appears to be active against most R5 HIV-2 isolates [83, 251, 252]; however, there is little clinical experience with this CCR5 antagonist in HIV-2 infected patients and until recently no approved assay to determine HIV-2 co-receptor tropism existed [253-255].

Current clinical guidelines recommend the use of formulations with two NRTIs, as the preferred backbone, associated with an INI or a ritonavir-boosted PI/r in HIV-2 first- and second-line therapy [225, 229, 232, 256, 257]. Therefore, preferred regimens for initial treatment include combinations of tenofovir (TDF) with emtricitabine (FTC), zidovudine (AZT) with lamivudine (3TC) or abacavir (ABC) with 3TC associated with one PI (LPV/r, DRV/r) or one INI (RAL, DTG or EVG/cobicitast) [229, 232, 233, 257] (Table 2).

Limited options are available for second-line treatment in HIV-2, such as TDF with FTC (Truvada) in combination with an INI or a boosted PI.

In this context, when first line treatment with LPV fails with the emergence of V47A mutation, SQV is suggested due to the hypersusceptibility conferred by this mutation [229, 232]. As few treatment options exist for HIV-2, a genotypic resistance test should be performed to optimize the choice of second line antiretroviral drugs. However, drug resistance assays for HIV-2 are not commercially available and knowledge on drug resistance mutations in HIV-2 is still very limited because it is based on data from a small number of clinical and *in vitro* studies [232].

Although few data is available about MVC efficacy in clinical practice it should be considered as part of a third-line regimen for treatment-experienced patients infected with R5 viruses [232].

Treatment of individuals co-infected with HIV-1 and HIV-2 should be carried out using an active regimen against both viruses to ensure that the drugs used can effectively treat both viruses. One possible initial regimen for co-infected patients is LPV/r, SQV or DRV plus two NRTIs. The British HIV association guidelines recommend a baseline genotypic resistance test for HIV-1 and HIV-2, if possible, before treatment initiation in order to ensure that an active regimen is chosen against both viruses [232].

Table 2. Possible regimens for treatment of HIV-2 infection.

A*	B*
PI/r	**N(t)RTI**
LPV DRV SQV	TDF/FTC or ABC/3TC
INI	**N(t)RTI**
RAL DTG	TDF/FTC or ABC/3TC
EVG/c	TDF/FTC

(From Direcção Geral da Saúde. Recomendações Portuguesas para o tratamento da infeção por VIH-1 e VIH-2. Programa Nacional para a Infeção VIH/SIDA. 2016 [229]).

*The antiretroviral regimen will result from the association of an ARV from column A with a coformulation from column B.Note: EVG/c - elvitegravir/cobicistat.

Monitoring of treatment response

Response to treatment in HIV-2 infected patients has to be assessed using the same markers for HIV-1 infection, particularly RNA levels, CD4+ T lymphocyte cell count and clinical stage [232]. However, monitoring therapy in HIV-2 infection is more challenging than for HIV-1 because until recently viral load and ART resistance assays for HIV-2 were not commercially available and the standard methods and interpretation protocols that are used to monitor ART and resistance for HIV-1-infected patients may not apply for HIV-2-infected patients [138, 258].

In addition, as previously mentioned, HIV-2 infection is usually characterized by higher CD4 cell counts and low or undetectable plasma viral loads than that seen in HIV-1 infection. Therefore, viral load monitoring per se may not be as useful in HIV-2 clinical setting as it is in HIV-1 to assess treatment response. Therapeutic failure in HIV-2 treated patients occurs when viral load is detectable in two consecutive occasions or, in case of undetectable viral load, when there is a significant decrease in the number of CD4+ T lymphocytes [229, 231].

HIV-2 drug resistance

Combination antiretroviral therapy has proven to be effective in controlling the progression of disease and prolonging survival of HIV infected individuals, however these benefits can be compromised by the emergence of drug resistance leading to treatment failure. Resistance is a consequence of the interaction of viral, pharmacologic and host factors, such as drug potency, host genetics and patient adherence to treatment [259, 260].

HIV resistance to antiretroviral therapy can be divided into two categories, namely primary or transmitted drug resistance and secondary or acquired drug resistance. Primary resistance reflects acquisition of a drug-resistant strain of HIV by a newly infected person, while secondary resistance develops by residual viral replication during HIV treatment [261].

Drug resistance mutations can be characterized as either primary or major mutations, that usually decrease the susceptibility of the virus to an ARV, or accessory/minor or secondary mutations that further decrease susceptibility to an ARV in the presence of a major mutation or act as compensatory mutations that restore the viral replicative capacity [262]. Frequently a mutation that confers resistance to one drug can confer resistance to other drug(s) from the same class which is termed as cross-resistance. By contrast, some mutations that confer resistance to one ARV may increase viral susceptibility to another agent, referred to as hypersusceptibility [263, 264].

For some compounds, multiple mutations are required to decrease susceptibility (high genetic barrier), while others require only a few (or sometimes just one) mutations (low genetic barrier).

In case of treatment interruption, viruses with resistance mutations and lower replicative capacity are replaced by fitter wild type viruses [265-267]. In the absence of drug pressure, the resistant and less fit strains become present as minority variants within the virus population of the patient and are undetectable with the current genotypic assays [268]. These minority mutants are often archived in reservoirs and can re-emerge upon drug pressure leading to treatment failure [265, 269].

As mentioned before, HIV-2 is naturally resistant to NNRTIs and enfuvirtide. Natural resistance to NNRTIs is associated to the presence of natural polymorphisms Y181I and Y188L in RT, which confer resistance to this drug class in HIV-1 [270, 271]. On the other hand, differences in HR1/HR2 regions in gp41/gp36 between HIV-1 and HIV-2 are responsible for the lack of antiviral activity of enfuvirtide on HIV-2 [85].

NRTI resistance in HIV-2 comprise different mutational pathways than those seen in HIV-1. HIV-2 preferentially uses the exclusion pathway for acquiring resistance to NRTIs while the acquisition of thymidine analogue resistance mutations (TAMs), which is the principal route to NRTIs resistance in HIV-1, is rarely observed in HIV-2 [272-274]. This difference between both viruses is due to a lower ability of HIV-2 RT to excise zidovudine monophosphate than HIV-1 RT [172, 275]. Moreover, HIV-2 has a lower genetic barrier to this drug class compared with HIV-1. In HIV-2, Q151M mutation confers resistance to all NRTIs, with exception of TDF, and K65R and M184I/V confer class-wide resistance [274, 276, 277].

Regarding PIs, as previously mentioned, HIV-2 shows a decreased susceptibility to this drug class, when compared with HIV-1, retaining full susceptibility only to SQV, LPV and DRV [243, 244, 278]. HIV-2 displays several natural polymorphisms that may confer resistance to various PIs [279-281].
Indeed, recently it was demonstrated that four amino acid residues located in the PR binding pocket (positions 32, 47, 76 and 82) are the primary determinants of HIV-2 intrinsic resistance to PIs [282]. Furthermore, the genetic barrier for PI resistance in HIV-2 seems to be lower than in HIV-1. For example, the acquisition of V47A substitution that confers resistance to LPV requires only one nucleotide change in HIV-2 whereas two nucleotide substitutions are required in HIV-1 [278, 283, 284].

In the following sections the mutations conferring HIV-2 resistance to PIs and Entry inhibitors will be reviewed. No further mention will be made to mutations causing resistance to NRTIs or integrase inhibitors as these were not specifically addressed in the remaining chapters of this thesis.

Mutation profiles conferring resistance to protease inhibitors

Protease inhibitors mimic the structure of the viral substrates of PR competing with them for the binding to the enzyme´s active site. PIs inhibit the catalytic activity of the PR therefore preventing the maturation of virions [280, 285].
Protease has two identical monomers with 99 amino acids each and contains three functionally important domains: active site (which includes the conserved motif Asp-Thr-Gly), substrate binding domain and flap region (Figure 3). Each monomer comprises an aspartic acid residue, Asp25, in the substrate binding domain, which are important for the proteolytic cleavage reaction [286-288].

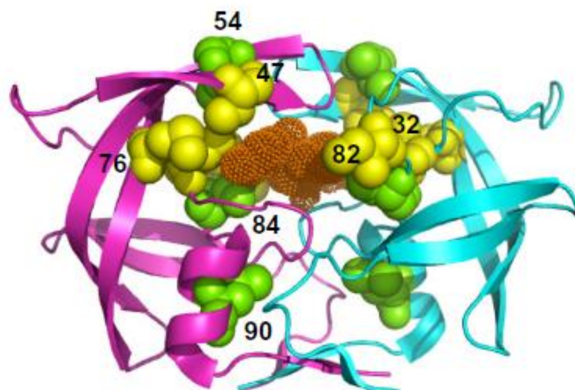

Figure 3. Ribbon representation of the secondary structure of HIV-2 PR complexed with darunavir. Two subunits (in blue and magenta) and darunavir (in brown) are shown. Yellow spheres represent residues at positions 32, 47, 76 and 82 that are located in the binding site of the enzyme and are different in HIV-1 and HIV-2. Ile^{54}, Ile^{84} and Leu^{90} (green spheres) when substituted by Met^{54}, Val^{84} and Met^{90} confer resistance to multiple PIs. (From Menendez-Arias, L. and M. Alvarez. Antiretroviral therapy and drug resistance in human immunodeficiency virus type 2 infection. Antiviral Res. 2014; 102:70-86 [243]).

HIV-1 and HIV-2 PRs share about 50% of amino acid sequence identity and both enzymes appear to be similar in structural terms with the regions essential to the enzyme function being conserved between the two viruses [279, 286, 289, 290]. However, HIV-2 PR displays several natural polymorphisms that may have impact in the mutational patterns of resistance to the PIs (Figure 4). In addition, some of these polymorphisms correspond to major (e.g. V32I/L, M46I/V, I47V, V82I) and minor (e.g. L10V, G73A, M36I) PI resistance mutations in HIV-1. The presence of these natural polymorphisms in HIV-2 can reduce the time to the emergence of resistance to certain PIs [273, 279, 281, 291].

As previously mentioned, it was recently demonstrated by site-directed mutagenesis that amino acid residues at positions 32, 47, 76 and 82 in the ligand binding pocket of PR are the primary cause of HIV-2 resistance to PIs [282]. In HIV-2, the combination of single amino acid substitutions, corresponding to the wild type amino acids in HIV-1, particularly I32V, V47I, M76L and I82V, conferred a PIs susceptibility pattern similar to that observed for HIV-1. The presence of a substitution at any one of these positions in HIV-1 confers multi PI resistance [292].

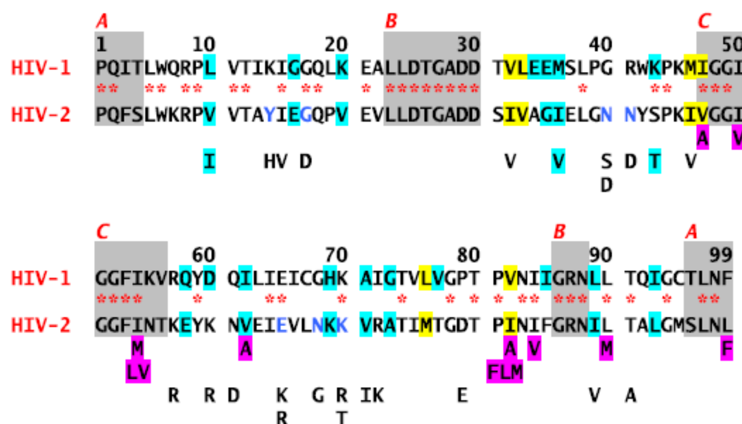

Figure 4. Amino acid sequence comparison of HIV-1 and HIV-2 proteases of reference strains HIV-1_{HXB2} and HIV-2_{ROD}. Identical residues are marked with red asterisks. Gray boxes, A, B and C, indicate conserved structurally regions, particularly, the boundaries of the dimerization domain, the active-site/carboxy-terminal triad, and the flap region of protease, respectively. Major and minor PR resistance mutations are highlighted in yellow and blue, respectively. Blue letters represent highly polymorphic positions in HIV-2 subtype A. Other residues that occur frequently are shown below. Amino acids highlighted in magenta were selected under treatment with PIs. (From Menendez-Arias, L. and M. Alvarez. Antiretroviral therapy and drug resistance in human immunodeficiency virus type 2 infection. Antiviral Res. 2014; 102:70-86 [243]).

Although nine PIs have been approved to treat HIV-1 infection (Table 1) only three (SQV, LPV and DRV) are recommended in treatment of HIV-2 infection as they are the only PIs fully active against this virus [232, 244, 246, 281, 293]. HIV-2 shows varying degrees of resistance to APV, FPV ATV, NFV and IDV [241, 244, 281, 294, 295]. For instance, HIV-2_{ROD} and HIV-2 clinical isolates show high level resistance to APV (17 to 30-fold change) and moderate to low level resistance to ATV, NFV and IDV (3 to 8-fold change) when compared to HIV-1_{BRU} or HIV-1 clinical isolates [244, 281].

Contradictory results about TPV efficacy have been reported, with one study demonstrating natural resistance [244] and another reporting full efficacy [246].

The information regarding mutational pathways in HIV-2 resistance to PIs is still limited and sometimes leads to contradictory results. Data has been collected from clinical studies reporting the emergence of mutations in HIV-2 patients under PIs therapy and also from phenotypic studies using HIV-2 mutants in PR and from the selection of mutations under PI pressure *in vitro*.

In HIV-1, the development of PI resistance is believed to be a stepwise process whereas in HIV-2, in general, it seems that the development of resistance mutations is easier and faster than in HIV-1. Thus, HIV-2 shows a lower genetic barrier to this class of inhibitor when compared to HIV-1 [293].

Saquinavir resistance

As with HIV-1, saquinavir´s signature mutation in HIV-2 appears to be L90M [296, 297]. This mutation alone confers low-level resistance to SQV (~3.5-fold increase in IC_{50}) [278, 298] whereas when combined with other mutations (e.g. I54M mutation alone or I54M plus I84V) can confer moderate level of resistance to this inhibitor (10-fold increase in IC_{50}) when compared to HIV-2_{ROD} reference strain [278].

L90M has been reported *in vivo* from patients receiving SQV but also other PIs such as IDV, RTV, ATV, NFV, usually in association with other mutations as described above or with I82F and I54L [143, 272, 289, 290, 299-301]. In addition, the HIV-1 major mutation G48V was observed in one HIV-2 patient receiving SQV without low-dose RTV [302]. In contrast, V47A, I82F and the combinations of I54M/I82F and G17N/V47A have been associated to hypersusceptibility to SQV [245, 278, 284].

Lopinavir resistance

The most well documented HIV-2 resistance pathway to LPV involves the V47A mutation. Several studies have reported the selection of V47A in patients under LPV based regimens [43, 245, 273, 278, 283, 300].

Phenotypic studies showed that this mutation confers moderate to high level resistance to LPV (between 6.3 to 41-fold increase in IC_{50}) relative to HIV-2_{ROD} or clinical isolates [245, 278, 284]. In HIV-1, I47A mutation also leads to high-level resistance to LPV [303, 304].

Other single mutations such as I50V and I54M and multiple combinations of mutations containing I54M have also been associated to moderate to high-level resistance to this inhibitor [278, 281, 284].

Conflicting data exists regarding I82F mutation that has been show to confer high level resistance to LPV (36.3-fold increase) compared to HIV-2 clinical isolates in one study [281] while another study showed that this mutation did not confer any resistance to this inhibitor [278].

<u>*Darunavir resistance*</u>

DRV was the latest PI to be approved and data about resistance to this drug is still scarce. The I54M and I50V mutations seem to be related to DRV resistance in HIV-2 [297]. In HIV-1, both mutations are also involved in resistance to this inhibitor [305]. I54M alone or in combination with I84V or L90M confer moderate level HIV-2 resistance to this PI [278] while the presence of the triple mutant I54M/I84V/L90M can lead to high level resistance to DRV (31-fold-increased IC_{50} compared to HIV-2_{ROD}) [278].The single mutations I54M and I50V or the combination of both have been observed in patients with virologic failure under DRV treatment [301]. In contrast, hypersusceptibility to DRV is associated with the presence of I82F mutation [278]. This mechanism of hypersusceptibility is probably due to an increased efficiency of inhibitor binding to PR and diminished binding of its natural substrates.

Activity of entry inhibitors on HIV-2

<u>*Fusion inhibitors*</u>

Enfuvirtide (T-20) is a polypeptide of 36 amino acids whose sequence corresponds to that of the HR-2 region of the TM glycoprotein of HIV-1. Binding of T-20 to HR-1 region prevents the association of HR-1 and HR-2, thereby inhibiting fusion of viral envelope and cellular membrane [306].This inhibitor exhibits reduced efficacy against HIV-2 primary isolates, showing up to 100-fold increase in IC_{50} values *in vitro* comparing to HIV-1 primary isolates [83]. Therefore, the use of T-20 in HIV-2 infection is not recommended [232].

T-1249 is a second generation peptide of 39 amino acids based on gp41/gp36 sequences derived from HIV-2, HIV-1 and SIV strains [86]. Although it exhibits potent antiviral activity against HIV-1 (IC_{50} range:0.08-10.3 nM) and HIV-2 isolates (IC_{50}: 0.9-21.9 nM), including enfuvirtide-resistant strains [83, 86], the clinical development of T-1249 was discontinued due to formulation difficulties [307].

P3 is a new HR-2 based peptide with improved anti-HIV-2 activity [84]. This peptide has 34 residues and is based on HR-2 ancestral sequences of HIV-2 and SIVmac.

P3 is a strong inhibitor of HIV-1 (IC_{50}:0.3-62.2 nM), including enfuvirtide-resistant strains (IC_{50}: 0.15-11.8 nM), and also demonstrates improved antiviral activity against HIV-2 isolates (IC_{50}:13.3-369.4 nM) [84, 85]. So far it has not been possible to obtain HIV-2 isolates resistant to P3 suggesting a high barrier to resistance to this peptide.

CCR5 antagonists

Maraviroc is a small molecule allosteric inhibitor that binds to the coreceptor CCR5, altering the conformational state of this receptor and thus inhibiting the binding of the SU envelope glycoprotein to CCR5 [308]. MVC is the first and the only licensed CCR5 antagonist that is approved for clinical use in HIV patients infected only with CCR5-tropic viruses [309]. Therefore, coreceptor usage of the infecting viruses needs to be determined before starting therapy with MVC (see Coreceptor tropism testing) [310].

MVC inhibits the replication of HIV-2 clinical isolates *in vitro* with a similar IC50 relative to HIV-1 (IC_{50} values between 0.175 to 2.1 nM) [83, 251, 252]. However, 90% inhibition of HIV-2 isolates requires much higher concentration of MVC relative to HIV-1 (42.7 nM vs 9.7 nM) [83]. Consequently, higher doses of MVC may be necessary for full clinical efficacy in HIV-2 infected patients.

MVC has been successfully used in the treatment of HIV-1 infection [311]. So far, only three clinical studies have reported the use of MVC in salvage therapy in HIV-2 heavily treated patients with limited therapeutic options [253-255]. It was demonstrated that the three regimens, MVC and RAL or MVC and Foscarnet or MVC, TDF and DRV/r allowed CD4+ T cells count to increase and viral load to decrease to undetectable levels. However, these clinical cases do not provide valuable information about the absolute efficacy of MVC as salvage therapy in HIV-2 infected patients since this inhibitor was used in combination with other antiretroviral drugs and a tropism test was only performed in one of the three studies before MVC administration [254].

Therefore, additional data on the clinical effectiveness of MVC in HIV-2 infected patients is needed. One important factor preventing the use of MVC in HIV-2 infected patients was the absence, until recently, of a genotypic assay to predict co-receptor use.

Failure of MVC-based therapeutic regimens can occur by two routes. The first pathway is through the emergence of X4-tropic strains that were present at very low levels prior to initiation of CCR5 antagonist therapy and that were not detected by tropism tests at baseline [312]. These X4 variants are unresponsive to MVC-based regimens.

The second pathway involves adaptive alterations in Env glycoprotein enabling recognition and continued use of the drug-bound conformation of CCR5 [313, 314]. These alterations were only reported for HIV-1 and mainly include substitutions that occur in the V3 loop of the SU glycoprotein (e.g. A316T and I323V) [315, 316], although, in some cases, mutations in the fusion peptide of TM glycoprotein (e.g. G516V, M518V and F519) were also described to be involved in this mechanism [317, 318].

However no signature mutational pathway has been identified so far that would enable to predict resistance to CCR5 antagonists.
In addition, HIV-1 and HIV-2 Envs with V3 deletions were reported to be resistant to CCR5 antagonists [319, 320], and this resistance apparently resulted from the interaction of other Env domains (e.g. bridging sheet) with the amino terminus of CCR5 without the presence of V3 region to mediate viral entry [319].

Transmitted drug resistance in HIV-2

Transmitted drug resistance (TDR) is an important public health issue as it may limit future treatment options, decrease efficacy of subsequent regimens and increase the risk of virologic failure [321-323].
In general, drug resistant variants when transmitted to newly infected individuals not receiving ARV tend to revert to wild-type, especially if those mutations reduce the replicative capacity of the virus (e.g. M184V) [324, 325]. However, TDR can persist for years as dominant or minority quasispecies in plasma or in PBMCs [326-329]. In addition, the transmitted drug resistant viruses can also evolve to other variants (e.g. T215D/C), representing intermediates between mutant and wild type strains, which are rarely observed in treated HIV patients [330, 331].
TDR has been widely documented among treatment-naive HIV-1 infected patients, with prevalence ranging from 9 to 11.5% in Europe and North America, 7.6% in Latin America/Caribbean, 2.9 to 5.5% in South/Southeast and Upper-income Asian countries and 2.8% in Sub-Saharan Africa [332]. The highest prevalence of TDR in developed countries is consistent with the long term use of antiretroviral drugs.
Information about the occurrence of TDR in HIV-2 is still scarce (Table 3). Accessing TDR in HIV-2 has been difficult because of the lack of clinically validated resistance tests, the limited spread of HIV-2 infection worldwide and the relatively recent availability of ARV to treat HIV-2 infections in West Africa [299].
In RT, resistance mutation Q151M was described in three antiretroviral naïve patients, from Guinea-Bissau, Burkina Farso and Belgium [139, 299, 333]. The M184I/V was reported in one naive patient from Senegal and another from Belgium and in four patients from Portugal [139, 299, 302, 334-336] while K65R was found in one patient from Portugal [335].

In HIV-2, K65R, Q151M and M184V are signature mutations associated with resistance to NRTIs. Q151M and K65R are major mutations associated to different levels of resistance to this class of inhibitors whereas M184I/V leads to 3TC and FTC resistance [274, 276, 277, 337].

In PR, I50V was observed in one Portuguese patient and I54M was found in two and one patient from French and Portuguese Cohorts, respectively [290, 302, 336]. The L90M mutation was detected in two individuals from Portugal and France whereas V47A and I82F were identified in two and one patients, respectively, from the French National HIV-2 Cohort [290, 335, 338].

The I54M mutation confers different levels of resistance to PIs, with the exception of SQV; V47A, I50V and I82F mutations are associated with moderate to high-level resistance to LPV and L90M is the SQV signature resistance mutation in HIV-2, as previously described [243, 278, 281, 284]. TDR is expected to increase as more antiretroviral drugs are being used in HIV-2 infected individuals. The current low level of TDR recommends against drug resistance testing before initiating therapy in HIV-2 naive patients [257].

Table 3. Transmitted drug resistance mutations described in HIV-2 antiretroviral-naïve patients.

NRTIs		**PIs**	
Mutation	**Study**	**Mutation**	**Study**
K65R	Duarte *et al*. 2016 [335]	**V47A**	Charpentier *et al*. 2013 [338]
Q151M	Ruelle *et al*. 2007[139], 2008[299]; Jallow *et al.* 2009[333]	**I50V**	Parreira *et al*. 2006[336]
M184I/V	Parreira *et al*. 2006[336]; Ruelle *et al*.2007[139],2008[299]; Cavaco-Silva *et al*. 2010[302]; Duarte *et al*. 2016[335]	**I54M**	Damond *et al*. 2005[290]; Cavaco-Silva *et al*. 2010[302]
		I82F	Charpentier *et al*. 2013[338]
		L90M	Damond *et al*. 2005[290]; Duarte *et al*. 2016[335]

Drug resistance testing

Resistance testing provides valuable information to guide the selection of the antiretroviral regimen more likely to achieve and maintain viral suppression [339-343]. Genotypic and phenotypic assays are currently available in clinical practice to detect drug resistance mutations [344, 345].

Genotypic assays require the amplification of regions of the HIV genome whose proteins are target by antiretroviral drugs (PR, RT and IN regions in *pol* and *env*), followed by population sequencing of the amplified products and subsequent identification of differences by comparison with a reference wild-type virus [346].

These assays can be performed "in-house" or using commercial kits. "In-house" tests are cheaper and more flexible allowing the sequencing of any region of the genome in HIV. However, in general these tests are not standardized and are more susceptibility to variability.

Genotypic assays are usually based on plasma virus but they can also be based on proviral DNA, particularly in the setting of virologic failure with very low level viremia and for those cases who present undetectable viral loads and need to change the regimen for simplification purposes or toxicity problems [347-349].

Phenotypic assays measure the ability of the virus to grow *in vitro* under different concentrations of antiretroviral drugs in comparison with a known susceptible reference virus [346]. Usually, phenotypic commercial assays involve the generation of recombinant virus by polymerase chain reaction (PCR) amplification of the region of the interest from patient´s plasma and inserting it into the backbone of a laboratory clone of HIV from which the region has been removed. Recombinant viruses and a wild-type reference strain are then produced and used to infect susceptible cells in the presence of increasing concentrations of antiretroviral drugs [350]. The drug concentration that inhibits 50% of viral replication (IC_{50}) is calculated and the ratio of the IC50 of the tested (drug resistant) and the wild-type viruses is reported as the fold increase in IC50 (i.e. fold resistance) [351].

Genotyping is preferable to phenotyping in the clinical routine because of lower cost, faster turnaround time and is less laborious to perform. Phenotypic assays can provide additional information when genotypic results are not sufficient in specific situations such as newer drugs, complex mutational patterns or in the case of HIV-2 [352].

However, the commercial available genotypic and phenotypic tests are unable to detect minority variants below 20% of the total viral quasispecies [261].

Technologies continue to evolve with the ability to sequence and detect extremely small minority populations. Although the clinical significance of these populations is not well determined, these minority drug resistant variants may impair the virological outcomes of antiretroviral therapy [268, 353, 354].

The European HIV Drug Resistance Guidelines Panel recommends both genotypic and phenotypic resistance testing, if available, when treatment changes are needed after therapy failure in HIV-2 infected patients [257].

However, until recently, no validated HIV-2 genotypic or phenotypic antiretroviral resistance assays were available for clinical use. In house genotypic and phenotypic methods are available in some specialized laboratories and recently an automated Internet tool for analyses of HIV-2 drug resistance was developed (HIV-Grade) [297, 355].

For HIV-1/HIV-2 coinfected individuals resistance testing should be done for both viruses and interpretation of resistance should consider the results from both viruses when selecting a new regimen [257].

Co-receptor usage testing for treatment with MVC

The use of coreceptor antagonist MVC in clinical practice requires a tropism testing to predict when such agent will be active by excluding clinically relevant CXCR4-using strains or dual/mixed tropic variants in a patient’s viral population [257].

X4 or dual tropic viruses can occur as minority variants in the population and may impact the virologic outcome of patients under treatment with MVC based regimens leading to therapy failure [311, 356-358]. Through the course of antiretroviral treatment, viral tropism can evolve and patients can experience a switch from R5 to X4 and, conversely, a reverse shift can also occur with the discontinuation of MVC therapy [359, 360].

Phenotypic and genotypic methodologies are available to determine coreceptor usage in HIV-1. Phenotypic tropism testing use recombinant viruses pseudotyped with patient derived HIV-1 Env proteins to infect cell lines expressing CD4 and either CCR5 or CXCR4 [357, 361].

In contrast, genotypic tropism testing is based on amplification and sequencing of the V3 region which is then analysed by a specific bioinformatic tool [362-364].

For HIV-2, until recently, no validated genotypic or phenotypic tropism assays were available for clinical use.

Nonetheless, a genotypic approach was recently developed for estimation of HIV-2 coreceptor use in clinical isolates based on V3 loop sequence from HIV-2 primary isolates with previously defined coreceptor use [103]. According to this algorithm the presence of at least one of the following determinants in V3 predicts CXCR4 tropism: a global net charge superior to 6 and the presence of mutations in positions 18, 19 and amino acid insertions at position 24. As most HIV-2 infected individuals have undetectable or low viral loads, prediction of HIV-2 coreceptor use based on proviral DNA was also studied using this algorithm [99, 100, 365].

The European HIV Drug Resistance Guidelines Panel indicate a coreceptor tropism test in all cases in which MVC is being considered as part of the subsequent regimen, such as virological failure or the need to change to a successful regimen because of toxicity or inconvenience complications [366, 367]. In addition, tropism testing may be useful in patients under MVC-containing regimens to determine whether failure is associated with a potential non-R5 tropic variant [257].

Thus European guidelines suggest the use of either genotypic or phenotypic tests for tropism determination, being enhanced sensitivity trofile assay (ESTA) and Geno2pheno $_{[coreceptor]}$ the most frequently used in HIV-1 infection [362, 368]. When a treatment change is needed in patients who present supressed viral loads, it is suggested that tropism testing may be performed in the latest plasma sample (if sufficient viral RNA is present) or in proviral DNA. However no clinical validation has been performed for these approaches [369-373].

Since there is limited experience with MVC in HIV-2 clinical setting, no recommendations were expressed concerning tropism testing for this virus.

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CHAPTER II

Aims and work plan

HIV-2 is an important cause of disease in West Africa and in countries with socio-historical links to this region, such as Portugal and France [1].

HIV-2 and HIV-1 share many similarities including their genomic and structural organization, modes of transmission and intracellular replication pathways. However, as seen before, when compared with HIV-1, HIV-2 infection is characterized by much lower plasma viral loads, slower decline in CD4+ T cell counts, slower disease progression to AIDS and reduced mortality rates [2]. Moreover, unlike HIV-1, HIV-2 infected patients usually show a lower state of immune activation, which might be responsible for the slower disease progression [3-5]. Another important difference between the two viruses is the production of potent and broad neutralizing antibodies in HIV-2 infected patients, in comparison to HIV-1 infected individuals [6-8]. In terms of treatment there are limited options for HIV-2 infected patients since HIV-2 is intrinsically resistant to NNRTIs and to T-20 and displays differential degrees of susceptibility to NRTIs and PIs [9].

To infect T lymphocytes, macrophages and other cell types, HIV SU glycoprotein must first interact with the CD4 receptor and CXCR4 or CCR5 coreceptor on these target cells. The V3 region on the surface envelope glycoprotein of HIV-2 plays an important role on coreceptor usage, host cell tropism and susceptibility to antibody neutralization [10-13]. However, the amino acid residues in V3 contributing to these phenotypic features remain to be determined. Therefore, in the first study (**Chapter 3**) we used site-directed mutagenesis to dissect the role of individual amino acid residues in V3 in: (1) CCR5 and CXCR4 use; (2) replication capacity in $CD4^+$ T lymphocytes and macrophages and (3) susceptibility to antibody neutralization.

Six V3 mutants were created on pROD10, an infectious molecular clone of HIV-2_{ROD} which is X4, with amino acid substitutions in residues 18 and/or 29 and/or single deletions at positions 23 and 24. These variants and HIV-2_{ROD} were used to infect TZM-bl cells that express CD4+, CCR5 and CXCR4, in the presence of a CXCR4 antagonist (AMD3100) or CCR5 antagonist (TAK-779) to investigate the impact of the amino acid substitutions in coreceptor usage.

The V3 region has long been described as a potent neutralizing domain in HIV-2 [13-15]. On the other hand, CXCR4 usage by primary HIV-2 isolates has recently been associated with higher resistance to antibody neutralization when compared with CCR5 usage [6-8].

This correlation has been associated with major changes in sequence, charge, size and secondary structure of the V3 region but there was no attempt to identify the amino acid residues that directly contribute to the susceptibility to antibody neutralization. Hence, the second objective of this study (**Chapter 3**) was to evaluate the impact of the V3 mutations in suceptibility of HIV-2 to antibody neutralization. Viruses bearing the V3 mutations and wild type ROD were analyzed against a panel of plasma samples collected from HIV-2 infected patients and Env-specific monoclonal antibodies obtained from HIV-2 infected patients using a luciferase reporter gene assay in TZM-bl cells. Furthermore, with the purpose of determining the potential impact of these specific mutations in the HIV-2 V3 secondary structure (third objective), three dimensional structure models of V3 loops of HIV-2_{ROD} and mutants were generated by homology modelling based on crystallographic structure of the gp125 of HIV-2_{ST}.
Macrophages and CD4+ T lymphocytes are the major targets of HIV infection. For HIV-1, several determinants in the viral envelope have been associated to macrophage tropism, particularly in gp120 [e.g. N283 (C2), I326 (V3), D386 (V4)] [16]. Contrary to HIV-1, studies addressing the potential role of V3 region in HIV-2 cell tropism are scarce. The fourth aim of this study (**Chapter 3**) was to evaluate the impact of the V3 mutations in the replication capacity of HIV-2_{ROD10} in macrophages and CD4+ T lymphocytes. Thus, wild type ROD10 and V3 mutant viruses were used to infect macrophages and CD4+ T lymphocytes and viral replication was monitored in culture supernatants by reverse transcriptase (RT) activity using an enzyme-linked immunosorbent assay (ELISA) for 12 days.

MVC is a CCR5 coreceptor antagonist that is currently used to treat patients infected with R5 isolates [17]. Determining coreceptor usage is critical before initiating MVC to ensure treatment efficacy and to prevent a switch to CXCR4 coreceptor [18]. Currently there are no standardized phenotypic or genotypic assays to predict HIV-2 coreceptor usage. Consequently, in the second study (**Chapter 4**), we firstly intended to provide a genotypic tool for predicting whether an HIV-2 V3 amino-acid sequence originates from an R5 or an X4-capable variant. Secondly, we aimed to determine which V3 amino-acid mutations confer the X4-capable phenotype.

A data set of 126 pairs of HIV-2 amino acid sequences and phenotypic coreceptor usage annotations were collected in order to generate statistical models capable of predicting HIV-2 coreceptor usage. Support vector machines (SVMs) were then trained and validated based on this data to identify the most predictive models according to their areas under the ROC curve (AUCs). A linear SVM based on the V3 amino acid sequence were used for all further analyses due to its high predictive accuracy. The existing rules-based genotypic approach for HIV-2 coreceptor identification was then validated and compared with SVMs in terms of predictive accuracy [10].
To identify which substitutions in the V3 amino-acid sequence are associated with the X4-capable phenotype according to the linear SVM, the model weights were investigated and the discriminatory strength of individual substitutions in the V3 loop were statistically tested. Lastly, the linear SVM was implemented as a web service denominated geno2pheno[coreceptor-hiv2]. To assess its predictive accuracy, the web service was validated with a set of nine HIV-2 isolates, which were not previously used for training the model, including the six V3 variants of HIV-2_{ROD10} described in **Chapter 3**.

As described before, currently available antiretroviral drugs were specifically developed to inhibit HIV-1 entry and replication, and consequently some drugs have limited or no activity against HIV-2. For example, the fusion inhibitor T-20, approved as the first and so far the only fusion inhibitor for clinical use in HIV-1 infection, doesn't work on HIV-2 isolates [19]. Considerable efforts have been made to develop new fusion inhibitors with improved pharmaceutical profile*s* against this virus. Therefore, the main purpose of the third study (**Chapter 5**) was to generate short fusion inhibitor peptides active against both HIV-2 and HIV-1 isolates. Fifteen novel M-T hook-modified peptides were synthesized and their antiviral activity was determined against a panel of HIV-1 and HIV-2 primary isolates and SIV. The activity of the new peptides was also tested against T-20 resistant HIV-1 isolates and the panel of V3 variants of HIV-2_{ROD10} described in **Chapter 3**.

PIs are commonly used in combination therapy with NRTIs or INIs to treat HIV-2 infected patients in Portugal [20]. The long-term evolution of resistance to the PIs in HIV-2 infected patients has not been investigated so far.

The main objectives of the last study (**Chapter 6**) were to characterize PR diversity and genotypic resistance to PIs of HIV-2 infected individuals living in Portugal and to evaluate the impact of resistance mutations to PIs in treatment outcome eight years post-therapy.

Blood samples were collected from 27 HIV-2 infected patients living in Lisbon. Fifteen were on ART and 12 were untreated. The PR region was amplified from proviral DNA present in PBMCs (in one case from RNA), cloned and sequenced. PI resistance mutations were identified using HIV-Grade internet tool and transmitted and acquired drug resistance were estimated in this population. In patients who exhibited DRM we investigate the impact of those mutations in patient response to therapy eight years after study entry. Additionally, we searched correlations between amino acid entropy and the existence of CTL epitopes, as an interaction between PI treatment and CTL immune response may occur in HIV-2 infected patients as it has been reported for HIV-1 [21].

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CHAPTER III

Determinants of coreceptor usage, tropism and susceptibility to antibody neutralization in the V3 region of HIV-2

Determinants of coreceptor use, tropism and susceptibility to antibody neutralization in the V3 region of HIV-2

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Abstract

The V3 loop is a key functional domain of the HIV-1 envelope as it determines coreceptor use, cellular tropism and antibody neutralization. Little is known about the functional role of V3 in HIV-2, a virus that is endemic in Western Africa and a few other countries in Europe and can cause AIDS and death.

In this study we investigated the role of the V3 region in HIV-2: (1) coreceptor use; (2) replication capacity in CD4+ T cells and macrophages and (3) susceptibility to antibody neutralization. Six V3 mutants were produced in pROD10, an infectious molecular clone of HIV-2_{ROD} which is CXCR4-tropic, replicates well in CD4+ T lymphocytes and is resistant to neutralization. Mutated variants carried amino acid substitutions in residues 18 and/or 29 and/or single deletions at positions 23 and 24 in the V3 region. TZM-bl cells and CXCR4 or CCR5 inhibitors were used to investigate coreceptor usage patterns; susceptibility of V3 mutants to antibody neutralization was evaluated using plasma samples from HIV-2 infected individuals and a panel of human monoclonal neutralizing antibodies. Finally, viral replication capacity in CD4+ T cells and macrophages was examined by monitoring viral reverse transcriptase activity in culture supernatants. The data showed that the H18L mutation was sufficient for full X4-to-R5 tropism switch in the context of the short version of the V3 loop (H23Δ+Y24Δ). R5/X4 mutants H18L and H23Δ + Y24Δ were easier to neutralize when compared to HIV-2_{ROD} (P=0.0411; P=0.0152, respectively). Like HIV-2_{ROD}, H18L mutant was able to replicate efficiently only in CD4+ T cells. In contrast, K29T, H23Δ+Y24Δ and H18L+ H23Δ+Y24Δ mutants gained the ability to replicate in macrophages albeit at the cost of some capacity to replicate in CD4+ T cells when compared with ROD wild-type. This study identifies the main determinants of coreceptor use, tropism and susceptibility to antibody neutralization in the V3 region of HIV-2.

Keywords: V3; HIV-2; Coreceptor usage; CCR5, CXCR4; Antibody neutralization; Macrophages

Introduction

The V3 loop of HIV is one of the key domains of viral envelope as it determines coreceptor use, cellular tropism and antibody neutralization [1-13].

Currently, the predominant coreceptor used by HIV to infect target cells can be predicted by phenotypic and genotypic tropism tests [14, 15]. Genotypic assays are based on the analysis of V3 sequence of HIV and represent an attractive choice compared to phenotypic tests as they are more accessible, rapid and economical. These tests can help to select patients for treatment with CCR5 antagonist maraviroc (MVC) [16, 17].

MVC has been successfully used in the treatment of experienced HIV-1 infected patients [18]. Though data on the efficacy of MVC in HIV-2 is still limited, this drug appears to be an interesting new option for HIV-2 treatment according to phenotypic studies and *in vivo* case reports [19-24].

Studies have investigated the potential role of the V3 region on HIV-2 coreceptor use [1-3, 25-29]. Several genetic determinants in C-terminal domain (aa.18 to aa.36) of HIV-2 V3 have been associated to the use of CXCR4 such as any substitution at positions 18, insertions at position 24 and the presence of positively charged amino acids at positions 19 and 27 [1-3, 30]. In addition, a genotypic tropism approach based on HIV-2 V3 sequences has been recently described [2]. However, with one exception [3], site directed mutagenesis has not been used to investigate the specific role of selected amino acid residues in V3 on HIV-2 coreceptor use. Phenotypic studies of coreceptor usage using site-directed V3 mutants are required to obtain a definitive knowledge on the molecular determinants of HIV-2 coreceptor usage. A better genetic identification of coreceptor use by HIV-2 will be crucial to increase the safe use of MVC to treat HIV-2 infected patients.

In contrast to HIV-1, coreceptor usage has also been associated to HIV-2 susceptibility to antibody neutralization. Indeed we have found that X4 HIV-2 primary isolates are generally more resistant to antibody neutralization than R5 isolates [6, 31, 32]. Major alterations on HIV-2 V3 sequence, particularly, the charge, size and structural conformation of this region have been suggested to explain the relation between coreceptor usage and escape to neutralization [6, 31, 32]. Further studies are required to investigate the molecular and structural determinants in the V3 region of HIV-2 linking coreceptor usage and escape to antibody neutralization.

CCR5 and CXCR4 coreceptors are both expressed in macrophages and CD4+ T lymphocytes [12]. Several determinants that influence HIV-1 tropism for macrophages have been reported in different regions of viral envelope, mostly in the CD4-binding site but also in the variable loops including V3 [33, 34]. Contrary to HIV-1, studies addressing the potential role of V3 region in HIV-2 tropism for macrophages are scarce.

In this study we aimed to identify the role of selected amino acid residues in the envelope V3 region in the following phenotypic features of HIV-2: (1) coreceptor usage; (2) replication capacity on CD4+ T cells and macrophages and (3) susceptibility to antibody neutralization. Our results indicate that H18L mutation is sufficient for a switch from X4 to R5 tropism and loss of X4 tropism while K29T substitution allows moderate to high levels of replication in CD4+ T cells and macrophages and confers resistance to antibody-mediated neutralization. We also demonstrate that Nab resistance is an intrinsic feature of CXCR4 tropic HIV-2 isolates that is acquired over the course of infection in close association with sequence changes in V3 that favour R5-to-X4 switch.

Material and methods

Virus isolates and plasma samples

Five X4 primary isolates were obtained from five adult chronically infected patients in late disease stage (median $CD4^+$ T cells/µl=78; interquartile range=31.5-221) and two additional X4 variants were obtained from two vertically infected children at age 5 (early infection) (median $CD4^+$ T cells/µl=319.5; interquartile range=44-595). Three primary R5 isolates were obtained from chronically infected patients with median $CD4^+$ T cells/µl=275; interquartile range=66-615). All of these primary isolates have been described previously [6, 7, 19, 31, 32]. Sixteen plasmas from unrelated HIV-2 infected patients (median $CD4^+$ T cells/µl=333; interquartile range=194.5-480) were used to neutralize the X4 and R5 viruses.

HIV-2_{ROD} stocks were prepared by transfecting full-length pROD10 DNA into 293T cells using JetPrime transfection reagent (Polyplus-transfection SA) according to manufacturer's instructions.

The viral supernatants were collected 48 hours after transfection and were stored at -80ºC. The 50% tissue culture infectious dose ($TCID_{50}$) of each viral stock was determined in a single-round viral infectivity assay in TZM-bl reporter cells using a luciferase assay system (Promega) according to manufacturer's instructions as described elsewhere [19].

Cells, antiretrovirals, plasmid and antibodies

293T cells were purchased from American Type Culture Collection (ATCC) and TZM-bl cells were obtained through the National Institute of Health (NIH) AIDS Research and Reference Reagent Program. Peripheral blood mononuclear cells (PBMCs), from HIV uninfected donors, were obtained from buffy-coats by Ficoll–Hypaque density gradient centrifugation and maintained in RPMI 1640 medium supplemented with 15% inactivated fetal bovine serum (FBS). 293T and TZM-bl cells were grown in Dulbecco minimal essential medium (DMEM) complemented with 10% inactivated FBS.

The coreceptor antagonists TAK-779 and AMD3100 and full-length infectious clone of HIV-2 (pROD10) were obtained from the NIH AIDS Research and Reference Reagent Program. Monoclonal antibodies targeting V3 (6.10F and 1.4B), V4 (1.7A), CD4 binding site (6.10B) and CD4 induced (1.4H), and a non-neutralizing antibody used as a control (2.6C) were obtained from James Robinson [35].

Site-directed mutagenesis

Single and multiple V3 amino acid substitutions H18L, H23Δ + Y24Δ, K29T, H18L+ H23Δ + Y24Δ, H18L+ K29T and H18L+ H23Δ + Y24Δ+ K29T were introduced into full-length infectious molecular clone of HIV-2_{ROD} strain (pROD10) using QuickChange II XL site-directed mutagenesis kit (Stratagene) and adequate primers; mutant plasmids were sequenced to confirm the presence of the mutations and exclude additional mutations. Mutant ROD10 plasmids were purified using JetStar Plasmid Purification Kit (Genomed).

Determination of coreceptor use

CCR5 and CXCR4 coreceptor usage were determined using a single round viral infectivity assay performed with TZM-bl cells (CD4+, CCR5+, CXCR4+) in the presence of CCR5 antagonist TAK-779 and/or CXCR4 antagonist AMD3100 as previously described [19]. Briefly, the day prior to assay, 10.000 TZM-bl cells/well were seeded in 96 well plates and incubated overnight. After 24 hours, the medium was removed and the cells were replaced with new complete growth medium containing 10 µM TAK-779 and/or 1.2 µM AMD3100 and were incubated for 1 hour at 37ºC. A mixture of complete growth medium with DEAE-Dextran and 200 $TCID_{50}$ of each virus was then added to the cells and incubated at 37ºC with 5% CO_2 for 48 hours. After 48 hours, the cells were lysed and the luciferase activity was measured using a luciferase assay system (Promega).

Infectivity assays on CD4+ T lymphocytes and macrophages

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors. Monocytes were obtained from PBMC by immune-magnetic separation. To obtain monocytes-derived macrophages, monocytes were cultured in complete medium with macrophage colony-stimulating factor (M-CSF). Cells were cultured for additional 7 days in 24 well plates at a density of $3x10^5$ cells/well. Autologous CD4+ T cells were purified by negative selection with CD4+ T Cell isolation kit II (Miltenyi), activated with PHA-L during 3 days and maintained in culture medium supplemented with IL-2 at a density of $5x10^5$ cells/well. Monocytes-derived macrophages and CD4+ T cells were infected with different HIV molecular clones during 3 hours at 37ºC with 5% of CO_2, or left uninfected as controls. Viral inocula were determined by reverse transcriptase (RT) activity using an enzyme-linked immunosorbent assay (ELISA) (Lenti-RT kit, Cavidi, Uppsala, Sweden). During 12 days, culture supernatants were collected and monitored by RT activity using the same assay.

Antibody neutralization assays

Antibody neutralization assays were performed using a luciferase reporter gene assay in TZM-bl cells as described previously [6, 36]. Briefly, 10,000 TZM-bl cells/well were seeded in 96 well plates. After 24 hours a mixture of 200 $TCID_{50}$ of each virus and 2-fold serial dilutions of heat inactivated patients plasma was made in complete growth medium with DEAE-dextran and incubated for 1 hour at 37ºC with 5% CO_2.

After 1 hour viruses were used to infect TZM-bl cells seeded in the day before. After 48 hours, luciferase expression was quantified using a luciferase assay system (Promega). Medium-only control wells were measured as background, and virus-only control wells were included as 100% infection. IC_{50} values were estimated by the sigmoidal dose–response (variable slope) equation in Prism (version 5.0; GraphPad Software). All statistical analyses were performed with Prism with a level of significance of 5% (P value < 0.05).

Modelling 3D V3 Structures

The structure of the V3 region of wild-type and mutant isolates was modeled by homology modelling as no three-dimensional (3D) experimental structure of the HIV-2 V3 loop is currently available. The first crystallographic structure of HIV-2_{ST} gp125 has been released recently but, unfortunately, this crystallographic structure does not present the complete V3 loop [37].

To generate the V3 loop model a traditional homology modeling procedure has been used. Firstly, the sequence of HIV-2 gp160 envelope glycoprotein containing 858 amino acids (ROD isolate) was identified from UniProt, the Universal protein resource data bank (http://www.uniprot.org/) under the code P04577. Then, to identify the most informative template a search on a database of all known protein structures and sequences (http://www.rcsb.org/pdb/) was performed in order to obtain proper templates based on this sequence. The same procedure was repeated using the Molecular Operating Environment program (MOE) version 2014.09 (http:// www.chemcomp.com/software/) to ensure the quality of the potential templates. The candidate lists were reduced by elimination of all hits having low statistical significance (BLAST *E*-value greater than 0.01) or alignment length shorter than 70% of the target sequence. Three crystallographic structures were selected as proper templates: an HIV-1 envelope glycoprotein gp160 with the V3 core complexed with CD4 (PDBID: 2QAD, resolution 3.3 Å) [38], an HIV-1 gp120 envelope glycoprotein with the V3 core complexed with CD4 (PDBID: 2B4C, resolution 3.3 Å) [39], and the recently resolved HIV-2_{ST} gp125 envelope glycoprotein complexed with CD4 (PDBID: 5CAY, resolution 3.0 Å) [37]. Alignments of target (C2-V3-C3 region) and selected template sequences were performed using MOE-Align feature using default settings.

A preliminary model construction of the C2-V3-C3 region of the HIV-2 sequence was performed with MOE software using the corrected crystallographic 3D structures of the two template HIV-1 envelope glycoproteins and a set of several intermediate models was generated and refined with Amber99 forcefield resulting in the corresponding homology models. The quality of the models was analyzed using the Ramachandran plots. The models with the least amount of outliers were selected. The models based on HIV-1 templates 2B4C and 2QAD, both obtained after correction of a deletion of two residues at positions 15 and 16 of V3, were selected and subjected to energy minimization using MOE software. An additional model based on HIV-2_{ST} gp125 envelope glycoprotein crystallographic structure (5CAY) was generated using the same procedure. After correction of 5CAY structure with the correspondent portion of the 2B4C minimized model (five residues from the N-terminus and seven residues from C-terminus were conserved in the V3 loop and the missing portion of residues was replaced by our previously model), a new homology model of the C2-V3-C3 portion of gp125 was generated. This new model was minimized (and optimized) and used as template to generate a set of six new models with mutations and/or deletions at the V3 loop (H18L, H23Δ + Y24Δ, K29T, H18L+ H23Δ + Y24Δ, H18L+ K29T and H18L+ H23Δ + Y24Δ+ K29T). In order to analyse the constructed models a secondary structure prediction was run in Robetta and PSIPRED servers. Robetta server (http://robetta.bakerlab.org/) uses a fully automated implementation of the Rosetta software package for protein structure prediction. PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/) uses a highly accurate secondary structure prediction method.

Results

Resistance to antibody neutralization evolves over time in X4 isolates of HIV-2

We have previously found that X4 HIV-2 isolates more frequently resist plasma antibody neutralization than R5 isolates both in adults patients [32] and children infected perinatally [31].

To better determine if resistance to antibody neutralization is an intrinsic feature of primary isolates of HIV-2 that use the CXCR4 co-receptor, we characterized the neutralization phenotype of a panel of X4 isolates obtained during early infection in children (2 isolates) and late stage infection (5 isolates) and compared it with three R5 isolates obtained from patients with long-term chronic infection (Figure 1A).

The neutralization studies were done against 16 heterologous plasmas from unrelated HIV-2 infected patients. We found that X4 isolates were significantly more resistant to antibody neutralization compared to R5 viruses [mean log10 IC50 (standard deviation-SD) for all X4 isolates: 2.130 (0.7221) vs R5 isolates from chronically infected patients: 3.796 (1.311), $P<0.0001$] (Figure 1B). Moreover, X4 isolates from late stage infection patients were significantly more resistant to neutralization than X4 isolates from early infection [mean log10 IC50 (SD) for late stage infection X4 isolates: 1.784 (0.5002) vs early infection X4 isolates: 2.997 (0.3797), $P<0.0001$] (Figure 1B). These results provide definitive evidence that Nab resistance is an intrinsic characteristic of primary isolates of HIV-2 that use CXCR4 and that this feature evolves over the infection period. To look for sequence correlates of Nab resistance we compared the V3 sequences of X4 isolates with those of R5 isolates. Compared to the R5 isolates, the V3 region of all X4 strains had a mutation at position 18, the mutation V19K/R, an insertion in position 24 and a global positive net charge ≥7. Moreover, the V3 region of early infection X4 isolates had a lower mean global net charge compared to late infection X4 isolates (+8 and +9, respectively) (Figure 1C).

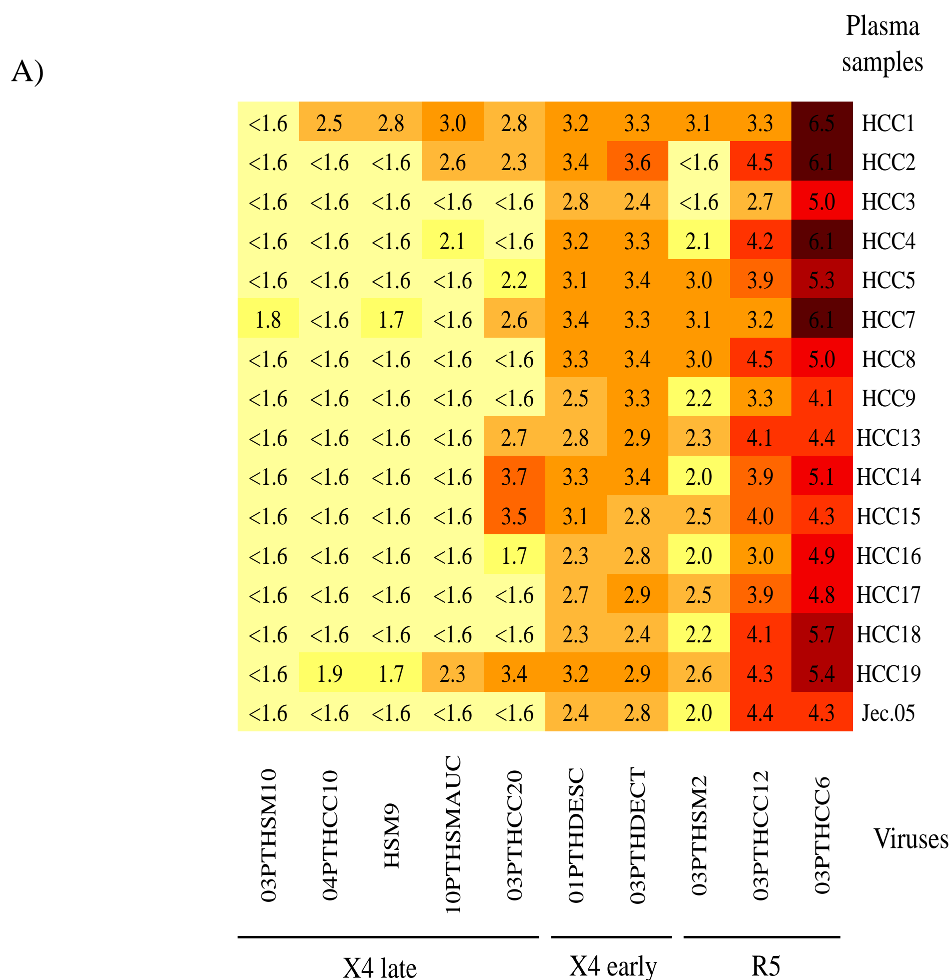

Figure 1A. Susceptibility of X4 and R5 primary isolates of HIV-2 to antibody neutralization. A heat map of the median reciprocal log10 IC50 neutralizing titer of each heterologous plasma sample (right) against the seven X4 isolates and the three R5 isolates (bottom) is shown. The reciprocal log 10 IC50 value is colour-coded. The darkest colour represents the highest neutralizing titer; the lightest colour indicates that there was no detectable neutralization above 50% with the lowest plasma dilution tested (1/40);

B)

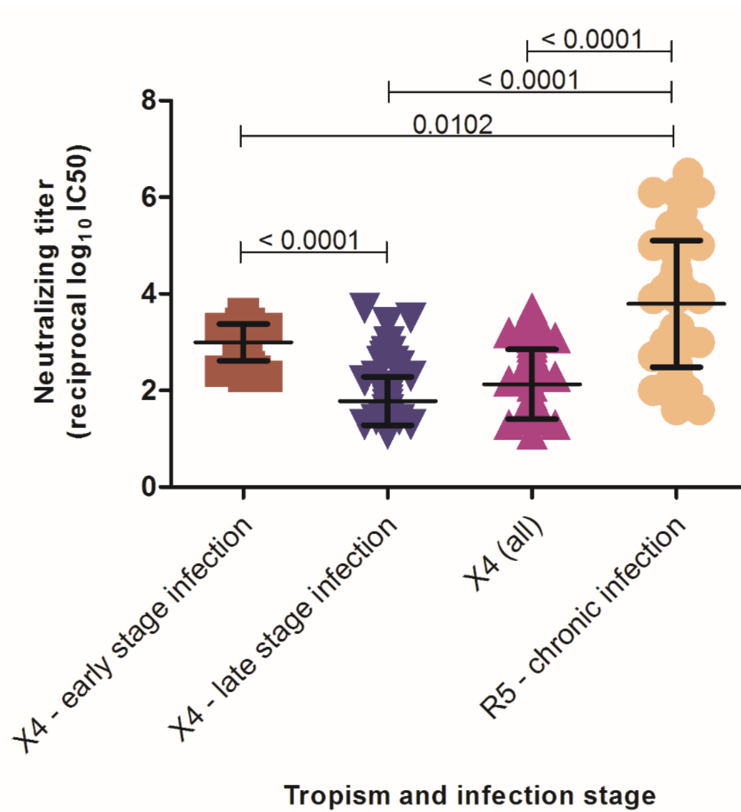

Figure 1B. Susceptibility of X4 and R5 primary isolates of HIV-2 to antibody neutralization. Dot-plot graphic showing the median reciprocal log_{10} IC50 neutralizing titers and standard deviation of 16 heterologous plasma samples against primary X4 isolates from early and late stage disease and R5 isolates. Mann-Whitney U test was used to compare the median log_{10} reciprocal IC50 values;

C)

```
                                  V3 region
03PTHCC6    : CRRPGNKTVVPITLMSGLVFHSQP---INRRPKQAW
03PTHCC12   : ........................---.........   R5
03PTHSM2    : ........................---..T......   (+6/+7)
03PTHCC20   : .............G..RR..G.RFHS.........
03PTHSM9    : .............G..QR..FR.--R.....M...
10PTHSMAUC  : ................FK.....--V.........   X4 late stage
03PTHCC10   : ................YK...R.--V..Q..M...   (+7/+8/+9/+11)
04PTHSM10   : .............Q..KR..FR.--V..Q......
03PTHDECT   : ................YR....A--V..T......   X4 early stage
01PTHDESC   : ................RK...R.--I..E......   (+7/+ 9)
```

Figure 1C. Susceptibility of X4 and R5 primary isolates of HIV-2 to antibody neutralization. Alignment of the amino acid sequences of the V3 loop of the X4 and R5 isolates. Dots in the alignments indicate sequence identity to the first R5 isolate sequence (03PTHCC6); dashes indicate deletions; amino acids in red boxes are involved in co-receptor use as determined phenotypically and genotypically based on V3 loop sequence patterns. Disease stage and global net charge of the V3 region are indicated at the right of the alignment.

X4-to-R5 tropism switch requires a single amino acid substitution in V3

The HIV-2_{ROD} reference strain [40] uses CXCR4 as an entry coreceptor [3, 19, 41]. HIV-2_{ROD10} V3 region shows almost all of the genetic determinants previously associated with X4 tropism namely, the L18H and T29K mutations, a double insertion and a V3 charge of 7 (Figure 2A) [2, 3]. Substitutions at position V19K/R were also described to influence CXCR4 usage but in case of HIV-2_{ROD10}, a valine is present at this position. We aimed to map the amino acid residues in V3 that determine coreceptor use in HIV-2 using HIV-2_{ROD} as a model. To this end, single reverse substitutions at positions 18 (H18L) and 29 (K29T) and deletion of the two insertions at positions 23 and 24 were done by site-directed mutagenesis in the V3 region of pROD10. Mutant isolates were produced by transfection in 293T cells and were used to infect TZM-bl cells in the presence of a CXCR4 (AMD3100) or a CCR5 inhibitor (TAK-779). Consistent with its CXCR4 tropism, HIV2_{ROD10} wild-type was fully sensitive to AMD3100 and fully resistant to TAK779 (Figure 2B). The K29T variant was the only mutant with a susceptibility profile to the coreceptor antagonists similar to wild-type ROD. Mutants H18L, H23Δ + Y24Δ and H18L+ K29T showed significant loss of CXCR4 usage compared to the wild type ROD, as determined by the increased ability to replicate in the presence of AMD3100 (P=0.022, for all three mutants), and gain of CCR5 usage as determined by the lower infectivity in the presence of TAK779. Finally, replication of mutants H18L+ H23Δ + Y24Δ and H18L+ H23Δ + Y24Δ+ K29T was completely blocked by TAK-779 but not by AMD3100 (P=0.0022 for differences in infectivity of both mutants relative to both antagonists). In summary, these results have shown that: 1) lysine at position 29 *per se* is not important for CXCR4 usage of HIV-2_{ROD}; 2) amino acid insertions at positions 23 and 24 of the V3 loop play a role in CXCR4 usage; 3) amino acid at position 18 in V3 is an important determinant of coreceptor switch; and 4) full X4-to-R5 tropism switch of HIV-2 requires only histidine to lysine change (with concomitant charge removal) at position 18 in the short version of V3.

A)

```
                                       324/325
                  302 (1)     319 (18)  (23/24)  330 (29)   337 (36)
ROD WT                     CKRPGNKTVKQIMLMSGHVFHSHYQPINKRPRQAWC
ROD H18L                   .................L..................
ROD K29T                   ...........................T........
ROD H23Δ+Y24Δ              ......................--............
ROD H18L+K29T              .................L.........T........
ROD H18L+H23Δ+Y24Δ         .................L....--............
ROD H18L+K29T+H23Δ+Y24Δ    .................L....--...T........
```

B)

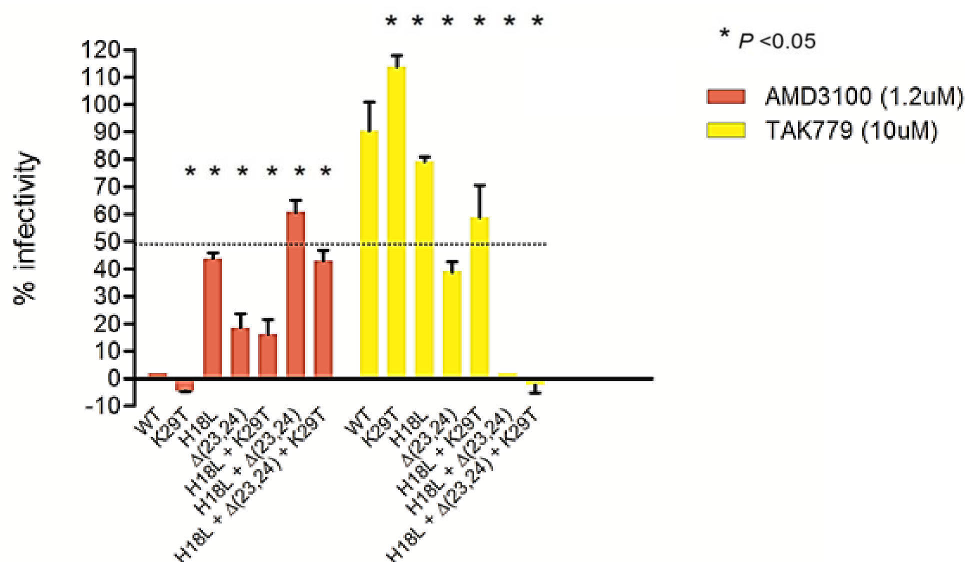

Figure 2. Amino acid residues in HIV-2_{ROD} V3 region associated with coreceptor use. A) Amino acid sequence alignment of V3 of HIV-2_{ROD10} wild type and mutant clones. Amino acids residues were numbered according to $HIV2_{ROD10}$ reference strain. Dots indicate identity with wild type, letters represent differences relative to wild type and dashes indicate gaps introduced to align the sequences. Red boxes indicate the amino acid alterations in V3 mutants compared with wild type; B) Bar graph showing the infectivity of V3 mutants as compared to HIV-2_{ROD10} wild-type in the presence of excessive amounts of co-receptors antagonists AMD3100 (1.2 μM) and TAK-779 (10 μM). Only *P* values <0.05 that represent significant differences between V3 variants and wild-type are shown. Mann-Whitney U test was used to compare the median values. Bars represent the standard error of the mean.

X4-to-R5 tropism switch increases HIV-2_{ROD} sensitivity to antibody neutralization

The susceptibility of HIV-2_{ROD} wild type and V3 variants was investigated against a panel of plasma samples from HIV-2 infected patients (n=6) with proven neutralization ability at 1:100 dilution (Figure 3A). Mean infectivity of ROD wt with the plasma panel was 62.64% (standard error, 7.79%). Remarkably, mutants H18L and H23Δ + Y24Δ were much easier to neutralize when compared to HIV-2_{ROD} [mean (SE) infectivity H18L: 33.14% (7.64%), *P*=0.0411, and H23Δ + Y24Δ: 26.14% (7.59%); *P*=0.0152]; the remaining mutants exhibited similar neutralization profiles to wild-type.

A)

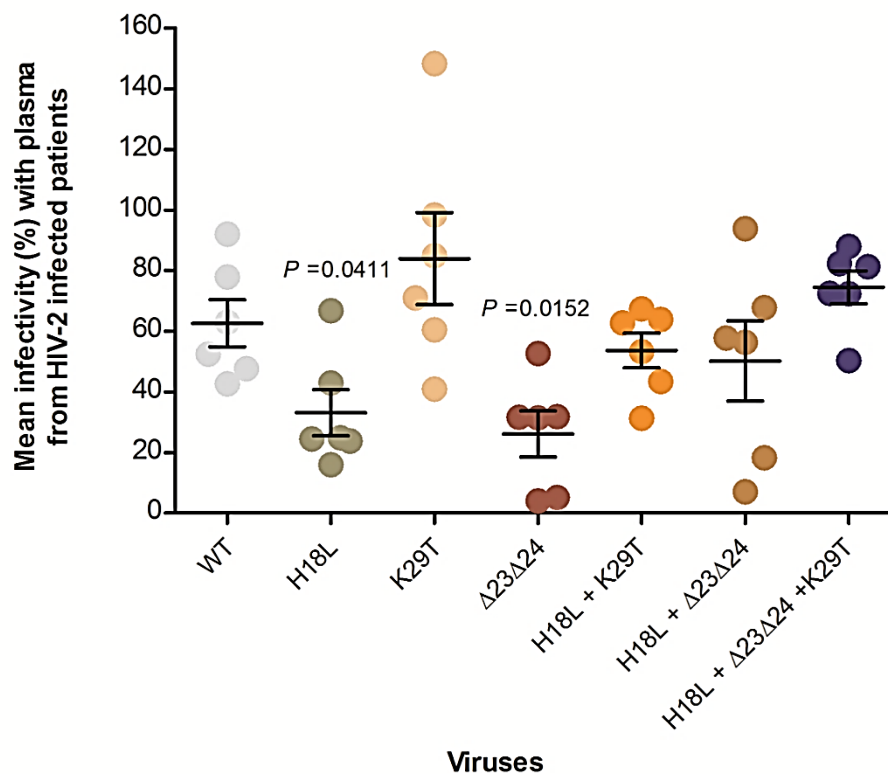

Figure 3A. Neutralization of mutant viruses with plasma samples from HIV-2 infected patients. Dot-plot graph showing the median infectivity of HIV-2_{ROD} wild type and V3 mutants in the presence of 1:100 dilution of multiple plasma samples (n=6) from HIV-2 infected patients. Only *P* values <0.05 that represent significant differences between V3 variants and wild-type are shown. Mann-Whitney U test was used to compare the median values. Bars represent the standard error of the mean.

A panel of human monoclonal neutralizing antibodies (HuMNabs) targeting V3 (6.10F and 1.4B), V4 (1.7A), CD4 binding site (6.10B) and CD4 induced (1.4H) was used to further analyse the susceptibility of wild-type and mutated virus to antibody neutralization (Figure 3B) [35]. A non-neutralizing monoclonal antibody (2.6C) and primary isolates HCC19 (CCR5-tropic) and HSM10 (CXCR4-tropic) were used as controls.

B)

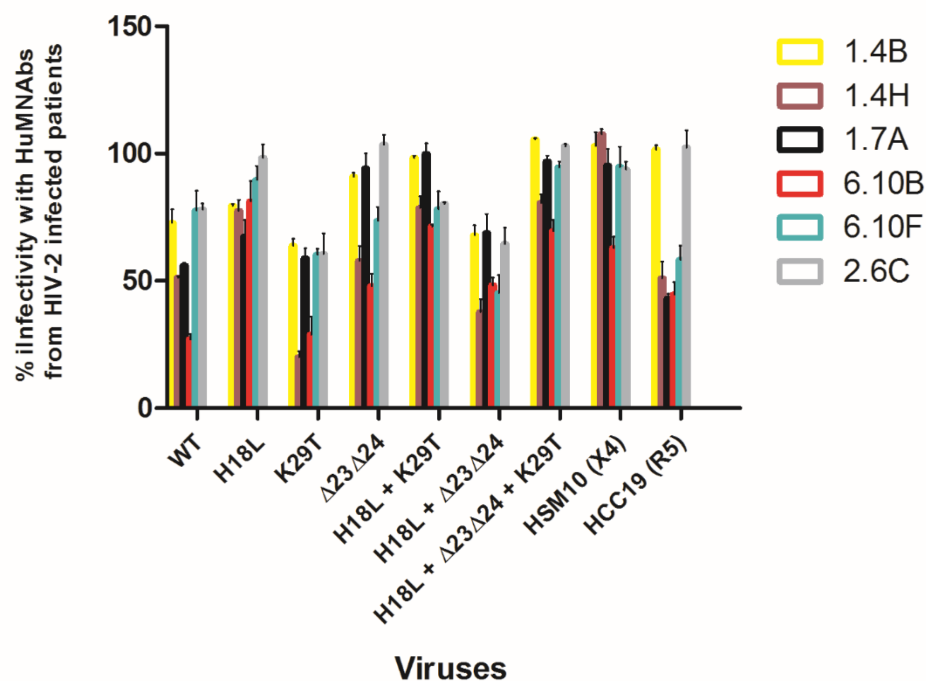

Figure 3B. Neutralization of mutant viruses with plasma samples and human monoclonal antibodies from HIV-2 infected patients. Bar graph showing the mean infectivity of HIV-2_{ROD} wild type and V3 mutants in the presence of a panel of human monoclonal neutralizing antibodies (HuMNabs) from HIV-2 infected patients targeting V3 (6.10F and 1.4B), V4 (1.7A), CD4 binding site (6.10B) and CD4 induced (1.4H). A non-neutralizing antibody (2.6C) and viruses HCC19 (CCR5-tropic) and HSM10 (CXCR4-tropic) were used as control. Mann-Whitney U test was used to compare the median values. Bars represent the standard error of the mean.

Interestingly, Nabs targeting CD4, 1.4H and 6.10B, were the most potent antibodies against the studied viruses. These Nabs neutralized mutants H18L+ H23Δ + Y24Δ (*P*=0.0152 for both Nabs), K29T (*P*=0.0022; *P*=0.9372, respectively) and H23Δ + Y24Δ (*P*=1.000; *P*=0.0043). Mutants H18L (*P*=0.0022 for both Nabs), H18L+K29T (*P*=0.0022 for both Nabs) and H18L+ H23Δ + Y24Δ+ K29T (*P*=0.0022; *P*=0.0043) were more resistant to antibody neutralization than wild-type virus.

The V3 Nabs 6.10F and 1.4B failed to neutralize the mutants and wild-type, with the exception of H18L+ H23Δ + Y24Δ mutant that was neutralized (~50%) by Nab 6.10F, although with no significant change compared to wild-type.

Moreover, all mutants, with the exception of K29T and H18L+ H23Δ + Y24Δ were more difficult to neutralize with 1.4B than the wild-type (*P*=0.0260 for H18L, H23Δ + Y24Δ and H18L+K29T mutants and *P*=0.0022 for H18L+ H23Δ + Y24Δ+ K29T variant). A similar neutralization pattern was found with Nab 1.7A.

Therefore, we have shown that all Mabs failed to neutralize the wild type and V3 mutants, with the exception of CD4-specific antibodies, 6.10B and 1.4H. The V3 substitutions did not appear to impact on neutralization sensitivity or resistance of wild type strain and variants to V3 human antibodies, 6.10F and 1.4B.

A single substitution in V3 makes HIV-2_{ROD} able to replicate in macrophages

HIV-2_{ROD} replicates efficiently in T lymphocytes but it doesn't in macrophages and this can be associated with ability to bind to the different coreceptors (Figure 4). We therefore analysed the replication capacity of ROD mutants in CD4+ T cells and macrophages by monitoring reverse transcriptase (RT) activity in culture supernatants during 12-days of infection (Figure 4). Like HIV-2_{ROD}, H18L mutant was able to replicate efficiently only in CD4+ T cells. In contrast, mutants K29T, H23Δ+Y24Δ and H18L+ H23Δ+Y24Δ gained the ability to replicate in macrophages at the cost of some capacity to replicate in CD4+ T cells when compared with wild-type. The H18L + H23Δ + Y24Δ+ K29T variant was unable to replicate in both types of cells indicating severe fitness problems and low likelihood for detection in infected individuals.

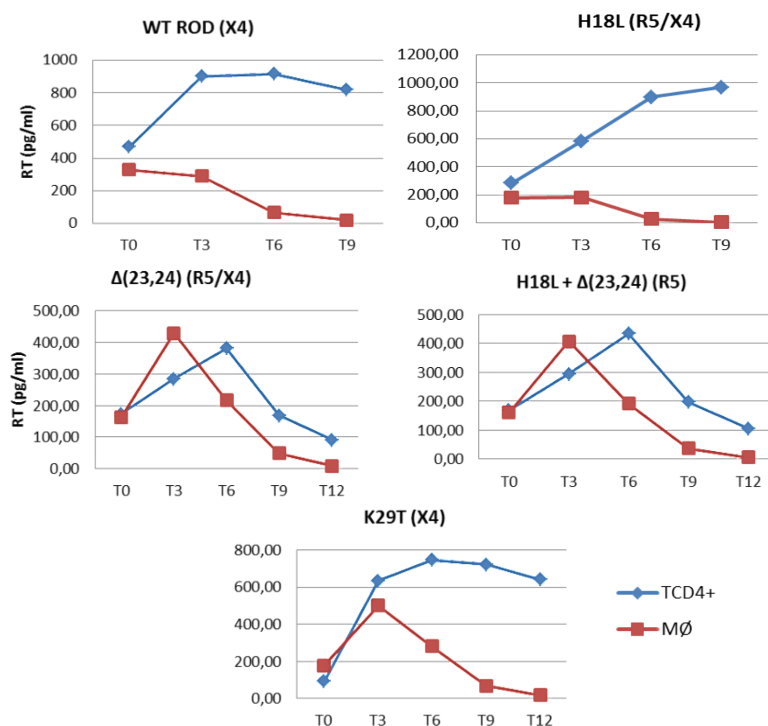

Figure 4. Viral replication in CD4+ T cells and macrophages of V3 mutants and HIV-2_{ROD10} wild type was quantified by RT activity in culture supernatants during 12-days after infection.

Modeling of HIV-2 gp125 V3 loop

Aiming to pinpoint specific structural features that could correlate with the known genotypic determinants of HIV-2 tropism located in the gp125 V3 loop region a three-dimensional (3D) structure of C2V3C3 fraction of HIV-2 gp125 was generated by homology modelling. To assess the importance of these structural features and compare with our experimental results, wild type HIV-2_{ROD} gp125 V3 loop model was generated besides, six other models, incorporating the previously described modifications (mutations and/or deletions). After a careful search and selection process three crystallographic structures were identified as proper and promising templates based on the sequence identity with the target: two HIV-1 envelope glycoprotein gp160 with the V3 core complexed with CD4+ (PDBID: 2QAD and PDBID: 2B4C) [38,39], and the recently resolved crystallographic data (2015) of HIV-2_{ST} gp125 envelope glycoprotein complexed with CD4 (PDBID: 5CAY) (Table 1) [37].

Table 1. V3 sequences in the three wild type virus templates used to generate the models, as well as the V3 sequence in HIV-2_{ROD} obtained from Uniprot (P04577).

Virus	V3 sequence
HIV-1 (2QAD)	CTRPNNNTRKSINIQRGPGRALYTTGEIIGDIRQAHC
HIV-1 (2B4C)	CTRPNQNTRKSIHIQRGPGRAFYTTGEIIGDIRQAHC
HIV-2_{ST}	CKRPGNKTVVPITLMSGLVFHSQPINRRPRQAWC
HIV-2_{Rod}	CKRPGNKTVKQIMLMSGHVFHSHYQPINKRPRQAWC

Preliminary models construction of the C2V3C3 region of the HIV-2_{rod} sequence was generated using MOE program. The models with a minor of outliers were primarily selected for subsequent treatment. A set of several intermediate models were generated, energy optimized and refined using Amber99 forcefield resulting in the corresponding three final homology models (Figure HM1). All these models took into account the spatial restrictions imposed by the presence of a disulphide bridge between Cysteine at position 1 and Cysteine at position 36 of V3.

The models quality was analyzed and satisfies the general criteria commonly used for assessing the quality of local geometry of protein structures. MOE program were used to analyse structures and confirm that the V3 are mainly localized in energetically favourable regions of the Ramachandran plot as well as their dihedral $\chi 1$ angles (in line with the information appearing in the literature).

The best models based on HIV-1 templates 2B4C (Figure HM1, a) and 2QAD (Figure HM1, b), obtained after correction of a deletion of two residues on position 15 and 16 of V3 in the crystallographic structure, were selected and subjected to energy minimization using MOE software. In addition, another model was generated based on HIV-2 gp125 envelope glycoprotein crystallographic structure (5CAY) using the same procedure (Figure HM1, c).

After the correction of 5CAY structure with the correspondent portion of the 2B4C minimized model (five residues from the N-terminus were conserved as the seven residues from C-terminus on the V3 loop on the original 5CAY structure) and the missing portion of residues was replaced by our previously model. Several models were generated and a new homology model of C2V3C3 portion of gp125 from an HIV-2 as template sharing 88 % of identity.

Figure HM1 shows each of the 3D structures of the V3 loops from HIV-2_{ROD} gp125 V3 loop generated by homology modelling. as well as an overlay of all these models (Figure HM1, d).

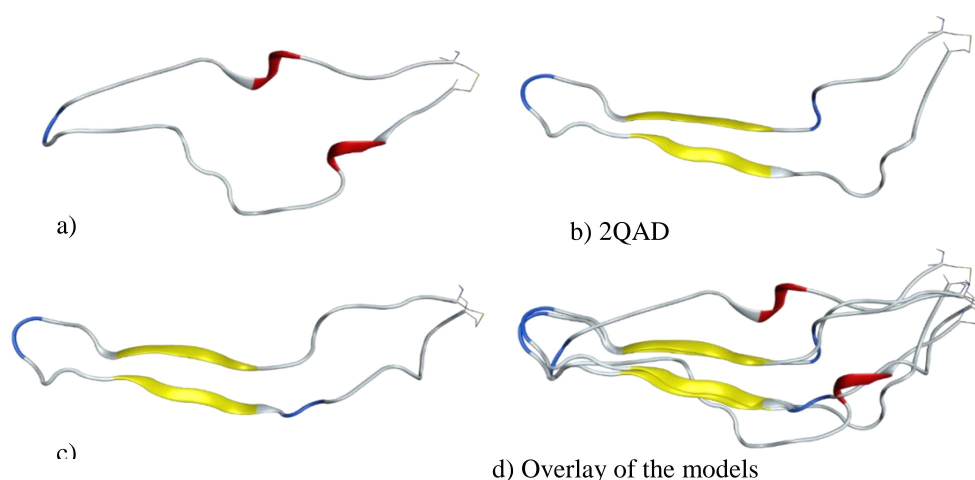

Figure HM1. HIV-2 gp125 V3 loop model based on a) 2B4C.PDB template; b) 2QAD.PDB template; c) 5CAY.PDB template; D) overlay of the three models.

The three models of V3 loop structures indicate that there are significant differences between them (supported by the values of root-mean-square deviation (RMSD) in the range 2.264 to 5.160 Å).

Figure HM2 provides an overview of the mutation and/or deletion made in the HIV-2_{ROD} gp125 V3 loop by showing the respective residues in sticks.

This new model was used as the template for a set of six new models (Figure HM2) with mutations and/or deletions on V3 loop amino acid sequence (H18L, H23Δ + Y24Δ, K29T, H18L+ H23Δ + Y24Δ, H18L+ K29T and H18L+ H23Δ + Y24Δ+ K29T).

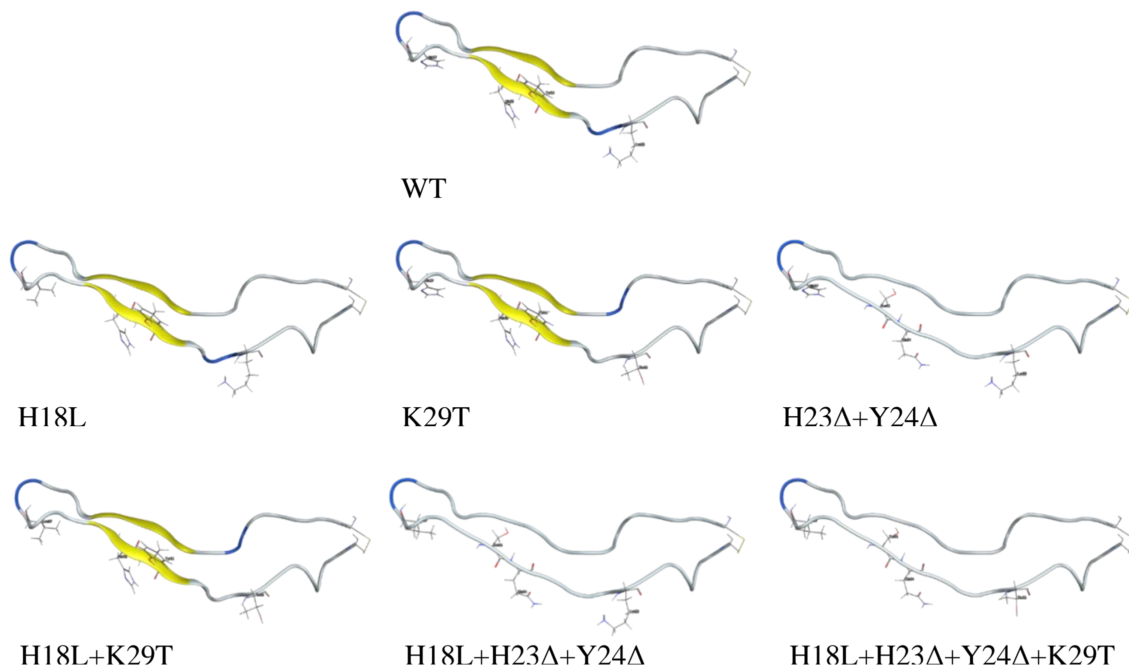

Figure HM2. 3D structure of model 5CAY and the correspondent variants.

In our homology model, the structure of V3 loop wild type is characterized by 23 % β sheet and a null percentage of α helix. In the case of H23Δ+Y24Δ, H18L+H23Δ+Y24Δ and H18L+H23Δ+Y24Δ+K29T variants this percentage drops to zero in both values. Based on the C2C3V3 sequence of HIV-2_{ROD} a structural prevision was made on PSIPRED server and Robetta server.

The H18L variant replaces a basic residue by an hydrophobic residue. This interchange leads to the loss of an aromatic moiety and abrogate any possibility to the establishment of π-π interactions in this position with the V3 environment. In the V3 wild-type, H18 forms H-bonds with M at position 15 and F at position 20. These interactions could be significant to interactions with the co-receptor usage.

The binding of the glycoprotein with CD4 leads to conformational changes and determines the co-receptor specify. The aromatic system at position 18 on V3 can influence this specificity [42]. The substitution of a lysine, a charged residue, at position 29 by a threonine, a polar uncharged residue, reduced the charge of V3 and lead to the loss of the interactions with Isoleucine at position 27. Beside this, no other significant deviations were identified. This suggests a similar behavior compared with the wild type.

H23 and Y24 fit on the β sheets present in V3 wild type. These two residues establish important interactions, H23 interacts with glycine at position 25 and at position 11, moreover tyrosine at positon 24 is a residue that could not only establish π-π interactions but also the exposition of its hydroxyl group could promote other interactions with the environment. This deletion results in a loss of the aromatic system formed by these two residues and leads to the elimination of the parallel β sheets presented in the V3 loop. Loss of this aromatic system could interfere with the hydrophobic equilibrium. The receptor binding is highly sensitive to modifications on the aromatic system [42]. If modifications were made in V3 the binding could be different and specificity to the co-receptor could be modificated. However, our model shows that the deletions of these two important residues H23 and Y24 (H23Δ+Y24Δ) lead to the formation of new set of interaction between Serine at position 22 and Glutamine at position 11 and Lysine at positon 10 and also between Glycine at position 25 and Proline at position 26.

When the double H18L and K29T mutations are present, the V3 loop maintains the same pattern observed in the presence of the single mutations.

In H18L+H23Δ+Y24Δ and H18L+H23Δ+Y24Δ+K29T variants, a loss of β sheet structure was also observed. We could not find other significant differences in the V3 loop.

Calculations of the accessible solvent area (ASA) show mostly a decrease of the accessible solvent area when compared with the wild type unless for the variant H18L (Table HM1). The charge of the V3 system was in agreement with our experimental results. No significant deviations were observed on the length of the V3 loop with the performed mutations, however, a substantial increase on the width of the V3 loop was observed in particular for the structures containing the H23 and Y24 deletions.

Table HM1. Calculations of the accessible solvent area (ASA).

V3	**RMSD**	**ASA Å^2**	**Δ(ASA)**	**Charge**	**Length**	**Width**
WT	0	3852.3	0	+7	47.50	5.30
H18L	0.096	3853.1	0.8	+7	47.67	5.34
k29T	0.144	3834.4	-17.9	+6	47.67	5.34
ΔH23Y24	2.812	3790.8	-61.5	+7	47.67	6.17
H18L_K29T	0.150	3821.9	-30.4	+6	47.68	5.34
H18L_ΔH23Y24	1.107	3778.5	-73.8	+7	47.50	6.13
H18L_K29T_ΔH23Y24	1.128	3760.5	-91.8	+6	47.50	6.13

Discussion

The first aim of this study was to evaluate the phenotypic effects of some of these molecular determinants in HIV-2 coreceptor usage. Site-directed mutagenesis was used to create six variants with single and multiple amino acid substitutions at positions 18, 23, 24 and 29 (number refers to V3 of HIV-2_{ROD}) and six homology models were generated.
We found that H to L substitution at position 18 associated with the deletion of amino acids H and Y at positions 23 and 24 in V3 domain, respectively, are sufficient to confer CCR5 usage in HIV-2_{ROD}. This is in agreement with the findings of Isaka et al.[3] who showed the role of HIV-2 V3 region in coreceptor use, as exchange of the C terminal half (18aa-36 aa) of the V3 loop between the HIV-2 strains ROD and GH-1 changed the coreceptor use reciprocally. However, this study did not determine the exact positions in V3 that influenced coreceptor usage between the chimeric viruses.
Our findings are also in line with Visseaux et al. [2] that demonstrated an association between any substitution at residue 18 with CXCR4 tropism based on V3 sequences from HIV-2 isolates. Other studies claimed an association between V3 and coreceptor usage in HIV-2, however genetic signatures were only found in the N terminal of V3 (1aa-17aa) [25, 26].
In contrast, studies performed by Kulkarmi et al.[28], Owen et al.[27] and Santos-Costa et al.[29] did not find any genetic association for this or other amino acids positions in V3 of HIV-2.

Those studies were performed using different methods of phenotypic tropism assays (mainly based in GHOST cell lines), with a restricted number of HIV-2 isolates and few X4 viruses.
Structurally, H18L leads to a loss of an aromatic moiety, very important for the establishment of π-π interactions, which could induce a structural preference by a specific coreceptor. Modifications on the aromatic residues at position 18 and at positions 23 and 24 of V3 suggest an important feature to determine co-receptor usage. It was seen that modifications at these positions in different variants originate an increment in the CCR5 usage and if these alterations occur in the same variant there was specificity to co-receptor CCR5, therefore suggesting that the presence of aromatic systems increment CXCR4 usage.

The histidine and tyrosine at positions 23 and 24 of HIV-2_{ROD} are apparently unusual in V3 sequences from HIV-2 isolates or reference strains [2, 3, 28]. The deletion of these two residues resulted in a slight increase in CCR5 usage and in a decrease of more than 50% in the ability to use CXCR4 comparing with the wild type. These insertions may promote efficient entry with CXCR4 coreceptor in HIV-2_{ROD}. Histidine at position 23 was present in two parallel hydrogen bridges with Q11 which could be essential to promote the spatial arrangement of the loop and maintained both of β sheets. However, in variants with these two residues deleted, both sides of the loop loss β sheet definition. Variant H23Δ+Y24Δ presented biggest variation of width of the V3 loop and accessible surface area. These alterations reinforce the hypothesis of both of interactions between H23 and Q11 are important to position residues that could promote interaction with the receptor or lead to alteration of its usage. Results showed a major distance of the hydrogen bond between Histine at position 18 and Phenylalanine at position 20 on variant H23Δ+Y24Δ. This fact promotes the importance of the position 18 and interactions made in the neighbour residues and its role on coreceptor usage.

A global net charge of V3 superior to 6, defined by the presence of positively charged amino acids (K, lysine; R, arginine) mostly at residues 19 or 27, has been associated to the ability of HIV-2 to use CXCR4 coreceptor [1-3].

However, this association was not found in our study as a substitution of a lysine to a threonine at position 29 (number refers to ROD), that decrease the net charge of HIV-2_{ROD} variants from 7 to 6, had no effect on coreceptor usage comparing to wild type. Structurally, no significant deviations were observed.

The lack of association between V3 charge and tropism for CXCR4 in this study is in line with previous study by Kulkarmi et al.[28].

Therefore in our study we identified three critical V3 determinants of CCR5 coreceptor use in HIV-2_{ROD}: mutation H to L at residue 18 and deletions of H and Y amino acids at positions 23 and 24. In contrast, we found no association between substitution T at residue 29 of HIV2_{ROD} in coreceptor usage. Moreover, our study also demonstrated the lack of association between decreasing global net charge of V3 from 7 to 6 and CCR5 usage.

We found that HIV-2_{ROD} and the K29T mutant are resistant to neutralization by plasma from HIV-2 infected individuals which is consistent with their X4 phenotype. In contrast, H18L and H23Δ + Y24Δ variants, both with dual tropism for CCR5 and CXCR4 were easier to neutralize, indicating a possible role of these three amino acid positions in HIV-2_{ROD} susceptibility to Nabs. It is of note that the variant with the both deletions exhibited a decrease in the V3 length and in this case both length and coreceptor usage may have contributed to the response to Nabs. Thus, the sensitivity of HIV-2_{ROD} to antibody neutralization was increased with the ability to use CCR5. However, the same behaviour did not occur with R5 only tropic variants (H18L+ H23Δ+Y24Δ and H18L+ H23Δ+Y24Δ+ K29T) at it would be expected despite the fact that these variants also presented shorter V3 loops. Therefore, these results are partially in agreement with the findings of previous studies [6, 31, 32], although we did not find any potential relationship between the charge and size of V3 of our variants and susceptibility to antibody neutralization as it was demonstrated by Marcelino et al [32] and Rocha et al. [31].

The sensitivity of wild type virus and V3 variants was also analyzed against a panel of anti-HIV-2 Env human monoclonal antibodies (Mabs) targeting V3 (6.10F and 1.4B), V4 (1.7A), CD4 binding site (6.10B) and CD4 induced (1.4H).

Our results demonstrated that in general all Mabs failed to efficiently neutralize wild type viruses and V3 variants, with the exeption of CD4-specific antibodies, 6.10B and 1.4H. These findings are in concordance with a previous study by de Silva et al.[43] that also revealed that HIV-$2_{ROD\ A}$ was resistance to all these anti-HIV-2 Env Mabs.

We showed that V3 substitutions did not alter the susceptibility of HIV-2_{ROD} to Mabs targeting this region, 6.10F and 1.4B. Curiously, some V3 mutations tested in our study were located within the potential epitopes recognized by these antibodies [44]. The Mab 6.10F recognizes a sequence 319-LMSGLVF-325 (positions according to HIV-2_{7312A} and positions 315-321 of HIV-2_{ROD}) in which the residues L319, G322, L323, and F325 are essential for binding [44]. Although, H18L mutation lies in this region (corresponding to L323 position), we could not find any different patterns of neutralization of this variant comparing with wild type in the presence of 6.10F.

Similarly, it was demonstrated that 1.4B probably targets a V3 peptide in the same region, 319- LMSGLVFHSQPINKR-333 (positions according to HIV-2_{7312A} and positions 315-321 of HIV-2_{ROD}). Residues G322, V324, N331, and R333 were indicated to be crucial for 1.4B neutralization [44]. Even though, mutation H18L and deletions at positions 23 and 24 are located within this epitope, 1.4B failed to neutralize all the mutants and wild-type strain. It was expected that the removal of amino acids at positions 23 and 24 would confer more sensitivity to neutralization by this Mab, as the sequence would be similar to the original epitope.

In contrast, anti-CD4 6.10B and 1.4H showed some efficient neutralization on K29T and H18L+ H23Δ + Y24Δ variants.

The Mab 1.4H is defined as a CD4-induced antibody. Apparently, its epitope is exposed or become more accessible to 1.4H antibody following interactions between Env and CD4 receptor, suggesting that coreceptor binding site may be a possible target. Although the sequence is still unknown, and there is no evidence for V3 or V4 to act as epitope [44], our results demonstrated that two V3 variants, K29T and H18L+ H23Δ + Y24Δ, showed a neutralization-sensitive phenotype in the presence of 1.4H, indicating that the epitope may be located in V3 region.

The Mab 6.10B was isolated from the same subject as 1.4H and recognizes an unidentified epitope that overlaps the CD4 binding site [44]. K29T, H23Δ + Y24Δ and H18L+ H23Δ + Y24Δ were neutralized by 6.10B, suggesting that the location of the epitope is also in V3, near the target of 1.4H.

These results suggested that V3 region is the potential target for the CD4-antibodies 1.4H and 6.10B, indicating the proximity between CD4 receptor and coreceptor binding site. Moreover, those interactions may be dependent on the conformation of V3 [44, 45].
H18L and ROD X4 variants replicated only in $CD4^+$ T cells whereas variants that also used CCR5 replicated also in macrophages. Moreover, K29T variant gained the ability to replicate in macrophages. The tropism plasticity of this variant along with its high resistance to antibody neutralization can be a major advantage for transmission and persistence. On the other hand, H23Δ + Y24Δ and H18L + H23Δ + Y24Δ variants replicated in macrophages with a similar capacity than in $CD4^+$ T cells. Taken together, we did not find a correlation between coreceptor usage and replication capacity in these types of cells. Probably biological determinants other than viral coreceptor may be involved in cellular tropism.
We showed that higher levels of replication occurred earlier in macrophages (3 days post infection) than in $CD4^+$ T cells (6 days post infection). This is in agreement with Marchant et al.[46] who demonstrated that infection in macrophages by HIV-2 primary isolates occurred with lower replication levels (compared to HIV-1) and with a transient peak of virus production 2 days postinfection, followed by an apparent state of latency.

Conclusions

This is the first molecular study on the role of the V3 region on HIV-2 (1) coreceptor use, (2) replication in CD4+ T cells and macrophages, and (3) susceptibility to antibody neutralization.
Our data shows the H18L mutation is sufficient for a switch from X4 to R5 tropism and loss of X4 tropism in the context of the short version of V3. In addition, we also demonstrated that K29T substitution allows moderate to high levels of replication in CD4+ T cells and macrophages and confers resistance to antibody-mediated neutralization. This substitution at position 29 in the context of HIV-2_{ROD} background may impact in the transmission and progression of disease.
Our results also show that Nab resistance is an intrinsic feature of CXCR4- tropic HIV-2 isolates and that is acquired over the course of infection in close association with sequence changes in the envelope V3 loop that also favour R5-to-X4 switch.

These results have important implications for vaccine development since a potential vaccine targeting only the transmitted R5 strains will likely not be effective on X4 isolates and might determine early R5 to X4 tropism switch in infected individuals.
Further phenotypic studies are needed with additional mutations in V3 loop and other regions of Env to assess the impact of these determinants in HIV-2 CCR5 and CXCR4 usage and susceptibility to antibody neutralization.

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CHAPTER IV

A genotypic method for determining HIV-2 coreceptor usage enables epidemiological studies and clinical decision support

A genotypic method for determining HIV-2 coreceptor usage enables epidemiological studies and clinical decision support

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Abstract

CCR5-coreceptor antagonists can be used for treating HIV-2 infected individuals. Before initiating treatment with coreceptor antagonists, viral coreceptor usage should be determined to ensure that the virus can use only the CCR5 coreceptor (R5) and cannot evade the drug by using the CXCR4 coreceptor (X4-capable). However, until now, no online tool for the genotypic identification of HIV-2 coreceptor usage had been available. Furthermore, there is a lack of knowledge on the determinants of HIV-2 coreceptor usage. Therefore, we developed a data-driven web service for the prediction of HIV-2 coreceptor usage from the V3 loop of the HIV-2 glycoprotein and used the tool to identify novel discriminatory features of X4-capable variants.

Results: Using 10 runs of tenfold cross validation, we selected a linear support vector machine (SVM) as the model for geno2pheno[coreceptor-hiv2], because it outperformed the other SVMs with an area under the ROC curve (AUC) of 0.95. We found that SVMs were highly accurate in identifying HIV-2 coreceptor usage, attaining sensitivities of 73.5% and specificities of 96% during tenfold nested cross validation. The predictive performance of SVMs was not significantly different (p value 0.37) from an existing rules-based approach. Moreover, geno2pheno[coreceptor-hiv2] achieved a predictive accuracy of 100% and outperformed the existing approach on an independent data set containing nine new isolates with corresponding phenotypic measurements of coreceptor usage. geno2pheno[coreceptor-hiv2] could not only reproduce the established markers of CXCR4-usage, but also revealed novel markers: the substitutions 27K, 15G, and 8S were significantly predictive of CXCR4 usage. Furthermore, SVMs trained on the amino-acid sequences of the V1 and V2 loops were also quite accurate in predicting coreceptor usage (AUCs of 0.84 and 0.65, respectively)

Conclusions: In this study, we developed geno2pheno[coreceptor-hiv2], the first online tool for the prediction of HIV-2 coreceptor usage from the V3 loop. Using our method, we identified novel amino-acid markers of X4-capable variants in the V3 loop and found that HIV-2 coreceptor usage is also influenced by the V1/V2 region. The tool can aid clinicians in deciding whether coreceptor antagonists such as maraviroc are a treatment option and enables epidemiological studies investigating HIV-2 coreceptor usage. geno2pheno[coreceptor-hiv2] is available at http://coreceptor-hiv2.geno2pheno.org.

Background

Human immunodeficiency virus type 2 (HIV-2) is prevalent in Western Africa and specific European countries such as France and Portugal [1]. In comparison to HIV-1, HIV-2 exhibits a reduced infectivity [2], a lower replicative capacity [3], and an increased susceptibility to antibody-mediated neutralization [4]. During the course of HIV-2 infection, CD4 declines slowly and the clinically latent phase can last for decades [5]. Still, infection with HIV-2 can lead to acquired immune deficiency syndrome (AIDS) [6] and effective antiretroviral treatments are crucial for preventing disease progression.

Possible treatments for individuals infected with HIV-2 are limited because many antiretrovirals are less effective inhibitors of HIV-2 than of HIV-1 [7–9]. HIV-2 is intrinsically resistant to non-nucleoside reverse transcriptase inhibitors [10, 11] and to the fusion inhibitor enfuvirtide [7, 12]. Additionally, from the class of protease inhibitors, only saquinavir, lopinavir, and daruinavir are effective against HIV-2 [9]. Selecting an appropriate treatment regimen can be further exacerbated by the rapid development of HIV-2 drug resistance [9, 13, 14]. Maraviroc, a CCR5 coreceptor antagonist, poses a new treatment option for individuals infected with HIV-2 [15–18]. The drug prevents viral cell entry by obstructing the CCR5 coreceptor and should be administered only to patients infected with an R5-tropic virus to ensure treatment efficacy and to prevent a switch to viral usage of the CXCR4 coreceptor. Therefore, determining viral coreceptor usage is crucial before initiating treatment with coreceptor antagonists such as maraviroc [16]. Moreover, the identification of HIV-2 coreceptor usage can be useful for staging disease progression: CXCR4-using viruses, which are less susceptible to antibody neutralization than R5-tropic strains [19], are associated with low CD4+ T cell counts and progressed disease [20, 21].

Although some HIV-2 strains have been shown to infect cells without use of the CD4 receptor in vitro [1, 22, 23], HIV-2 enters cells *in vivo* by first binding to the CD4 receptor and then interacting with a coreceptor belonging to the family of chemokine receptors [24]. Similarly to HIV-1, CCR5 and CXCR4 are the major coreceptors that are used by HIV-2 *in vivo* [25, 26]. The variable loop 3 (V3) of the viral surface glycoprotein (known as gp125 or gp105) is crucial for coreceptor binding. Specific substitutions in the V3 loop are particularly indicative of X4-capability [27–30] and often bring forth an increased V3 net charge [21, 29, 31, 32].

Three viral variants can be delineated according to the coreceptor that is used during cell entry. R5-tropic viruses can use only the CCR5 coreceptor, X4-tropic viruses can use the CXCR4 coreceptor, and dual-tropic viruses can use both CCR5 and CXCR4. Patients harboring R5- and X4-tropic viruses simultaneously have mixed infections. Since mixed infections usually cannot be distinguished from infections with dual-tropic variants, the term dual/mixed (D/M) is used to denote patients with a dual infection or a dual-tropic virus. To simplify the terminology, we define a virus/viral population as R5 if it can use only CCR5, while X4-capable defines a virus/viral population that can use CXCR4 (possibly in addition to other coreceptors).

Viral coreceptor usage can be determined either phenotypically or genotypically. Phenotypic approaches often use engineered cell lines expressing only certain coreceptors on their surface such that they elicit a specific signal upon viral infection. For example, TZM-bl cells [33, 34] express firefly luciferase enzyme under the control of the HIV-1 promoter. Since TZM-bl cells express CD4, CCR5, and CXCR4, coreceptor usage can be measured by blocking one and/or both coreceptors with excessive amounts of coreceptor antagonists and evaluating the resulting luminescence [16, 35].

While phenotypic assays are accurate and engineered cell lines enable the detection of a broad range of coreceptor usage patterns, such assays are expensive, time-consuming, and their interpretation can be challenging. For example, when evaluating the results from an assay based on TZM-bl cells, the residual viral replication in the presence of the applied coreceptor antagonists needs to be interpreted. Moreover, TZM-bl cell based assays using different coreceptor antagonists (e.g. maraviroc and TAK-779 for CCR5) might not yield exactly the same results for the same isolate. Additionally, phenotypically determined coreceptor usage might not accord with *in vivo* coreceptor usage, because engineered cell lines exhibit larger surface densities of CD4 and HIV coreceptors than primary cells. Hence, a virus that cannot use a given coreceptor *in vivo* may be falsely reported to use that coreceptor if cell entry is enabled by the increased avidity of the interactions between virus and engineered cell. In contrast to HIV-1, where the enhanced sensitivity Trofile assay provides a standardized means for identifying coreceptor usage [36], there exists no standardized phenotypic assay for HIV-2. Instead, different phenotypic approaches are in use, which may lead to inconsistent results.

Genotypic methods, on the other hand, are not performed in a laboratory, but are based on detecting discriminatory features in the viral genome.

These approaches usually agree well with phenotypic tests [37], save time, and are much less expensive than phenotypic assays. The first genotypic approach for the identification of HIV-2 coreceptor usage was put forth by Visseaux et al. [28]. Their study identified nine markers in the V3 loop exhibiting significant associations with coreceptor usage. Four of these markers with sensitivities greater than 70% and specificities of 100% were selected to form the major genotypic determinants of X4-capable variants: the substitutions L18X (where X is any non-L amino acid) and V19K/R, any insertion after position 24, and a V3 net charge exceeding six. The other five substitutions (S22A/F/Y, Q23R, I25L/Y, R28K, and R30K) with significant associations were termed minor markers. Their rules-based system classifies an HIV-2 strain as X4-capable if its V3 amino-acid sequence contains at least one of the four major markers and otherwise as R5. Applying this approach to an independent data set yielded a sensitivity of 65% and a specificity of 100% for detecting X4-capable variants.

Our study had two goals. First, we wanted to provide a data-driven, genotypic tool for predicting whether an HIV-2 V3 amino-acid sequence originates from an R5 or an X4-capable variant. More specifically, we strove to improve on the rules-based approach to coreceptor identification introduced by Visseaux et al. [28]. Second, we wanted to investigate which V3 amino-acid mutations confer the X4-capable phenotype and determine whether amino-acid features in the V1/V2 region are also predictive of coreceptor usage.

We demonstrate that viral coreceptor usage can be accurately predicted from specific amino-acid substitutions in the HIV-2 V3 loop and provide geno2pheno[coreceptor-hiv2], a web service for HIV-2 coreceptor prediction. We were not only able to confirm previously established markers of X4-capability, but also found previously unreported V3 substitutions predictive of X4-capable viruses. Additionally, we found evidence indicating that the V1/V2 region also modulates HIV-2 coreceptor usage.

Results

To generate statistical models capable of predicting HIV-2 coreceptor usage, we gathered a data set of 126 pairs of HIV-2 genomic amino-acid sequences and phenotypic coreceptor usage annotations (either R5 or X4-capable). Based on this data set, we trained and validated support vector machines (SVMs) with various kernel functions on the amino-acid sequences of either the V1, V2, V3, or all three regions and the corresponding coreceptor usage annotations to identify the most predictive models according to their areas under the ROC curve (AUCs). Due to its high predictive accuracy, we decided to use a linear SVM based on the V3 amino-acid sequence for all further analyses. Next, we validated an existing rules-based approach for HIV-2 coreceptor identification [28] and compared the predictive accuracy of this approach with the accuracy of SVMs.

To identify which substitutions in the V3 amino-acid sequence impart the X4-capable phenotype according to the linear SVM, we investigated the model weights and statistically tested the discriminatory strength of individual substitutions in the V3 loop.

Last, we implemented the linear SVM as a web service, for which we transformed predicted X4-probabilities to false positive rates (FPRs), selected a suitable FPR threshold, and created a visualization representing the model weights associated with an input sequence. To validate the implementation of the web service, we evaluated the predictive accuracy of geno2pheno[coreceptor-hiv2] on an independent set of nine new HIV-2 isolates with phenotypically determined coreceptor usage, which were not previously used for training the model.

Model selection and validation of SVMs

To predict HIV-2 coreceptor usage, we trained SVMs on data involving several regions of the HIV-2 genome. We decided to train SVMs on the V1, V2, and V3 loops as those regions are known to impact HIV-2 coreceptor usage most [27–30, 38]. We also trained an SVM on a combination of all three variable regions. To estimate the predictive performance of SVMs on unseen data, we performed 10 runs of tenfold cross validation (CV) on the complete data set of 126 samples. Having partitioned the data set into 10 disjoint folds, the i-th ($i \in \{1, 2, \ldots, 10\}$) round of CV entails training a model using the samples contained in all folds except for the i-th fold and then validating the model on the i-th fold. Linear models based on the V1 and V2 loops (N = 62) achieved AUCs of 0.84 and 0.65, respectively.

SVMs trained on V3 amino-acid sequences (N = 126) achieved similarly high accuracies for all kernel functions considered with the exception of the SVMs based on the edit kernel, which had distinctly smaller AUCs (see Table 1). The best-performing SVM that was trained on the V3 loop outperformed the models based on the V1/V2 regions (AUC of 0.95).

Table 1. Classifier AUCs per run of cross validation.

CV Run	RBF (σ = 0.001)	Linear	Polynomial (degree = 2)	Edit Kernel (γ = 0.005, PAM70)
1	0.9475	0.9459	0.941	0.8629
2	0.9509	0.9506	0.9452	0.851
3	0.9504	0.9579	0.9444	0.8655
4	0.9449	0.947	0.9379	0.8634
5	0.9472	0.9467	0.9413	0.8744
6	0.9467	0.9467	0.9457	0.8689
7	0.9532	0.9535	0.9475	0.8377
8	0.9522	0.9532	0.9306	0.8623
9	0.9524	0.9524	0.9478	0.9012
10	0.9441	0.9431	0.9384	0.8672
M	0.949	0.9497	0.942	0.8654
Σ	0.0033	0.0045	0.0053	0.0162

The column names indicate the kernel function corresponding to each SVM and kernel parameters are indicated in brackets. Only the results for the best-performing kernel function (in terms of average AUC across all CV runs) for each set of evaluated parameters are shown. All of the classifiers performed best with a setting of $\nu = 0.3$.

We also evaluated the performance of SVMs trained on 62 samples using the amino-acid sequences of all three variable regions V1/V2/V3 and found that the best model performed worse (AUC of 0.89) than that based on the V3 loop alone. Due to the reduced predictive accuracy of models incorporating information from the V1/V2 region, we decided to use the linear ν-SVM trained on 126 V3 amino-acid sequences with the model parameter $\nu = 0.3$ (AUC of 0.95) for geno2pheno[coreceptor-hiv2]. We refer to this SVM as *the linear SVM* in the following.

To identify the predictive performance of SVMs trained on V3 amino-acid sequences under consideration of model selection bias, we also determined their tenfold nested CV performance. In the 10 inner runs, SVMs using a linear kernel were chosen seven times and SVMs using an RBF kernel were chosen three times using their AUCs as a selection criterion. The AUC of tenfold nested CV was 0.88 (sensitivity of 76.9% and specificity of 97.3%).

Evaluation of the rules-based approach for HIV-2 coreceptor identification

To evaluate the rules-based approach from Visseaux et al. [28] for identifying HIV-2 coreceptor usage, we determined the predictive accuracy of their approach on a subset of the complete data set called the *test data set*. The test data set was constructed to contain only those V3 sequences that had not been used for the identification of the predictive rules used in their approach. We evaluated the rules-based approach from Visseaux et al. [28] for different numbers of required major markers of X4-capability (either 1, 2, 3, or 4) on the test data set (N = 84) and found that the balanced accuracy of prediction decreased with increasing numbers of required major markers (balanced accuracies 0.89, 0.88, 0.85, and 0.81, respectively). Hence, our evaluations confirm that requiring one major marker for X4-capability is the most accurate rules-based strategy, but the presence of additional markers can corroborate a prediction (Additional file 1: Table S1).
To determine the predictive performance of individual markers of X4-capability, we applied a two-sided Fisher's exact test on the confusion matrices resulting from applying individual rules (Additional file 1: Table S2).

After correcting for multiple hypothesis testing using the Benjamini–Hochberg procedure [39] at a false discovery rate of 5%, we found that among the established discriminatory features only the substitutions R30K and I25L/Y were not significant predictors of X4-capability on the test data set at the 5% level.

Comparison of SVMs with the rules-based approach

To compare the predictive performance of SVMs and the rules based approach [28], we validated both approaches on the test data set (N = 84). The rules-based method from Visseaux et al. requiring just a single major rule to predict X4-capability [28] achieved a sensitivity of 85.3% and a specificity of 94% (balanced accuracy 89.6%). In comparison, tenfold nested CV of SVMs performed on the test data set resulted in a sensitivity of 73.5% and a specificity of 96% (balanced accuracy 84.7%), which is not significantly different (*p* value 0.37) to the rules-based predictions according to McNemar's test [40].

Discriminatory features in the V3 loop

To analyze discriminatory features in the V3 loop, we created a profile alignment of the V3 amino-acid sequences in the test data set and enumerated the positions in the V3 loop according to the HIV-2 reference strain M33262 [41–43]. Many sequences from X4-capable viruses exhibited more than one major marker for X4-capability. Of the 34 X4-capable sequences in the test data set, only 5 (14.7%) samples did not have any marker, 2 (5.9%) had a single marker, 2 (5.9%) had two markers, 4 (11.8%) had three markers, and 21 (61.8%) had four markers. Interestingly, the five X4-capable sequences without any markers for CXCR4 usage (accession numbers/isolate identifiers DQ213035 [27], GU204944 [32], consensus V3 loop from clones JX219591-JX219598, GB87 [31], 310248 [31]) could neither be identified as X4-capable by the rules-based method nor by geno2pheno[coreceptor-hiv2].

We investigated how well the linear SVM used for geno2pheno[coreceptor-hiv2] reproduces the nine previously described markers for X4-capability [28].

To this end, we visualized the predicted X4-probabilities of the linear SVM for sequences exhibiting these established discriminatory features (Fig. 1) and evaluated the SVM features contributing 75% of the total model weights (Table 2).

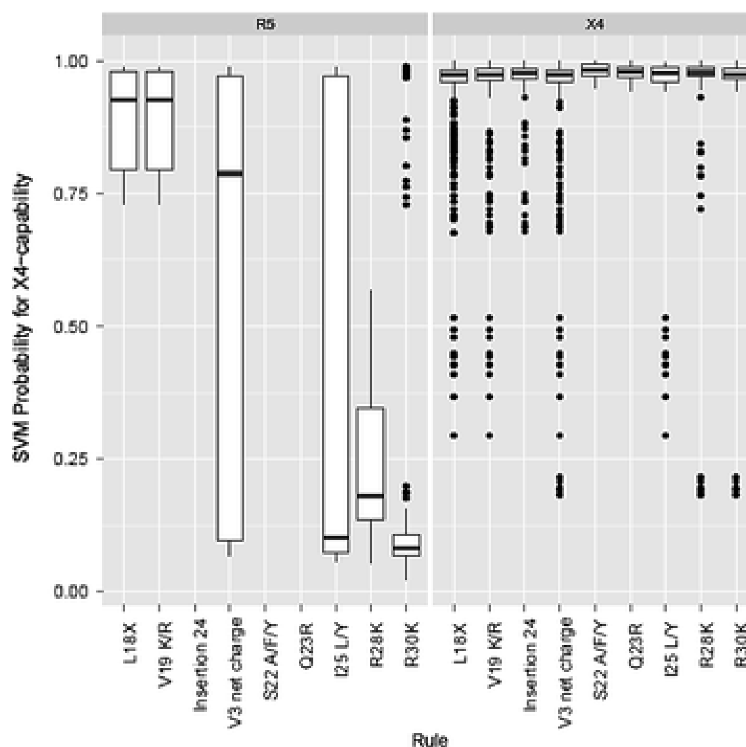

Figure 1. X4-probabilities predicted by geno2pheno[coreceptor-hiv2] for V3 amino-acid sequences exhibiting the established discriminatory features indicative of X4-capability listed on the x-axis. The *left-hand panel* shows the predicted X4-probabilities for sequences labeled as R5, while the *right-hand panel* shows the predicted X4-probabilities for sequences labeled as X4-capable. The *bottom line* of a *box* indicates the 1st quartile (Q1) of predicted X4-probabilities, the *bar* inside the *box* indicates the median, and the *top line* indicates the 3rd quartile (Q3). The *whiskers* extending from a *box* indicate predicted X4-probabilities that lie within 1.5× IQR (interquartile range, IQR = Q3 − Q1). Outlier values that are not within the *whisker* region are shown as *dots*. Note that some of the sequence characteristics indicated on the x-axis do not have a predicted X4-probability, because no sequences exhibiting the corresponding feature and phenotype were available.

Table 2. Features in the model with the strongest impact on predicted viral coreceptor usage.

Position	R5 feature	X4 feature	R5 weights	X4 weights
18	L	H, Q, F, M	0.69	−0.23, −0.15, −0.12, −0.1
Insertion after position 24	–	I, V	0.45	−0.22, −0.21
19	I	R, K, V	0.19	−0.25, −0.23, −0.19
Insertion after position 22	–	H, Y	0.36	−0.18, −0.18
24	P	*NA*	0.17	*NA*
23	Q	R	0.14	−0.14
27	Q	K	0.09	−0.12
13	T	R	0.11	−0.07
26	*NA*	N	*NA*	−0.09
10	A	K	0.09	−0.07
14	I	L	0.08	−0.08
22	S	*NA*	0.08	*NA*
15	A	G	0.08	−0.07
8	K	S	0.07	−0.07

Positions of discriminatory features that were not described previously are shown in bold italics.

We found that the SVM predicted high X4-probabilities for sequences from X4-capable viruses exhibiting established X4-markers, which indicates that the SVM captures the established features of X4-capable variants well. However, because some R5 sequences also exhibit markers of X4-capability (particularly L18X, V19K/R, or a V3 net charge >6), these isolates were falsely predicted to use CXCR4 with a high probability.

By analyzing the SVM model coefficients, we identified novel, discriminatory features associated with X4-capability. The substitutions 27K, 15G, and 8S were significantly predictive of X4-capability according to Fisher's exact test at the 5% level after multiple hypothesis testing correction with the Benjamini–Hochberg procedure (Table 2).

Predicted X4-probabilities and false positive rates

The distribution of predicted X4-probabilities resulting from applying the linear SVM on the complete data set (N = 126) using 10 runs of tenfold CV shows that V3 loops from R5- and X4-capable viruses are, for the most part, well separable (Additional file 1: Figure S1). The region of low X4-probabilities is interspersed with samples from X4-capable viruses, which indicates that the SVM falsely identifies X4-capable viruses as R5 in some cases.

To find an FPR cutoff producing a satisfactory separation of the predicted X4-probabilities from samples labeled as *R5* and *X4-capable*, we performed k-means clustering on the X4-probabilities after we had found $k = 2$ by applying the elbow test on the within sum of squares error [44]. From the cluster representing X4-capable viruses, we then selected the minimal predicted probability for X4-capability (53.4%) and determined the corresponding FPR (3.4%). For better memorability, we decided to set the recommended cutoff for HIV-2 coreceptor prediction to an FPR of 5%, which increases the number of false alerts only slightly (Additional file 1: Figure S2).

The geno2pheno[coreceptor-hiv2] web service

We implemented our predictive approach for the identification of HIV-2 coreceptor usage as a web service, which is available at http://coreceptor-hiv2.geno2pheno.org. After inputting one or multiple nucleotide/amino-acid sequences containing the V3 loop (at most 500) and selecting an FPR cutoff, the sequences are aligned to a profile of the V3 loop and coreceptor usage is predicted using the linear SVM. To interpret the results, the input sequences are compared to the HIV-2 reference strain M33262 [41–43].

The tool produces a PDF report showing the aligned V3 loops, provides a csv-file that tabulates the predictions for batch runs, and visualizes the model coefficients of the input sequences (Fig. 2). The visualization shows the extent to which individual amino-acid substitutions influence a prediction and enables users to gauge the evidence pointing towards a certain prediction.

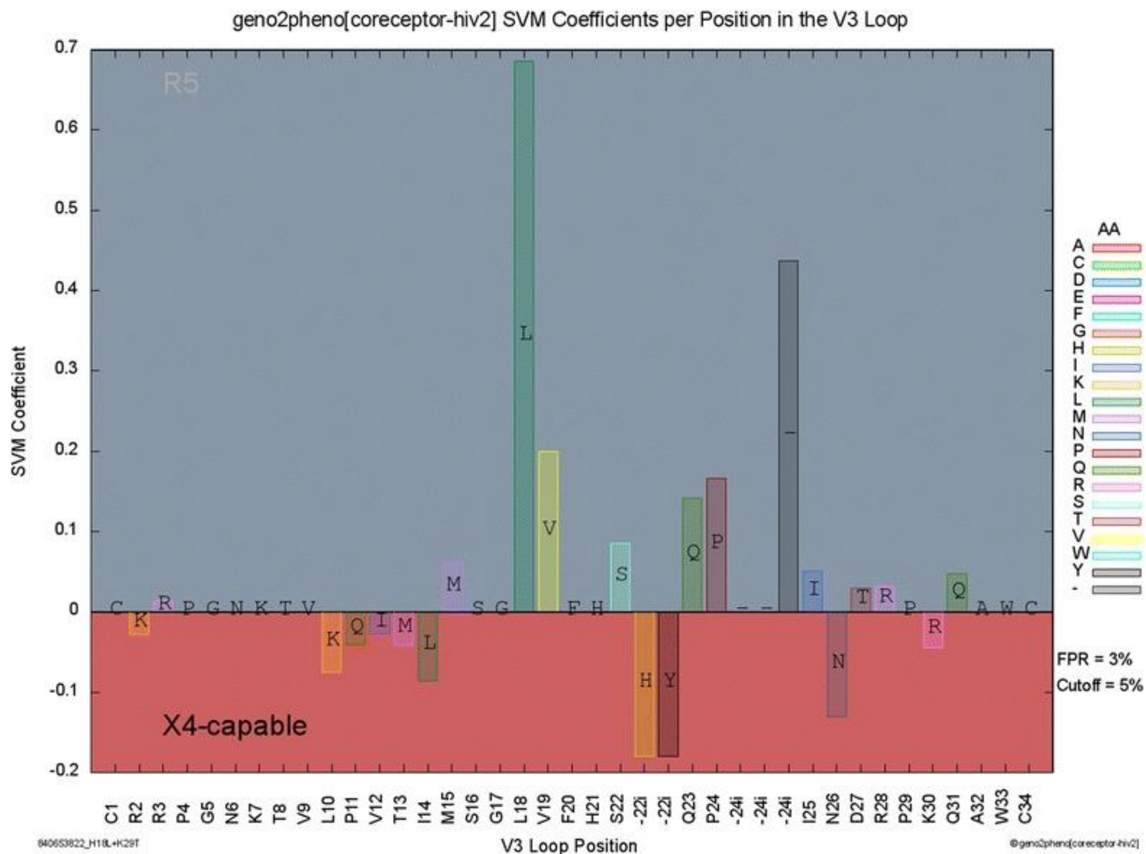

Figure 2. Visualization of the model coefficients for the V3 loop of the mutant ROD10 isolate (H18L + K29T). Amino acids with positive coefficients are associated with R5-tropic viruses, while negative coefficients are associated with X4-capable variants. The legend on the right indicates the color-coded amino acids and gives the FPR of the prediction. Because the predicted FPR is below the selected cutoff at 5%, the sequence is predicted to be X4-capable, which is indicated by the dark color of the X4-capable label in the bottom left corner. The labels of the x-axis refer to the positions and amino acids of the HIV-2 reference strain M33262. Note that since the input sequence contains two insertions relative to the reference (H and Y after position 22), the 29T mutation is visualized at the x-axis tick with the D27 label.

Validation of the geno2pheno[coreceptor-hiv2] web service on an independent test set

We validated the predictive performance of the geno2pheno[coreceptor-hiv2] web service on an independent test set containing nine additional V3 samples that were not contained in the data set (N = 126) that had been used to form the linear SVM of geno2pheno[coreceptor-hiv2].

Predictions from geno2pheno[coreceptor-hiv2] were compared to the phenotypically measured coreceptor usages for the nine samples, which had been determined using an assay based on TZM-bl cells. With the recommended FPR cutoff of 5%, all of the nine sequences were classified correctly (Table 3). The genotypic tool from Visseaux et al. performed slightly worse on these sequences: The R5-sequence ROD10 (H18L + H23Δ + Y24Δ) was incorrectly classified as X4-capable due to its net charge of +7 and the X4-capable sequence ROD10 (H18L + K29T) was classified incorrectly as R5, because it did not exhibit any of the major markers for X4-capability. Investigating the model coefficients of isolate ROD10 (H18L + K29T) in Fig. 2 reveals one of the strengths of geno2pheno[coreceptor-hiv2].

Table 3. Results from the validation of the web service on nine additional V3 sequences.

Isolate	FPR	Major markers	Minor markers	Visseaux prediction	geno2pheno [coreceptor-hiv2] prediction	Phenotype
ROD10 (Wildtype)	0.01	L18X, V3 net charge >6	*NA*	X4-capable	X4-capable	X4-capable
ROD10 (K29T)	0.01	L18X	*NA*	X4-capable	X4-capable	X4-capable
ROD10 (H18L)	0.03	V3 net charge >6	*NA*	X4-capable	X4-capable	X4-capable
ROD10 (H23Δ + Y24Δ)	0.01	L18X	*NA*	X4-capable	X4-capable	X4-capable
ROD10 (H18L + K29T)	0.03	NA	*NA*	R5*	X4-capable	X4-capable
ROD10 (H18L + H23Δ + Y24Δ)	0.11	V3 net charge >6	*NA*	X4-capable*	R5	R5
ROD10 (H18L + H23Δ + Y24Δ + K29T)	0.15	NA	*NA*	R5	R5	R5
15PTHSJIG	0.36	NA	*NA*	R5	R5	R5
15PTHCEC	0.01	L18X, V19K/R, Insertion24, V3 net charge >6	Q23R, R28K	X4-capable	X4-capable	X4-capable

Incorrect predictions are marked with an asterisk. ROD10 refers to the HIV2-group A reference strain, which uses both CCR5 and CXCR4 as entry coreceptors. Mutations from the ROD10 wildtype sequence are indicated in brackets, where Δ indicates deletions.

In contrast to rules-based approaches, geno2pheno[coreceptor-hiv2] takes into account all V3 amino acid positions, which enables the identification of coreceptor usage for viruses where a combination of substitutions enables CXCR4 usage. For example, for the ROD10 (H18L + K29T) mutant, the combination of multiple negative weights associated with the features R2K, P11K, V12K, T13M, I14L, insertions after position 22, and N26N resulted in the prediction of X4-capability, rather than fulfilling individual rules.

Discussion

We were able to confirm the role of the HIV-2 V3 loop as the major determinant for the usage of the CCR5 and CXCR4 coreceptors. On the largest data set for HIV-2 coreceptor usage available to us, high predictive performances of rules-based and data-driven approaches for coreceptor identification were demonstrated. Using SVMs, we were not only able to replicate all of the established markers of X4-capable variants, but could also identify additional markers with significant predictivity that have not been described previously.

Our results substantiate three characteristics differentiating the HIV-2 and HIV-1 V3 loops with respect to coreceptor usage. While individual mutations in the HIV-2 V3 loop by themselves are highly predictive of coreceptor usage (e.g. 18X has a sensitivity of 79% and a specificity of 96%), there is no discriminatory signal in the HIV-1 V3 loop that allows for the accurate identification of coreceptor usage by itself. For example, the 11/25 rule, which classifies HIV-1 as X4-capable if its V3 loop contains positively charged amino acids at the 11th or 25th position [45], is highly specific (93%) but severely lacks sensitivity (30%) [46]. Second, while the major discriminatory markers indicating CXCR4 usage of HIV-2 (18X, 19K/R, insertions after position 24) appear at the V3 C-terminus, discriminatory features of HIV-1 coreceptor usage occur along the full extent of the V3 region. Third, while a V3 net charge exceeding six is significantly associated with the usage of CXCR4 by HIV-2 (Additional file 1: Table S2) [28], there is no significant association between the overall charge of the HIV-1 V3 loop and coreceptor usage [47], although CCR5 and CXCR4 exhibit contrasting electrostatic potential surfaces [48].

Our analysis of the predictive performance of SVMs based on various kernel functions revealed that linear kernel functions are well suited for HIV-2 coreceptor usage prediction and that kernel functions capturing higher-order interactions do not offer additional benefits in this prediction scenario. These results suggests that HIV-2 coreceptor usage is largely based on individual amino-acid mutations in the V3 loop rather than on interdependent substitutions of amino acids as in HIV-1 [49]. This finding would be supported by the hypothesized open structure of the HIV-2 V3 loop, which might reduce the role of interactions among the amino acids in the V3 loop [27]. Determining and analyzing the structure of gp125 with an intact and ordered V3 loop would be a crucial step in confirming the independence of positions by elucidating the accessibility of the V3 loop [50].

We found further evidence [38] indicating that other envelope regions besides V3 might contribute to HIV-2 coreceptor usage. First, SVMs based on the V1 and V2 regions achieved substantial predictive accuracies. Second, the V3 sequences of some X4-capable viruses did not exhibit any known features indicative of CXCR4 usage (accession numbers/isolate identifiers: DQ213035 [27], GU204944 [32], consensus V3 loop from clones JX219591-JX219598, GB87 [31], 310248 [31]) and some V3 sequences of R5-tropic isolates exhibited markers of X4-capability (Fig. 1). Third, there are several samples sharing the same V3 loop, but exhibiting discordant measurements of phenotypic coreceptor usage (Additional file 1: Table S3). Note however that discordant phenotypic assignment of coreceptor use could also be the result of varying sensitivities among the different phenotypic assays (e.g. GHOST (3) cells, PBMCs with the Δ32 mutation, U87 cells) as well as experimental conditions. In case that phenotypically determined coreceptor usage is inconclusive, clarification could be obtained by genotypic approaches such as geno2pheno[coreceptor-hiv2].

R5-tropic HIV-2 viruses exhibiting X4-markers could also be explained by a switch from CXCR4 to CCR5 usage (X4-R5 reversion). X4-R5 reversions have already been reported in HIV-1-infected patients after immune reconstitution [51–54]. Because recent findings indicate that X4-capable HIV-1 viruses are less susceptible to neutralization by autologous antibodies than R5-using viruses from the same host [55], X4-R5 reversions could result from the normalization of naïve T-cell turnover following immunological recovery [56], after which the infection of naïve T-cells by X4-capable variants may not be productive enough [51].

Since X4-capable HIV-2 also seem to be less susceptible to neutralization than CCR5-using strains [19], X4-R5 reversions in HIV-2 could be explained by the same mechanism.

Besides these interpretations, discrepancies between the measured phenotypic coreceptor usage and features in the V3 amino-acid sequence could also be a by-product of the qualitative interpretation of phenotypic assays. *In vivo*, coreceptor usage is on a continuous scale and several, consecutive structural changes within the surface glycoprotein occurring along the viral evolutionary trajectory allow for increasingly effective coreceptor usage. However, this fact is neglected when the results of phenotypic assays are reported. Although the assays produce quantitative measurements (e.g. fluorescence, luminescence, or formation of syncytia), these measurements are typically converted to a qualitative scale for the sake of convenience regarding further analyses. Typical qualitative scales are the annotation of coreceptor usage (e.g. R5/X4-capable) or the efficiency of coreceptor usage (e.g. −/+/++/+++). For the sake of accuracy, however, it would be paramount to work on the raw, quantitative data. With quantitative measurements, it would be possible to place a virus onto the evolutionary continuum stretching from viruses using CCR5 highly efficiently to viruses capable of using CXCR4. Moreover, working on raw data from phenotypic assays would facilitate the application of established statistical techniques for the normalization of biased data arising from several experiments, which could improve the accuracy of large-scale studies on coreceptor usage considerably. Our genotypic analyses of several ROD10 mutants are a step in the right direction, because we were able to determine the impact of individual V3 substitutions on coreceptor usage quantitatively through the genotypic prediction of FPRs (Table 3).

To shed more light on the emergence of V3 amino-acid sequences with discordant phenotypic measurements, three aspects should be investigated. First, the agreement between different phenotypic assays should be validated or, even better, a standardized phenotypic assay should be developed. Second, further research investigating the intra-host evolution of HIV-2 with respect to coreceptor usage and its impact on viral fitness seems necessary to determine whether X4-R5 reversions do occur. Third and most importantly, it should be investigated whether amino acid substitutions in the V1/V2 region can impart the X4-phenotype independently of substitutions in the V3, a question for whose resolution more data is required [27].

In the following, we discuss the benefits of using geno2pheno[coreceptor-hiv2] for HIV-2 coreceptor identification. We could show that that geno2pheno[coreceptor-hiv2] outperformed the rules-based approach by Visseaux et al. [28] on an independent test set of nine V3 sequences (Table 3). Furthermore, the predictive performance of geno2pheno[coreceptor-hiv2] is at least as high as the predictive performance of geno2pheno[coreceptor] for HIV-1, whose established cutoffs (EU: 10%/20%, UK: 5.75%, Germany/Austria: 5–15%) exceed the optimized 5% cutoff that is employed by geno2pheno[coreceptor-hiv2] [57–59].

Since geno2pheno[coreceptor-hiv2] is based on an SVM, it considers all positions in the V3 loop when predicting coreceptor usage. Rules-based systems, on the other hand, use only a preselected set of discriminatory features from the V3 loop to identify coreceptor usage. This gives geno2pheno[coreceptor-hiv2] an edge over rules-based systems when coreceptor usage can only be discerned by considering combinations of multiple substitutions that together confer the X4-phenotype (Fig. 2).

The predictions by geno2pheno[coreceptor-hiv2] are not only accurate, but also interpretable. The web service visualizes the model coefficients of an input sequence to provide users a comprehensive view of the impact of individual positions on HIV-2 coreceptor usage. Additionally, geno2pheno[coreceptor-hiv2] outputs FPRs, which provide a measure of predictive confidence. Moreover, users are free to select the tradeoff between sensitivity and specificity by adjusting the cutoff for the FPR. For example, higher sensitivities (at the cost of more false alarms) can be obtained by increasing the FPR cutoff (e.g. from 5 to 20%).

Conclusions

geno2pheno[coreceptor-hiv2] is a highly accurate and interpretable online tool for the genotypic identification of HIV-2 coreceptor usage. Using our method, we were able to obtain a better understanding of the V3 amino-acid substitutions required for the usage of the CXCR4 coreceptor and to learn more about the impact of the V1 and V2 loops on HIV-2 coreceptor usage. geno2pheno[coreceptor-hiv2] can support the clinical management of HIV-2 infection because the tool can aid physicians in taking treatment decisions and enables researchers to undertake large-scale epidemiological studies on HIV-2 coreceptor usage.

Methods

Supervised learning with SVMs for HIV-2 coreceptor usage prediction

Our genotypic approach to coreceptor identification is based on supervised statistical learning, more specifically, on classification. Classification requires two types of data. The first type of data is a numeric input matrix $X \in \mathbb{R}^{N \times p}$, where N gives the number of observations and p gives the number of features. Due to the established association between the V3 loop and HIV-2 coreceptor usage [17, 27, 29, 30], we used the amino acids of the V3 loop as features (N = 126). The input matrix was constructed such that each row x_i contains the aligned, binary-encoded V3 amino-acid sequence of sample ***i***. The amino-acid sequences of the V1 and V2 loops were also considered as features (N = 62), but only investigated briefly due to lacking data and smaller predictive power of the V1/V2 region.

The second type of data required for binary classification is a vector of outcomes $Y \in \mathbb{Z}^N$, whose entries y_i contain the numeric representation of the phenotypically determined coreceptor usage of sample i, which is also called its label. We set $y_i = -1$ for sequences labeled as X4-capable and $y_i = 1$ for sequences labeled as *R5*.

Because SVMs [60] based on the amino-acid sequence of the V3 region have already been used successfully for identifying the coreceptor usage of HIV-1 [61], we also decided to use SVMs. In our setting, SVMs find a vector of coefficients α and an intercept β_0 that define a hyperplane maximizing the margin between observations from the two classes, X4-capable and R5. Predictions are generated by computing the decision function $f(xi)=\sum_{j=1}^{N} \alpha j y j K(xj,xi) + \beta_0$, where $K(x_i, x_j)$ is a kernel function representing the similarity of two V3 loops x_i and $x_j$in Hilbert space [62]. We used LIBSVM to determine the optimal hyperplane and transform decision values to the probability that a V3 originates from an X4-capable sequence [63, 64].

Data collection and sample labeling

The majority of the data were retrieved from the Los Alamos National Laboratory HIV database by gathering all available HIV-2 V3 sequences with annotations of phenotypic coreceptor usage [28, 32, 65–72].

Further data points were obtained from the literature [29–31] and complemented by our own phenotypic measurements, which were performed as described in the sections following Section Cells, plasmids, and coreceptor antagonists.

To differentiate sequences from R5-tropic strains from sequences of viruses that can use CXCR4, each observation was labeled either as R5 or X4-capable. Isolates for which CXCR4 usage was reported (X4-tropic or D/M) were annotated as X4-capable and isolates for which only the usage of the CCR5 coreceptor was reported were annotated as *R5*. All of the isolates capable of using coreceptors other than CCR5 or CXCR4 were also able to use the CXCR4 coreceptor and therefore labeled as X4-capable.

Next, to obtain a representative training data set for statistical learning, the initial data set of 314 genotype-phenotype pairs was filtered to remove duplicate V3 sequences. During duplicate removal, we found multiple sequences with discordant annotations of coreceptor usage (i.e. sequences sharing the same V3 amino-acid sequence but having different phenotypic measurements). For each set of discordant sequences sharing the same V3 loop, we considered two possibilities: either to include one of the discordant V3 sequences into the data set or to exclude all of the sequences (Additional file 1: Table S3). In the following, we discuss each decision in detail.

Each of the samples sharing the same V3 amino-acid sequence as DQ870430 [28, 30, 32, 65–67] and NARI-12 [28, 30, 31, 65] was phenotyped as X4-capable variant only once, while a decidedly larger number of identical V3 sequences was phenotyped as R5 (21 and 5 sequences, respectively). Hence, we regarded the X4-capable measurements as outliers and the respective sequences were included with the *R5* label. The sequence with the accession GU204945 [32] was identified as X4-capable once and as R5 thrice. Hence, due to lacking evidence of actual coreceptor usage, this sequence was removed from the data set. For the V3 sequence with the identifier 310248, usage of CCR5 and CXCR4 was reported in one study each.

The sequence had been identified in the X4-capable isolate 310248 [31], but also in an R5 isolate (JN230759/isolate 29) with the same V3 sequence except for an R/K ambiguity at position 27 [28]. Interestingly, the R5 isolate showed a marginal signal for the CXCR4 coreceptor, which was discarded because the signal was <5% of the signal for CCR5 usage. Further evidence pointing towards the usage CXCR4 was presented by Owen et al. [31], who reported a minor induction of syncytia for their isolate.

Additionally, applying a CXCR4 antagonist to cells lacking the CCR5 coreceptor revealed a reduction in infectivity between 40 and 90% for this strain [31], which suggests that the isolate actually seems to use CXCR4. Therefore, we included this sequence as X4-capable in our data set.

After duplicate removal and handling of sequences with discordant annotations, 126 genotype-phenotype pairs remained of which 74 (58.7%) were labeled as *R5* and 52 (51.3%) as X4-capable (Additional file 1: Table S4).The samples in the data set originate from diverse regions. In total, 87 (69%) samples were collected in Europe, of which 42 (48.3%) come from France, 33 (37.9%) from Portugal, and 12 (13.8%) from Sweden. All of the 10 (10.3%) Asian samples originate from India. Of the 24 (19%) West African samples, 15 (60%) were collected in Guinea-Bissau, 5 (20.8%) in Ivory Coast, 2 (8.3%) in Gambia, and 2 (8.3%) in Senegal.

Most isolates in the data set (84.9%) had been genotyped as HIV-2 group A. Only a minority of samples (13.5%) had been identified as group B and the remaining samples (1.6%) either had been identified as group D or had not been genotyped. The group distribution of the samples in our data set reflects the global distribution of HIV-2 groups: Groups A and B are the most prevalent genotypes and the majority of infections are caused by group A strains [42, 73, 74].

Sequence alignment

To align the V3 sequences in the data set, we modified the Smith–Waterman algorithm for pairwise alignments [75] to perform profile alignments in order to capture the diversity of the HIV-2 V3 region. In contrast to pairwise alignments, profile alignments compare the input sequence not with a single reference sequence, but with a profile corresponding to the expected amino-acid frequencies for every position in a genomic region. We retrieved all available amino-acid sequences of the HIV-2 envelope region from the LANL HIV database and selected the V3 region through pattern matching. If a sequence exhibited the highly conserved V3 start motif (CKRP or CRRP) and the end motif (QAWC), the corresponding subsequence was selected. In cases where either only the start or end motif could be found, a search for the substring of the missing motif was conducted and the corresponding subsequence was selected if a substring of the missing motif could be found.

The extracted 1979 V3 amino-acid sequences were aligned with ClustalW version 2.1 (using the accurate switch and default parameters) [76], which is an established tool that is sufficiently accurate for identifying an overall amino acid profile of the V3 loop. We then computed the frequency of each amino acid for every alignment position to obtain a profile of the V3 loop.

The profile alignment of the V3 amino-acid sequences was performed by computing the alignment scores under consideration of both, the frequency of amino acid substitutions given by the alignment profile and an amino acid substitution matrix [77].

Sequence encoding

Let AA be the set of 20 amino acids augmented with the gap character "-". To obtain the input matrix X, each aligned V3 amino-acid sequence s_i with $|s_i| = 39\ \forall i$ was encoded as a feature vector x_i with $21 * 39 = 819$ dimensions. Let $x_{i,j}[c]$ denote whether the character $c \in AA$ appears at position j in the V3 loop of observation $\boldsymbol{i}$. To deal with ambiguous positions, we disambiguate IUPAC ambiguity codes and define $s_{i,j}$ as the set of unambiguous amino acids occurring at position j in the i-th input sequence. For each position j in an aligned sequence s_i, we uniformly distribute the weight among all observed amino acids and set the value of non-observed amino acids to 0:

$$x_{i,j}[c] = \frac{1}{|si,j|} \quad \forall c \in s_{i,j}$$

$$x_{i,j}[c] = 0 \quad \forall c \notin s_{i,j}$$

Note that $x_{i,j}[c] = 1$ for unambiguous positions with $s_{i,j} = \{c\}$ and $|s_{i,j}| = 1$.

Model selection and validation

Based on the input matrix X containing the 126 aligned and encoded V3 amino-acid sequences as well as the vector of outcomes Y denoting phenotypic coreceptor usage, we trained several SVMs to identify which SVM performs best in terms of the AUC of the receiver operating characteristic [78]. The SVM parameter ν was varied in a range from 0.1 to 0.4 (higher values were not considered due to infeasible optimization problems) and different kernel functions (linear, radial basis function, polynomial, and edit kernel [79]) were used to form predictions.

To evaluate the performance of the SVMs, we conducted 10 runs of tenfold CV [80]. Additionally, to determine the expected performance of our approach taking into account the model selection procedure, we performed tenfold nested CV. In nested CV, two interlaced runs of CV were performed.

In the inner CV run, we computed the AUCs resulting from the predictions of each model and selected the model and kernel parameters maximizing the AUC. In the outer CV run, we trained a model with the selected parameters on the inner CV training data and predicted the outcomes of samples contained in an independent fold. After all outer fold predictions had been computed, the overall model performance was determined.

To compare the performance of the rules-based approach from Visseaux et al. [28] with our method, we set up a test data set (N = 84), whose observations where not used to identify discriminatory features by Visseaux et al. This test set was formed to determine the prediction performance of their model on independent data. We evaluated whether there exists a significant difference between the rules-based approach and our method by applying McNemar's test.

McNemar's test

McNemar's test [40] is based on the values contained in a 2 × 2 confusion matrix and can be used to determine whether two classifiers perform differently. The test can be applied on paired dichotomous variables that are mutually exclusive and identifies if there exists a difference in the distribution of the marginal frequencies of each outcome. In our case, we applied the test to the predicted and phenotypically determined coreceptor usages (*R5/X4*-capable). To compare the performance of SVMs for coreceptor prediction with the rules-based approach from Visseaux et al. [28], we computed the number of samples that were correctly or incorrectly predicted by each method and constructed a 2 × 2 contingency table. The null hypothesis assumes that both approaches have the same ratio of incorrect predictions. Let p indicate the probability of a certain outcome. Given the entries in Additional file 1: Table S5, the underlying assumption is that $p_a + p_b = p_a + p_c$ and $p_c + p_d = p_b + p_d$. Hence, the null hypothesis is that $H_0\colon p_b = p_c$ and, alternatively, $H_1\colon p_b \neq p_c$.

The test statistic, $X^2 = (b-c)^2 / b+c$, can be rejected when X^2 is sufficiently large, that is, indicates a significant difference between the predictive performance of both approaches.

Transformation of decision values to FPRs

We used SVMs that transform decision values to probabilities that indicate whether a V3 loop originates from an X4-capable virus (X4-probabilities) [64].

Although these probabilities give a measure of confidence, they does not afford insights into the accuracy of predictions, which is crucial for clinical applications, however. Since FPRs provide a useful measure for the confidence of a prediction and because they are an established measure for the quantification of HIV-1 coreceptor usage [61], we transformed the predicted X4-probabilities to FPRs.

Here, the FPR indicates the estimated rate at which an R5-tropic virus would be falsely predicted as X4-capable when using a given X4-probability as a cutoff for the two classes.

To transform X4-probabilities to FPRs, we constructed a mapping from predicted X4-probabilities to FPRs during the training stage. Each predicted X4-probability was used as a cutoff for classifying samples once: All samples with X4-probabilities below the cutoff were assigned *R5* and all samples with X4-probabilities greater or equal to the cutoff were assigned *X4-capable*. This cutoff-dependent class assignment in combination with the phenotypic labels for each observation yielded a 2×2 contingency table indicating false positives (FP) and true negatives (TN), from which we could compute the FPR as

$$FPR = \frac{FP}{FP + TN}$$

which results from applying every predicted X4-probability as a cutoff once. Using this transformation, low FPRs indicate confident predictions of X4-capable variants, while high FPRs designate R5-tropic viruses.

Determining the impact of amino acids in the V3 loop on HIV-2 coreceptor usage

LIBSVM outputs a weight vector $\alpha^* \in \mathbb{R}^n$. Its entries $\alpha^*_i = \hat{a}_i y_i$ indicate the estimated weight $\hat{a}_i$ of each support vector x^*_i scaled by the corresponding outcome y_i. The coefficients $\beta \in \mathbb{R}^p$, which reflect the impact of individual amino acids in the V3 loop on coreceptor usage, can be determined by $\beta = \alpha^{*T} X^*$. Hence, given a new input sequence, $x_i \in \mathbb{R}^p$, we can find its amino-acid specific weights $b(i)$ as the element-wise vector product of the coefficients and the encoded input features such that $b(i) = x_i * \beta$, which can be visualized in terms of a bar plot indicating the role of individual amino acids for HIV-2 coreceptor usage.

Modified feature encoding used by geno2pheno[coreceptor-hiv2]

To predict the label of a new input sequence, its V3 is modified in two ways in order to improve predictive performance. The first modification concerns gaps in the sequence and the second relates to ambiguous positions.

Errors during sequencing or problems with the alignment can lead to the introduction of gaps in the V3 loop, which have no functional meaning and can bias predictions. Therefore, our approach detects gaps that are not functionally relevant and are likely to represent artifacts in the following way. Let $\beta_j(c)$ be the coefficient that corresponds to character c at sequence position j and let $\varepsilon = 0.01$. For every position j with $c = -$, we consider the model weight associated with the gap, $\beta_j(c)$. If $|\beta_j(c)| < \varepsilon$, the gap does not affect HIV-2 coreceptor usage according to the model and it can be replaced with the encoded consensus amino acid a from position i contained in the V3 alignment profile by setting $x_j[c] = a$ before predicting coreceptor usage for the input sequence. Otherwise, if $|\beta_j(c)| \geq \varepsilon$, no modification is necessary.

Ambiguous positions in Sanger sequencing of viral populations indicate the presence of multiple viral variants within the same host. These variants might use different coreceptors for cell entry and a single position might indicate amino acids representative of both, R5 and X4-capable viruses. To be more sensitive towards X4-capable variants, every ambiguous position in an input sequence is replaced by the disambiguated amino acids that are most strongly associated with X4-capability. Note that, since the labels for training the SVM were encoded by -1 for X4-capable and 1 for R5, positive coefficients designate features associated with *R5* and negative coefficients designate features associated with X4-capable.

For every ambiguous sequence position j with observed amino acids s_j, we set $s_j = \arg\min_{c \in s_j} \beta_j(c)$ in order to construct a non-ambiguous sequence that is more predictive of X4-capability.

The fact that this worst-case scenario sequence might not exist *in vivo* when a sequence exhibits multiple ambiguous positions is only a minor concern. This is due to the following reason. Assume that a viral population consists of an R5- and an X4-capable quasispecies, which means that the prediction should be *X4-capable*.

In this case, every ambiguous position should contain an amino acid representing the X4-capable variant such that for every ambiguous position j we have $\beta_j(c) \leq 0$ for all amino acids c occurring at the ambiguous position. Selecting the observed amino acid whose weight contributes most strongly to X4-capability means choosing the character c obtaining the most negative weight $\beta_j(c)$. Consequently, the decision value of observation x, $f(x)$, enhances the prediction of X4-capable variants by reducing the decision value. The same logic can be applied to two distinct X4-capable variants. Assume now that there exist two variants that use only the CCR5-coreceptor. In this case, the prediction should be *R5* and the weights of ambiguous positions should be positive, because no amino acids associated with X4-capability are observable. Hence, the worst-case choice results in $\min \beta_j(c) \geq 0$ for all characters c at every ambiguous position j, which does not enhance the prediction of X4-capable and thus does not influence the likelihood of a correct prediction of R5 when the decision boundary is set to 0. Even for decision boundaries at values above zero, selecting the worst-case amino acid would only have a marginal effect on the prediction in the described scenario, because of the larger number and greater impact of non-ambiguous positions with positive weights.

Cells, plasmids, and coreceptor antagonists

HEK293T 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 cells [33, 34, 81–83], TAK-779 [84, 85], and bicyclam JM-2987, a hydrobromide salt of AMD-3100 [86–88]. The wild-type pROD10 plasmid was a gift from Keith Peden [89]. HEK293T and TZM-bl cells were cultured in complete growth medium consisting of Dulbecco's modified eagle medium (DMEM) supplemented with 10% of fetal bovine serum, 100 U/ml of penicillin–streptomycin, 2 mM of L-glutamine, 1 mM sodium pyruvate, and 1× of MEM non-essential amino acids (Gibco/Invitrogen, USA). All cell cultures were maintained at 37 °C in 5% of CO_2.

Virus isolates

Two new primary isolates, 15PTHSJIG and 15PTHCEC, were obtained from HIV-2-infected Portuguese patients by cocultivation with peripheral blood mononuclear cells from seronegative subjects, as described previously [90].

In addition, six new HIV-2_{ROD10} mutants were analyzed that contained the following mutations in the V3 loop: H18L, H23Δ + Y24Δ, K29T, H18L + H23Δ + Y24Δ, H18L + K29T, and H18L + H23Δ + Y24Δ + K29T [91]. HIV-2 ROD10 mutants were obtained by transient transfection of HEK293T cells. Transfections were performed with 10 μg of DNA in a 100 mm tissue culture dish, using the jetPrime transfection reagent (Polyplus) according to the instructions of the manufacturer. Cell culture supernatants were collected 48 h post-transfection, filtered, and stored at −80 °C.

The 50% tissue culture infectious dose (TCID50) of each isolate was determined in a single-round viral infectivity assay using a luciferase reporter assay with TZM-bl cells. First, 10,000 TZM-bl reporter cells were seeded in 96-well tissue culture plates and incubated overnight. On the next day, the growth medium was removed and replaced by 200 μl of fresh growth medium supplemented with 19.7 μg/ml of DEAE-dextran. A total of 100 μl of virus supernatant was added to the first well, from which serial threefold dilutions were prepared in the next wells. The assay was performed in quadruplets. After 48 h, luciferase expression was quantified by measuring luminescence with the Pierce Firefly Luciferase Glow Assay Kit (Thermo Fisher, USA) and the Infinite M200 luminometer (TECAN), according to manufacturer's instructions. Control wells containing only target cells and growth medium were used to measure background luminescence. The TCID50 was calculated using the statistical method of Reed and Muench [92].

Phenotypic determination of coreceptor usage

CCR5 and CXCR4 coreceptor usage was determined in a single-round viral infectivity assay with TZM-bl cells [16, 35]. First, 10,000 TZM-bl reporter cells were seeded in 96-well tissue culture plates and incubated overnight. On the next day, the growth medium was removed and the cells were incubated for 1 h (at 37 °C in 5% CO_2) with growth medium either in the presence or in the absence of excessive amounts of the CCR5 antagonist TAK-779 (10 μM) and/or of the CXCR4 antagonist AMD3100 (1.2 μM). A fixed amount of virus supernatant, corresponding to 200 TCID50 was added to each well and cells were cultured with a total volume of up to 200 μl of growth medium in the presence of 19.7 μg/ml of DEAE-dextran.

After 48 h, luciferase expression was quantified by measuring luminescence with the Pierce Firefly Luciferase Glow Assay Kit (Thermo Fisher, USA) and the Infinite M200 luminometer (TECAN), according to manufacturer's instructions. Control wells containing only target cells and medium were used to measure background luminescence. A viral population was classified as R5-tropic when viral infectivity was inhibited in the presence of TAK-779 but unaltered in the presence of AMD3100, and, as X4-tropic when infectivity was inhibited in the presence of AMD3100 but unaltered in the presence of TAK-779. When infectivity was completely inhibited only by the simultaneous presence of TAK-779 and AMD3100, the virus population was classified as dual/mixed (D/M) for viral isolates or as R5/X4 tropic for ROD10 mutants.

Authors´contributions

MD developed the predictive model and performed all computational analyses. PB, AM, and NT provided viral sequences and phenotypes and were major contributions in writing the manuscript. GF and JB developed the framework for the web service. RCC, JE, and RK helped in validating and developing the tool and contributed to the manuscript. TL and NP contributed by guiding the development of the method and to writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data used for the current study are mainly available from the Los Alamos National Library HIV databases (www.hiv.lanl.gov) [28, 32, 65–72]. Additional data were manually curated from the literature [29–31]. HIV-2 isolates 15PTHSJIG and 15PTHCEC are available under accession numbers KX911212 and KX911213, respectively.

The following isolates were annotated with their phenotypically determined coreceptor usages: AY913773-AY913794, EU358115-EU358567, EU360797-EU360799, GU591163, GU983917-GU983956, and HQ738338-HQ738350.

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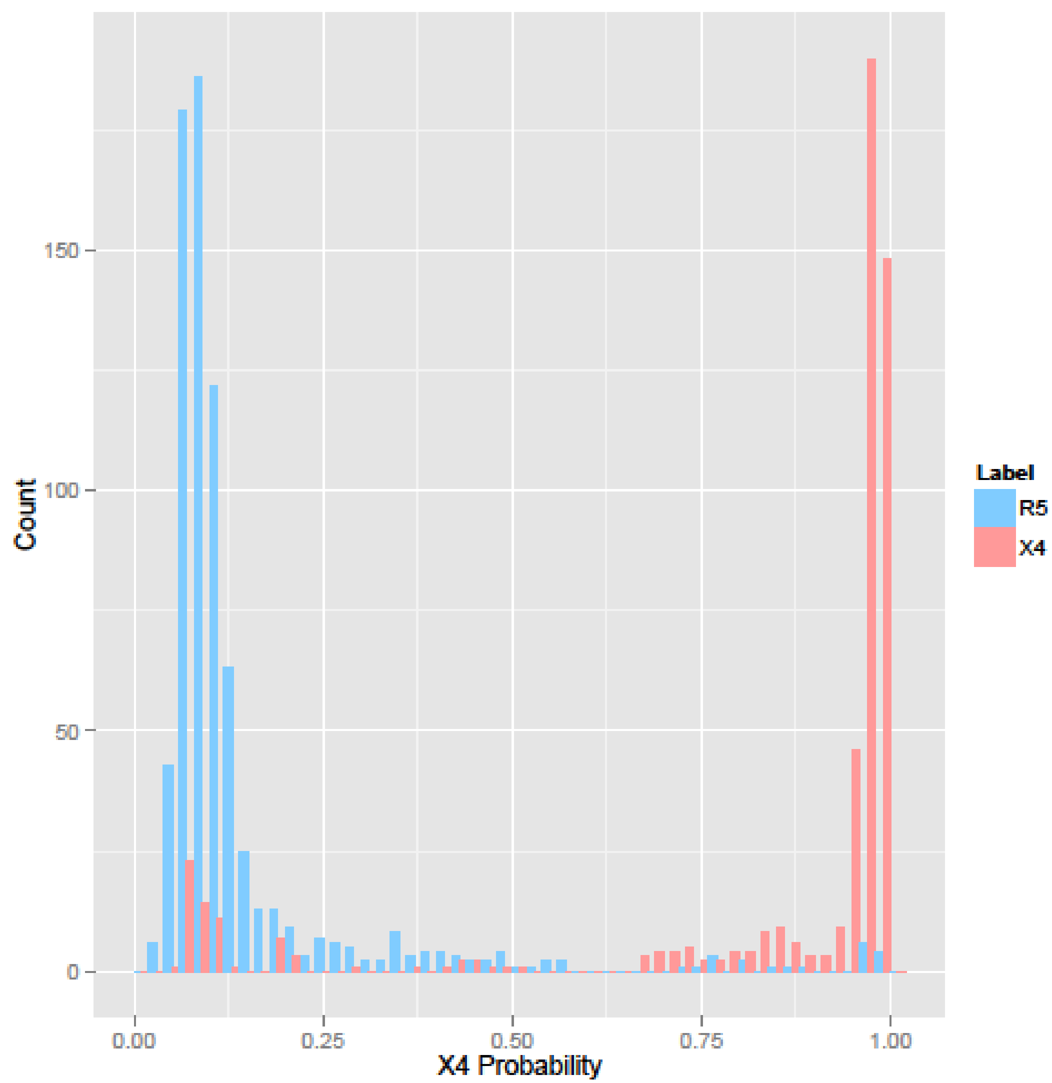
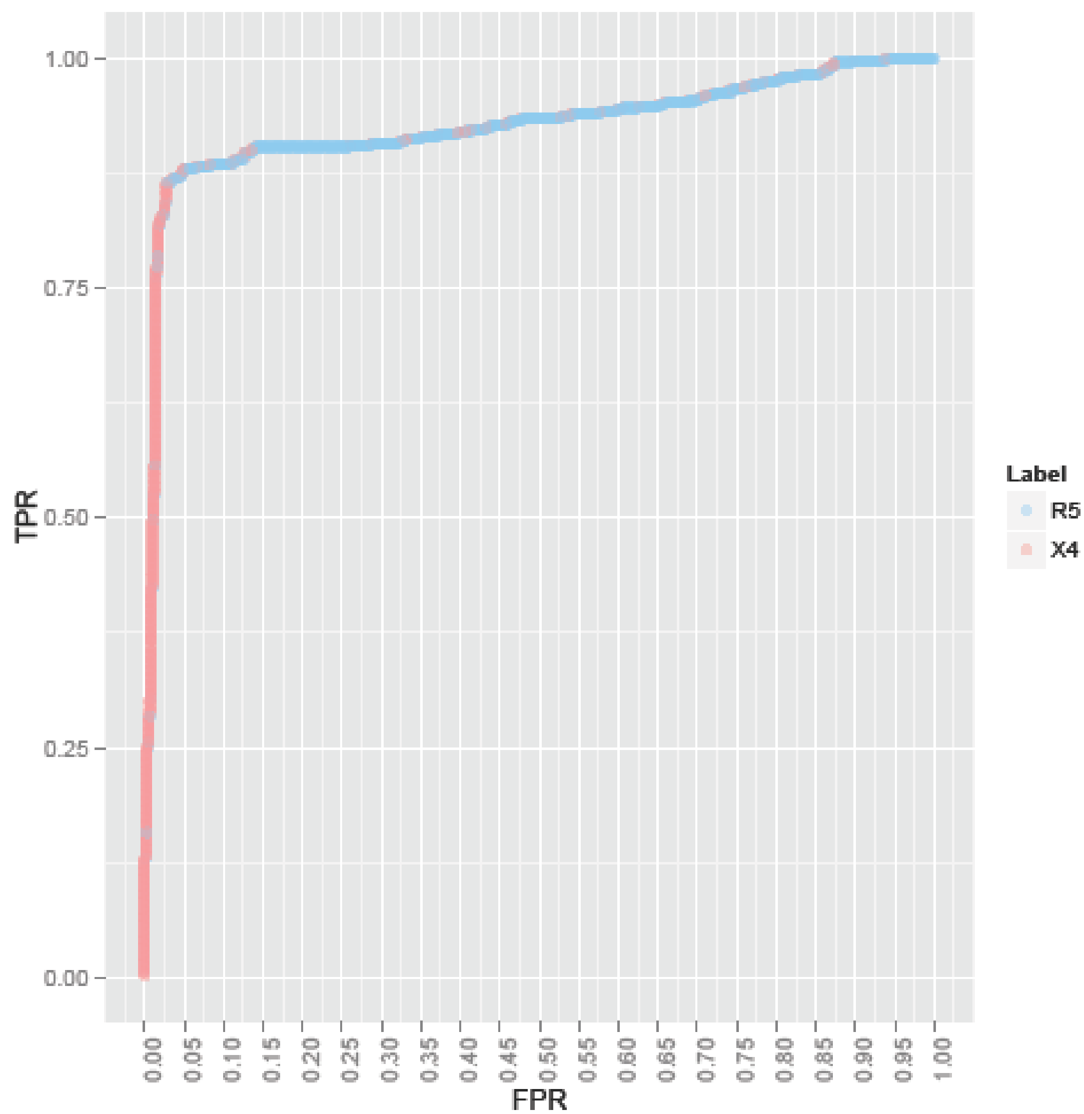
Supplementary Information



Supplementary Figure 1. Distribution of X4-probabilities predicted by geno2pheno[coreceptor-hiv2]. Blue bars indicate sequences labeled as R5, while red bars indicate sequences labeled as X4-capable.



Supplementary Figure 2. Estimated TPRs versus FPRs for predictions from geno2pheno[coreceptor-hiv2]. Each dot indicates a prediction of HIV-2 coreceptor usage and the color of the dot indicates the corresponding phenotypic coreceptor usage (blue: R5, red: X4-capable).

Supplementary Table 1. Predictive performance of the rules-based approach from Visseaux et al. on the test set.

Number of Rules	Sensitivity	Specificity	Balanced Accuracy
1	0.85	0.94	0.89
2	0.79	0.96	0.88
3	0.74	0.96	0.85
4	0.62	1	0.81

The column *Number of Rules* refers to the number of major rules (Visseaux et al.) in the V3 that were required for predicting X4-capability.

Supplementary Table 2. Predictive performance of individual rules identified by Visseaux et al. ordered by decreasing balanced accuracy as determined on the test set.

Rule	Sensitivity	Specificity	Balanced Accuracy	P-value
L18X	0.79	0.96	0.88	$2.3 \cdot 10^{-13}$ (*)
Insertion after position 24	0.74	1	0.87	$3.4 \cdot 10^{-14}$(*)
Net charge > +6	0.77	0.96	0.86	$6.8 \cdot 10^{-11}$ (*)
V19K/R	0.74	0.96	0.85	$8.7 \cdot 10^{-12}$ (*)
R28K	0.5	0.96	0.73	$8.9 \cdot 10^{-7}$ (*)
Q23R	0.29	1	0.65	$4.7 \cdot 10^{-5}$ (*)
R30K	0.47	0.7	0.57	0.17
S22A/F/Y	0.15	1	0.59	0.009 (*)
I25L/Y	0.08	0.97	0.53	0.47

The major discriminatory features are highlighted in bold. P-values were computed using a two-sided Fisher's exact test. P-values that were significant at the 5% level after correcting for multiple hypothesis testing using the Benjamini-Hochberg procedure are indicated by an asterisk (*).

Supplementary Table 3. Overview of observations with identical V3 loops, but discordant annotation of phenotypic coreceptor usage.

Identifier of X4-capable Isolate	No. of R5 isolates	No. of X4-capable isolates	V3 loop of the X4-capable sequence	Decision
DQ870430	21	1	**CKRPGNKTVVPITLMSGLVFHSQPINKRPRQAWC**	R5
NARI-12	5	1	CKRPGNKTVLPITLMSGLVFHSQPINTRPRQAWC	R5
GU204945	3	1	CKRPGNKTVRPITLLSGRRFHSQVYTVNPKQAWC	Exclude
310248	1	1	CRRPGNKTVVPITLMSGLVFHSQPINKRPRQAWC	X4-capable

Supplementary Table 4. Distribution of class labels and HIV-2 groups in the data set.

Class	Group	Frequency
R5	A	61 (48.4%)
X4-capable	A	46 (36.5%)
R5	B	12 (9.5%)
X4-capable	B	5 (3.9%)
X4-capable	D	1 (0.08%)
R5	Unknown	1 (0.08%)

Supplementary Table 5. Structure of the 2x2 contingency table required for McNemar's test.

Column total	a+c	b+d	N
	Rules-based Correct	**Rules-based Incorrect**	**Row total**
SVM Correct	A	B	a+b
SVM Incorrect	C	D	c+d

CHAPTER V

A helical short-peptide fusion inhibitor with highly potent activity against Human immunodeficiency virus type 1 (HIV-1), HIV-2, and Simian Immunodeficiency virus

A Helical Short-Peptide Fusion Inhibitor with Highly Potent Activity against Human Immunodeficiency Virus Type 1 (HIV-1), HIV-2, and Simian Immunodeficiency Virus

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Abstract

Human immunodeficiency virus type 2 (HIV-2) has already spread to different regions worldwide, and currently about 1 to 2 million people have been infected, calling for new antiviral agents that are effective on both HIV-1 and HIV-2 isolates. T20 (enfuvirtide), a 36-mer peptide derived from the C-terminal heptad repeat region (CHR) of gp41, is the only clinically approved HIV-1 fusion inhibitor, but it easily induces drug resistance and is not active on HIV-2. In this study, we first demonstrated that the M-T hook structure was also vital to enhancing the binding stability and inhibitory activity of diverse CHR-based peptide inhibitors. We then designed a novel short peptide (23-mer), termed 2P23, by introducing the M-T hook structure, HIV-2 sequences, and salt bridge-forming residues. Promisingly, 2P23 was a highly stable helical peptide with high binding to the surrogate targets derived from HIV-1, HIV-2, and simian immunodeficiency virus (SIV). Consistent with this, 2P23 exhibited potent activity in inhibiting diverse subtypes of HIV-1 isolates, T20-resistant HIV-1 mutants, and a panel of primary HIV-2 isolates, HIV-2 mutants, and SIV isolates. Therefore, we conclude that 2P23 has high potential to be further developed for clinical use, and it is also an ideal tool for exploring the mechanisms of HIV-1/2- and SIV-mediated membrane fusion.

Introduction

Currently, there are approximately 34 million people worldwide living with human immunodeficiency virus (HIV) [1]. Although HIV-1 is a major causative agent of the global AIDS pandemic, about 1 to 2 million people have been infected with HIV-2, mostly in West Africa. In recent decades, HIV-2 also spread to different countries in Europe, Asia, and North America, resulting in the relatively high prevalence of HIV-2 infection. For example, surveillance studies in Portugal and France showed that around 2% of the new infections during 2003 to 2006 were caused by HIV-2 [2, 3], raising additional concerns over the control of AIDS. Therefore, preventive vaccines and therapeutic drugs that are also effective on HIV-2 would be highly appreciated.

HIV-2 was first isolated from AIDS patients in West Africa, and its genome organization was determined from an isolate designated ROD [4, 5]. At present, HIV-2 strains are classified in nine groups, termed A to I, of which group A is by far the most disseminated worldwide (ROD is a prototypic HIV-2 group A strain) [6-8]. Previous studies demonstrated that HIV-1 and HIV-2 have different evolutionary histories and share only 50% genetic similarity [9, 10]. Unfortunately, all currently available antiretroviral drugs were specifically developed to inhibit HIV-1 entry and replication, and consequently some drugs in clinical use have limited or no activity on HIV-2, including all nonnucleoside reverse transcriptase inhibitors, some protease inhibitors, and the fusion inhibitor T20 (enfuvirtide; Fuzeon) [11-14]. T20, a 36-mer linear peptide derived from the native gp41 CHR sequence of the HIV-1 LAI isolate, was approved as the first and, so far, only HIV-1 fusion inhibitor for clinical use [15-17]. Mechanically, T20 inhibits HIV-1 entry by competitive binding to the complementary N-terminal heptad repeat (NHR) of gp41, thereby blocking the formation of the six-helical bundle structure (6-HB) that is essential for fusion of the viral and cellular membranes [18-21]. Despite its strong anti-HIV-1 activity, T20 easily induces drug resistance through mutations within its NHR-binding sites [22, 23]. Also, T20 has poor bioavailability, requiring large-dose injections (90 mg twice daily), which complicates patient adherence to treatment. Furthermore, we and others demonstrated that T20 displayed dramatically decreased activity in inhibiting HIV-2 isolates [14, 24, 25].

Considerable efforts have been made to develop new fusion inhibitors with improved pharmaceutical profiles [26–29]. T-1249 is a representative second-generation peptide fusion inhibitor that has 39 amino acids derived from the consensus CHR sequences of HIV-1, HIV-2, and simian immunodeficiency virus (SIV); however, its clinical development was hampered beyond phase I/II trials due to the drug formulation difficulties associated with its large size and elevated production costs [30, 31]. A number of new inhibitors were designed by using the CHR peptide C34 as a template, and the resulting peptides did show increased anti-HIV-1 activity; however, in most cases they had longer sequences (>34-mer) and still limited activity against HIV-2 isolates [26, 27, 32-34]. Finally, some peptides were designed using HIV-2 and/or SIV C34 as templates, generating inhibitors with somewhat improved anti-HIV-2 activity, such as $C34_{EHO}$ and P3 [24, 34].

We recently found that two residues (Met115 and Thr116) preceding the pocket-binding domain (PBD) of CHR peptides adopt a unique M-T hook structure that can greatly enhance the binding and antiviral activities [35-39]. Our crystal structures demonstrated that the residue Thr116 can redirect the peptide chain to position Met115 above the left side of the deep pocket on the trimeric coiled coil of N-terminal helices (NHR) so that its side chain caps the pocket to stabilize the inhibitor binding [37–39]. On the basis of the M-T hook structure, we generated short-peptide fusion inhibitors that mainly targeted the conserved pocket site of gp41 [37, 40, 41]. For example, MTSC22 and HP23 showed dramatically improved inhibition on diverse HIV-1 isolates and high genetic barriers to the development of resistance [37, 41]. In this study, we applied the M-T hook strategy to design fusion inhibitors that are also effective on HIV-2 isolates. A 23-mer helical peptide, termed 2P23, was generated that showed very potent inhibitory activities against distinct isolates of HIV-1, HIV-2, and SIV.

Results

The M-T hook structure can greatly improve the inhibitory activity of diverse inhibitors on HIV-2. To develop a fusion inhibitor that is effective for both HIV-1 and HIV-2, we synthesized and characterized a large panel of CHR peptides (Table 1), including 11 previously reported peptides as controls and 15 newly designed M-T hook-modified peptides as new inhibitors.

First, we verified that most of the HIV-1 sequence-derived peptides had markedly decreased activities in inhibiting HIV-2 infection, such as C34, SFT, and SC29.
They inhibited NL4-3 replication with 50% inhibitory concentrations (IC_{50}s) of 1.02, 1.1, and 1.13 nM, respectively, but they inhibited ROD with IC_{50}s of 387.93, 105.36, and 237.87 nM, respectively. Even specifically designed HIV-2 sequence-based peptides, such as $C34_{EHO}$ and P3, had much weaker anti-HIV-2 activities than anti-HIV-1 activities. Second, we showed that addition of the M-T hook residues to the N terminus of peptides could dramatically increase their inhibitory potency on both HIV-1 and HIV-2. For example, the M-T hook-modified MTC34, MTSFT, and MTSC29 inhibited NL4-3 with IC_{50}s of 0.5, 0.51, and 0.43 nM, while they inhibited ROD with IC_{50}s of 76.61, 33.93, and 22.9 nM, respectively. Therefore, these results have demonstrated that the M-T hook structure is a vital tool for optimizing an inhibitor against both HIV-1 and HIV-2 isolates.

Table 1. Inhibitory activity of peptide fusion inhibitors on HIV-1 and HIV-2 isolates [a].

Inhibitor	Sequence (no. of aa[b])	Source	IC_{50} (nM)	
			$HIV\text{-}1_{NL4-3}$	$HIV\text{-}2_{ROD}$
Classic peptides				
T20	YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF (36)	HIV-1	111.93 ± 3.45	305.55 ± 84.01
C34	WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL (34)	HIV-1	1.02 ± 0.28	387.93 ± 78.32
SFT	SWETWEREIENYTRQIYRILEESQEQQDRNERDLLE (36)	HIV-1	1.1 ± 0.52	105.36 ± 15.08
CP32 M	VEWNEMTWMEWEREIENYTKLIYKILEESQEQ (32)	HIV-1	2.01 ± 0.48	370.95 ± 108.97
SC29	WEEWDKKIEEYTKKIEELIKKSEEQQKKN (29)	HIV-1	1.13 ± 0.84	237.87 ± 74.81
SC22	WEEWDKKIEEYTKKIEELIKKS (22)	HIV-1	54.58 ± 6.94	527.66 ± 80.52
T2635	TTWEAWDR AIAEYAARIEALIRAAQEQQEKNEAALREL (38)	HIV-1	0.38 ± 0.1	18.56 ± 3.21
T1249	WQEWEQKITALLEQAQIQQEKNEYELQKLDKWASLWEWF (39)	HIV-1/HIV-2/SIV	0.97 ± 0.34	10.42 ± 1.96
$C34_{ROD}$	WQEWEKQVRYLEANISKSLEQAQIQQEKNMYELQ (34)	HIV-2	5.51 ± 2.84	48.26 ± 3.7
$C34_{EHO}$	WQQWERQVRFLDANITKLLEEAQIQQEKNMYELQ (34)	HIV-2	1.88 ± 0.35	33.67 ± 4.96
P3	WQEWEQQVRYLEANISQRLEQAQIQQEKNMYELQ (34)	HIV-2/SIV	6.34 ± 1.94	83.23 ± 34.08
M-T hook modified peptides				
MTC34	MTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELL (36)	HIV-1	0.5 ± 0.07	76.61 ± 2.77
MTSFT	MTWETWEREIENYTRQIYRILEESQEQQDRNERDLLE (38)	HIV-1	0.51 ± 0.21	33.93 ± 7.24
MTSC29	MTWEEWDKKIEEYTKKIEELIKKSEEQQKKN (31)	HIV-1	0.43 ± 0.16	22.9 ± 3.9
MTSC22	MTWEEWDKKIEEYTKKIEELIKKS (24)	HIV-1	1.32 ± 0.08	252.24 ± 12.48
$MTC34_{ROD}$	MTWQEWEKQVRYLEANISKSLEQAQIQQEKNMYELQ (36)	HIV-2	0.84 ± 0.29	20.57 ± 2.28
$MTC34_{EHO}$	MTWQQWERQVRFLDANITKLLEEAQIQQEKNMYELQ (36)	HIV-2	1.17 ± 0.16	34.55 ± 2.5
MTP3	MTWQEWEQQVRYLEANISQRLEQAQIQQEKNMYELQ (36)	HIV-2/SIV	1.38 ± 0.32	24.6 ± 2.37
HP23	EMTWEEWEKK IEEYTKKIEEILK (23)	HIV-1	0.19 ± 0.01	78.57 ± 3.02
HP23L	ELTWEEWEKK IEEYTKKIEEILK (23)	HIV-1	0.39 ± 0.06	126.33 ± 9
$P21_{ROD}$	MTWQEWEKQVRYLEANISKSL (21)	HIV-2	571.8 ± 41.3	>1,250
$P21_{EHO}$	MTWQQWERQVRFLDANITKLL (21)	HIV-2	402.35 ± 165.1	>1,250
$P21_{P3}$	MTWQEWEQQVRYLEANISQRL (21)	HIV-2/SIV	534.45 ± 295.78	>1,250
2P23	EMTWEEWEKKVEELEKKIEELLK (23)	HIV-1/HIV-2	0.22 ± 0.05	10.57 ± 0.27
2P23L	ELTWEEWEKK VEELEKKIEELLK (23)	HIV-1/HIV-2	0.59 ± 0.08	18.56 ± 1.31
2P23Q	EMTWQEWEQKVEELEKKIEELLK (23)	HIV-1/HIV-2	1.39 ± 0.45	31.87 ± 7.02

[a] The assay was performed in triplicate and repeated at least 3 times. Data are expressed as means standard deviations.

[b] aa, amino acids.

The M-T hook structure can greatly enhance the binding stability on HIV-2.

We previously demonstrated that the M-T hook structure can dramatically enhance the binding affinity of inhibitors to the target by using HIV-1 NHR-derived peptide N36 as a target surrogate [37, 38]. To get insights into the mechanism of action, we characterized the interaction between inhibitors and HIV-2 by performing CD spectroscopy. To this end, we synthesized the HIV-2 NHR-derived peptide $N36_{ROD}$as a target and then compared seven pairs of peptides (C34/MTC34, SFT/MTSFT, SC29/MTSC29, SC22/MTSC22, $C34_{ROD}$/$MTC34_{ROD}$, $C34_{EHO}$/$MTC34_{EHO}$, and P3/MTP3) for their binding stability. Interestingly, all of the M-T hook-modified peptides displayed significantly increased α-helicity compared to their templates (Table 2 and Figure 1). Thermal denaturation analyses showed that the addition of the M-T hook structure markedly increased the T_m (melting temperature) values of the 6-HB complexes formed between inhibitors and each of HIV-1 and HIV-2 N36 peptides (Table 2 and Figure 2).

Table 2. Interactions of inhibitors with HIV-1-, HIV-2-, and SIV-derived targets determined by CD spectroscopy [a].

	$N36_{NL4-3}$		$N36_{ROD}$		$N36_{SIV251}$	
Inhibitor	**% helix**	**T_m (℃)**	**% helix**	**T_m (℃)**	**% helix**	**T_m (℃)**
C34	84,5	63,62	30,88	NA	15,61	NA
MTC34	71,95	68,57	50,93	30,76	20,87	NA
SFT	81	69,18	65,64	32,96	25,48	NA
MTSFT	91,68	75,13	102,37	43,76	45,58	39,04
SC29	94,03	65,22	44,4	NA	32,33	NA
MTSC29	98,69	73,98	82,95	46,51	65,13	42,42
SC22	77,01	60,7	43,77	NA	22,09	NA
MTSC22	84,97	71,3	70,05	30,96	71,72	69,93
$C34_{ROD}$	70,87	59,06	50,52	32,91	21,82	NA
$MTC34_{ROD}$	70,22	68,39	100,36	50,4	56,61	45,84
$C34_{EHO}$	70,93	65,83	93,29	45,08	53,12	41,08
$MTC34_{EHO}$	70,18	73,52	107,34	58,24	61,65	53,95
P3	62,79	61,29	61,99	35,41	24,08	NA
MTP3	67,38	69,07	96,72	49,66	45,79	46,01
HP23	86,36	82,18	103,49	43,54	44,99	34,41
$P21_{ROD}$	70,39	48,77	40,51	NA	24,56	NA
$P21_{EHO}$	89,21	55,47	42	NA	18,08	NA
$P21_{P3}$	46,54	NA	32,92	NA	27,61	NA
2P23	102,26	78,79	117,85	55,26	62,51	47,35

[a]The assay was performed 2 times and results are expressed as means.

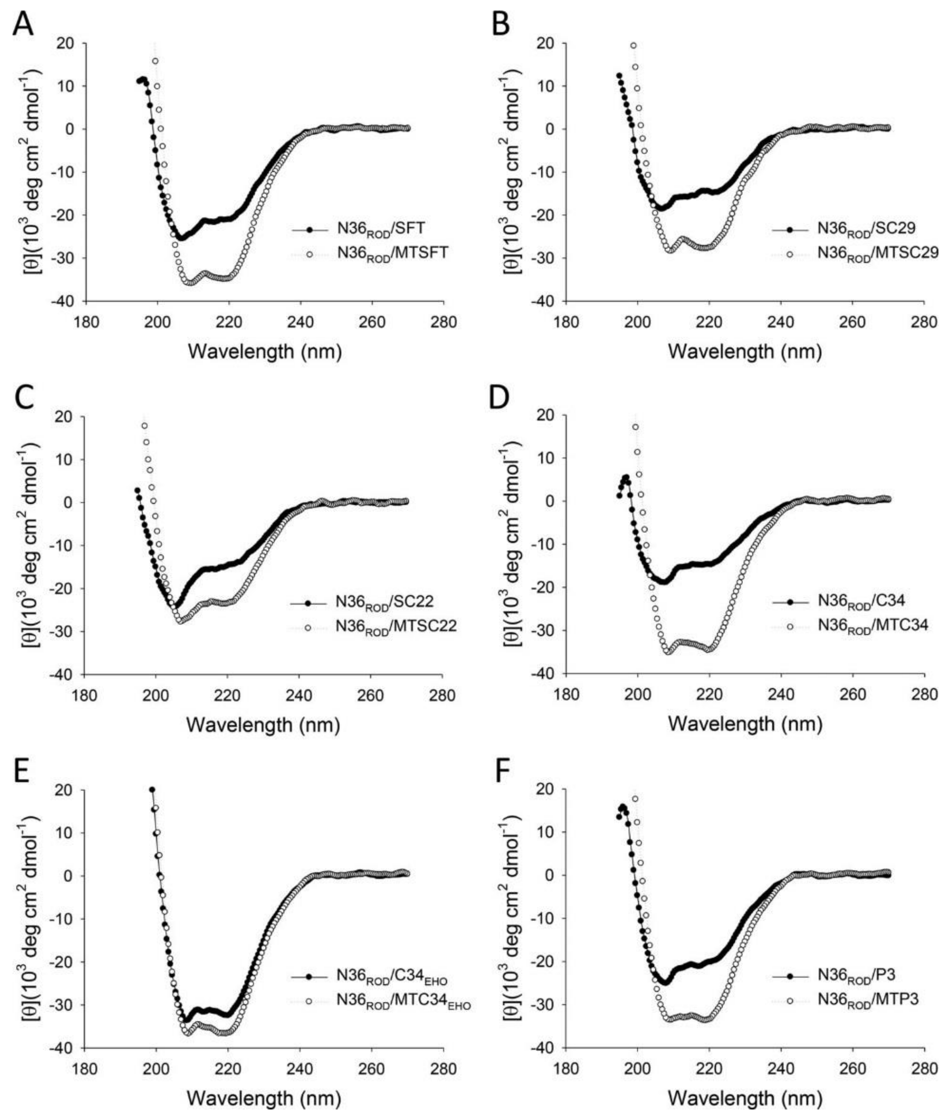

Figure 1. α-Helicity of peptide inhibitors with $N36_{ROD}$ determined by CD spectroscopy. (A) SFT and MTSFT; (B) SC29 and MTSC29; (C) SC22 and MTSC22; (D) $C34_{ROD}$ and $MTC34_{ROD}$; (E) $C34_{EHO}$ and $MT\text{-}C34_{EHO}$; (F) P3 and MTP3. The final concentration of each peptide in PBS was 10 M.

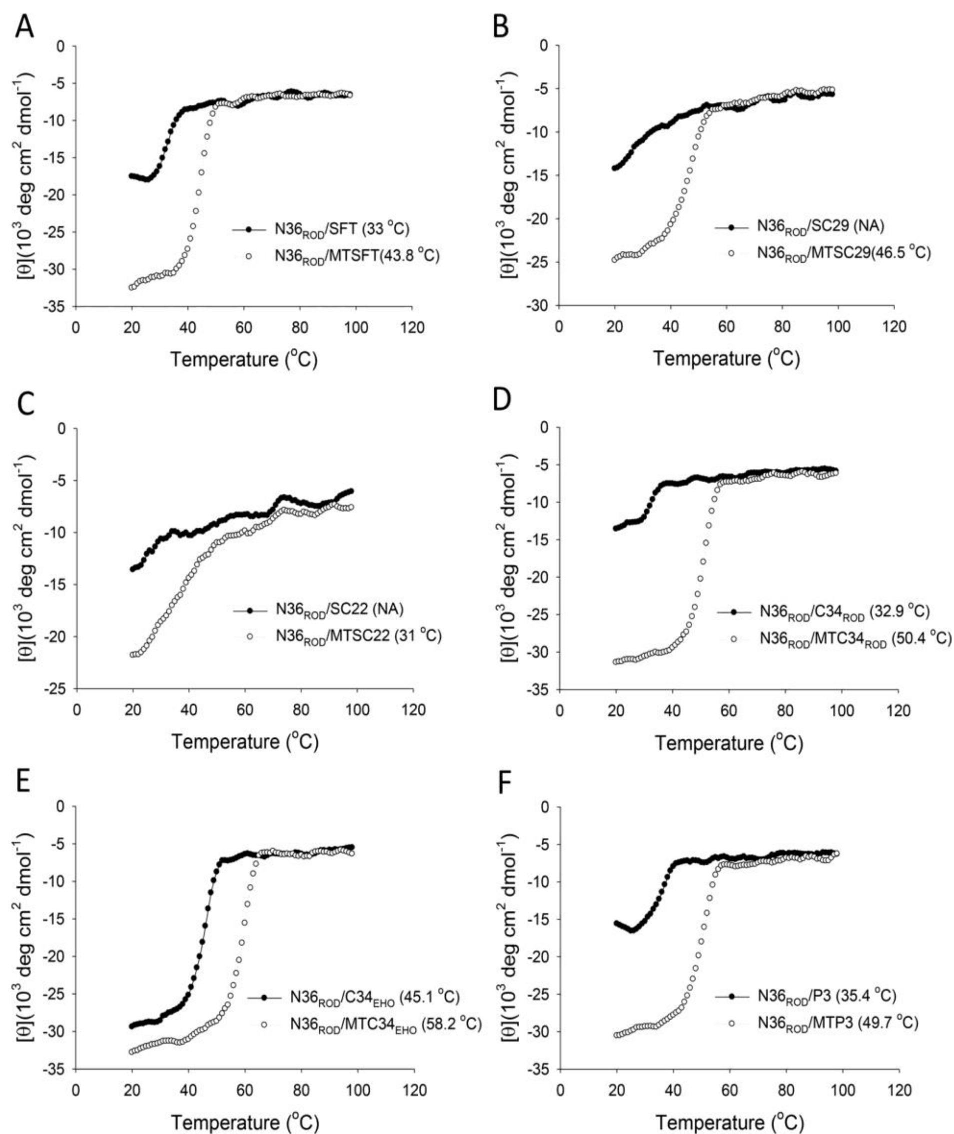

Figure 2. Binding stability of peptide inhibitors with $N36_{ROD}$ determined by CD spectroscopy. (A) SFT and MTSFT; (B) SC29 and MTSC29; (C) SC22 and MTSC22; (D) $C34_{ROD}$ and $MTC34_{ROD}$; (E) $C34_{EHO}$ and $MT\text{-}C34_{EHO}$; (F) P3 and MTP3. Final concentration of each peptide in PBS was 10μM.

Design of a novel short-peptide inhibitor effective on both HIV-1 and HIV-2. Recently, we demonstrated that a short-peptide fusion inhibitor with potent anti-HIV-1 activity could be developed on the basis of M-T hook structure [37, 40, 41]. HP23 and its mutant, HP23L, have only 23 amino acids, but they possess highly potent activity in inhibiting diverse subtypes of HIV-1 isolates and T20-resistant variants. However, our results shown here indicated that both HP23 and HP23L had dramatically reduced activities on HIV-2 ROD, with IC_{50}s of 0.19 versus 78.57 nM and 0.39 versus 126.33 nM, respectively (Table 1). We therefore decided to develop a short-peptide fusion inhibitor that is effective on both HIV-1 and HIV-2 isolates by using the M-T hook strategy and HIV-2 sequence. Disappointingly, three HIV-2-derived short peptides with the M-T hook residues ($P21_{ROD}$, $P21_{EHO}$, and $P21_{P3}$) exhibited poor inhibition on HIV-1 and no inhibition on HIV-2 (Table 1). Encouragingly, a 23-mer peptide, named 2P23, was successfully designed by introducing the critical residues for HIV-2 binding, the salt bridges for peptide stability, and an N-terminal capping residue. First, 2P23 had dramatically improved binding activities on both HIV-1 and HIV-2. As shown in Table 2 and Figures 3A to D, 2P23 bound HIV-1 N36 and HIV-2 N36 with $T_m$values of 78.79 and 55.26°C, respectively. Second, 2P23 had largely increased inhibitory activities. As shown in Table 1 and Figures 3E and F, it inhibited HIV-1 and HIV-2 with IC_{50}s of 0.22 and 10.57 nM, respectively, which were much better than those of HP23. Taken together, these results suggested that 2P23 has promising features as a novel fusion inhibitor peptide.

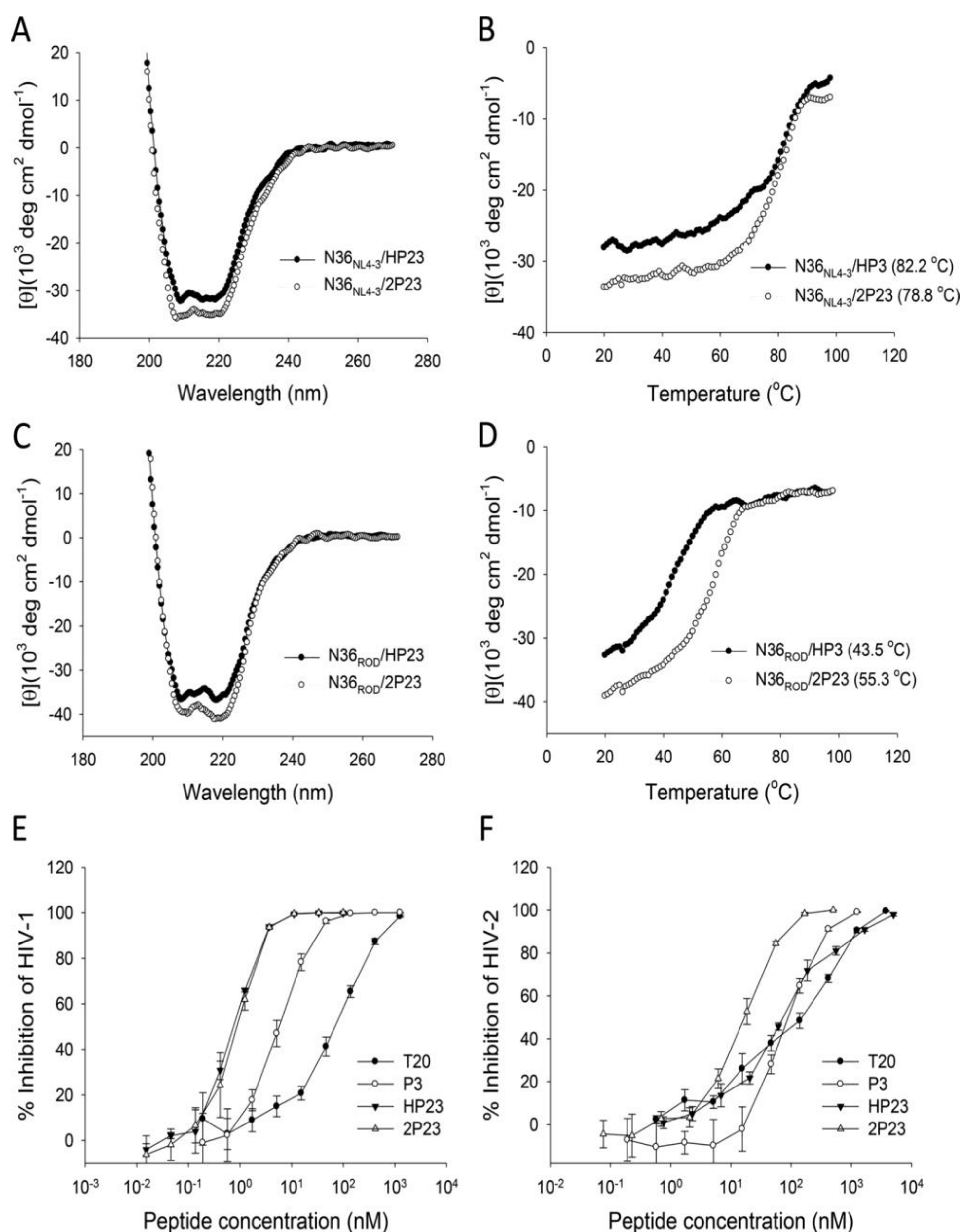

Figure 3. Biophysical properties and anti-HIV activity of 2P23 and control peptides. (A) The α - helicity of HP23 and 2P23 in complexes with N36NL4-3. (B) The thermostability of HP23 and 2P23 in complexes with N36NL4-3. (C) The α-helicity of HP23 and 2P23 in complexes with N36ROD. (D) The thermostability of HP23 and 2P23 in complexes with N36ROD. (E) Inhibition of 2P23 and control peptides (T20, P3, and HP23) on infection of HIV-1NL4-3. (F) Inhibition of 2P23 and control peptides (T20, P3, and HP23) on infection of HIV-2_{ROD}. CD experiments were performed with a final concentration of each peptide at 10 M. The inhibition assays were performed in triplicate and repeated 3 times. Percent inhibition of the peptides and IC50s were calculated as described in the text. Data are expressed as means standard deviations (SD).

2P23 efficiently inhibits SIV isolates. We sought to determine whether 2P23 was active against SIV isolates, which are believed to have crossed the species barrier into humans, resulting in HIV-2 and HIV-1. First, we synthesized the SIV NHR-derived peptide $N36_{SIV251}$ as a target and determined its interactions with HP23 and 2P23. As shown in Figures 4A and B, 2P23 could interact with $N36_{SIV251}$ much more effectively than HP23, with a T_m value of 47.35 versus 34.41°C. We then generated two SIV Env-pseudotyped viruses, SIV_{pbj} and SIV_{239}, and used them in single-cycle infection assays to evaluate the inhibitory activity of 2P23 and three control peptides (T20, P3, and HP23). As shown in Figures 4C and D, 2P23 efficiently inhibited SIV_{pbj} and SIV_{239} with IC_{50}s of 9.96 and 3.34 nM, respectively; in sharp contrast, T20, P3, and HP23 had dramatically decreased activities in inhibiting both SIV isolates. T20, P3, and HP23 inhibited SIV_{pbj} with IC_{50}s of 190.8, 121.8, and 247.7 nM, respectively, and inhibited SIV_{239} with IC_{50}s of 297.67, 17.5, and 105.65 nM, respectively.

2P23 efficiently inhibits HIV- and SIV-mediated cell-cell fusion. We next determined the inhibitory activity of 2P23 and three control peptides (T20, HP23, and P3) on viral Env-mediated cell-cell fusion by a DSP-based assay. In line with its inhibition on viral infection, 2P23 exhibited the most potent activity. As shown in Figure 5A, 2P23 inhibited HIV-$1_{NL4\text{-}3}$ Env-mediated cell fusion with a mean $IC_{50}$of 0.24 nM, whereas T20, P3, and HP23 had mean IC_{50}s of 7.89, 2.25, and 0.33 nM, respectively. Similarly, 2P23 inhibited SIV Env-mediated cell fusion efficiently, with an IC_{50} of 1.8 nM on SIV_{pbj} (Figure 5B) and an IC_{50} of 2.39 nM on SIV_{239} (Figure 5C). In sharp contrast, three control peptides had markedly decreased inhibitory activity on SIV Env. T20, P3, and HP23 inhibited SIV_{pbj} at IC_{50}s of 8.35, 3.94, and 7.8 nM, respectively, and they inhibited SIV_{239} with IC_{50}s of 217.33, 6.55, and 17.68 nM, respectively.

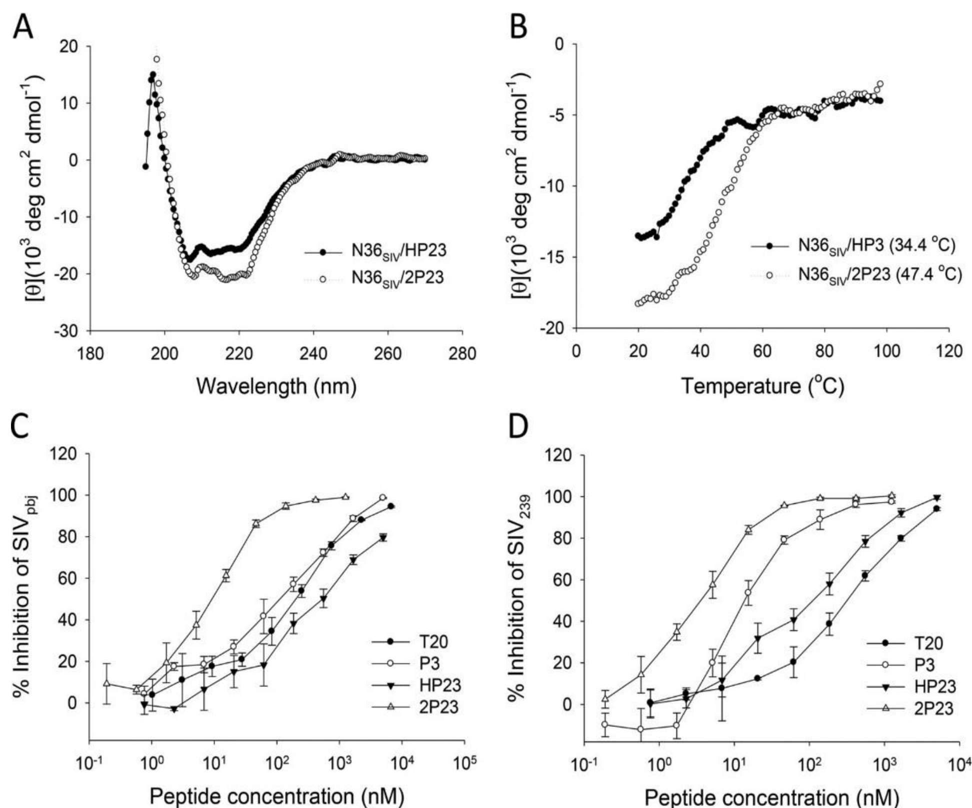

Figure 4. Biophysical properties and anti-SIV activity of 2P23 and control peptides. (A) The α-helicity of HP23 and 2P23 in complexes with $N36_{SIV251}$. (B) The thermostability of HP23 and 2P23 in complexes with $N36_{SIV251}$. (C) Inhibition of 2P23 and control peptides (T20, P3, and HP23) on SIV_{pbj} Env-pseudotyped virus in single-cycle assay. (D) Inhibition of 2P23 and control peptides (T20, P3, and HP23) on SIV_{239} Env-pseudotyped virus in single-cycle assay. CD experiments were performed with a final concentration of each peptide at 10 M. Single-cycle infection assays were performed in triplicate and repeated 3 times. Percent inhibition of the peptides and IC_{50}s were calculated. Data are expressed as means SD.

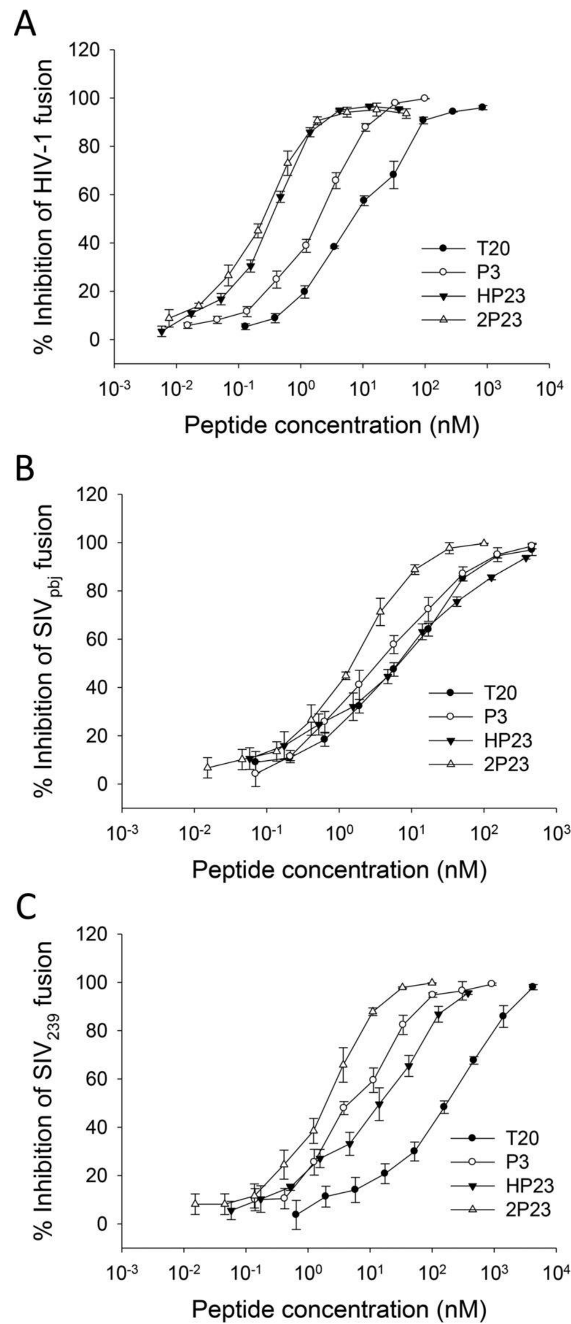

Figure 5. Inhibitory activity of 2P23 and control peptides on Env-mediated cell fusion. Inhibition of 2P23 and control peptides on HIV-1$_{NL4-3}$ Env (A), SIV$_{pbj}$ Env (B), and SIV$_{239}$ Env-mediated cell-cell fusion was measured by DSP-based assays. The experiments were performed in triplicate and repeated at least 2 times. Percent inhibition of the peptides and IC$_{50}$s were calculated. Data are expressed as means SD.

2P23 is a potent inhibitor of primary HIV-1 isolates and T20-resistant mutants. As a potential inhibitor for further development, we were intrigued to know whether 2P23 was active, like HP23, on distinct subtypes of HIV-1 isolates and the fusion inhibitor-resistant mutants. Therefore, we assembled a panel of 29 HIV-1 Envs (Table 3), including 3 from subtype A, 6 from subtype B, 3 from subtype B′, 6 from subtype C, 1 from subtype G, 1 from subtype A/C, 4 from subtype A/E, and 5 from subtype B/C. Among them, 12 Env proteins were recently described as a global panel reference that represents the genetic and antigenic diversities of HIV-1 [42]. All of the corresponding pseudoviruses were generated, quantified, and used in single-cycle infection assays. As shown in Table 3, 2P23 potently inhibited diverse subtypes of HIV-1 isolates with a mean IC_{50} of 5.57 nM, which was comparable with that of HP23 (4.7 nM). As controls, T20 and P3 inhibited HIV-1 isolates with mean IC_{50}s of 31.49 and 24.35 nM, respectively.

We also constructed a panel of 15 HIV-1_{NL4-3}-based pseudoviruses with Envs carrying T20- or HP23-resistant mutations [43, 44]. The inhibition data in Table 4 showed that (i) the long peptides T20 and P3 exhibited relatively higher resistance on T20-resistant mutants, but the short peptides HP23 and 2P23 could maintain their potency, and (ii) 2P23 also displayed improved inhibition over some HP23-resistant mutants (e.g., L57R and L57R/E136G). Taken together, these results indicated that 2P23 is a highly effective fusion inhibitor against diverse subtypes of primary HIV-1 isolates and T20-resistant mutants.

Table 3. Inhibitory activity of 2P23 and control inhibitors on diverse subtypes of HIV-1 isolates [a].

Pseudovirus	Subtype	IC_{50} (nM)			
		T20	P3	HP23	2P23
92RW020	A	10.83 ± 1.65	21.3 ± 3.63	3.28 ± 0.55	2.71 ± 0.92
92UG037.8	A	6.68 ± 1.15	8.56 ± 0.98	2.73 ± 0.26	2.16 ± 0.28
398-F1_F6_20[b]	A	30.48 ± 10.8	24.22 ± 3.4	2.48 ± 0.37	1.6 ± 0.31
SF162	B	7.17 ± 1.3	43.95 ± 1.22	22.10 ± 0.95	15.77 ± 2.48
JRFL	B	49.38 ± 28.4	24.69 ± 2.23	24.51 ± 3.54	8.27 ± 3.24
AC10.0.29	B	3.63 ± 0.38	1.8 ± 0.07	1.13 ± 0.18	1.74 ± 0.54
SC422661.8	B	13.28 ± 1.94	5.88 ± 0.01	1.45 ± 0.06	2.56 ± 0.49
TRO.11[b]	B	18.75 ± 6.37	30.77 ± 7.67	3.68 ± 1.41	5.88 ± 2.37
X2278_C2_B6[b]	B	10.86 ± 1.9	9.85 ± 1.45	2.27 ± 0.4	1.05 ± 0.07
B01	B′	54.48 ± 21.13	68.93 ± 7.19	2.88 ± 0.12	6.35 ± 0.21
B02	B′	42 ± 14.42	83.27 ± 0.04	4.37 ± 1.48	6.56 ± 3.86
B04	B′	12.25 ± 2.52	37.45 ± 18.49	3.09 ± 1.15	6.36 ± 3.02
CAP45.2.00.G3	C	161.36 ± 28.92	87.27 ± 5.19	8.14 ± 4.69	23.59 ± 9.03
ZM109F.PB4	C	12.80 ± 0.76	3.02 ± 0.4	0.91 ± 0.07	1.18 ± 0.21
ZM53 M.PB12	C	35.19 ± 2.13	4.06 ± 0.82	0.91 ± 0.18	1.22 ± 0.43
CE703010217_B6[b]	C	12.36 ± 1.66	8.3 ± 2.98	2.12 ± 0.41	4.52 ± 1.19
CE1176_A3[b]	C	13.56 ± 2.82	22.46 ± 5.54	3.59 ± 0.25	5.20 ± 0.26
HIV_25710-2.43[b]	C	23.38 ± 4.61	5.57 ± 0.44	2.38 ± 0.46	2.53 ± 0.7
X1632-S2-B10[b]	G	12.61 ± 1.77	13.71 ± 0.22	4.25 ± 0.08	4.40 ± 0.99
246_F3_C10_2[b]	A/C	28.59 ± 7.33	11.17 ± 3.15	5.15 ± 1.23	3.12 ± 1.43
AE03	A/E	5.09 ± 2.33	35.38 ± 5.15	1.43 ± 0.54	6.57 ± 0.17
AE04	A/E	9.25 ± 0.62	27.95 ± 6.13	4.33 ± 1.76	8.48 ± 0.67
CNE8[b]	A/E	40.76 ± 18.17	24.76 ± 2.17	4.99 ± 0.08	9.2 ± 1.38
CNE55[b]	A/E	30.82 ± 11.68	18.93 ± 2.04	2.26 ± 0.46	3.25 ± 0.06
CH64.20	B/C	20.15 ± 0.28	2.02 ± 0.32	0.6 ± 0.23	0.61 ± 0.14
CH070.1	B/C	176.6 ± 39.27	26.51 ± 8.71	6.05 ± 0.55	7.83 ± 1.39
CH120.6	B/C	30.25 ± 0.69	29.69 ± 7.2	7.33 ± 0.21	10.19 ± 0.71
CH119.10[b]	B/C	6.21 ± 0.86	13.69 ± 9.07	3.69 ± 0.13	7.33 ± 0.42
BJOX002000.03.2[b]	B/C	34.37 ± 17.2	10.95 ± 3.51	4.06 ± 1.21	1.37 ± 0.57
Mean IC_{50} (range)		31.49 (3.63~176.6)	24.35 (1.8~87.27)	4.7 (0.6~24.51)	5.57 (0.61~23.59)

[a]The assay was performed in triplicate and repeated at least 3 times. Data are expressed as means ± standard deviations.

[b]A global panel of HIV-1 isolates representing the genetic and antigenic diversities worldwide.

Table 4. Inhibitory activity of 2P23 and control peptides on drug-resistant HIV-1 mutants [a].

	T-20		P3		HP23		2P23	
HIV-1$_{NL4-3}$	**IC_{50} (nM)**	**n-fold**	**IC_{50} (nM)**	**n-fold**	**IC_{50} (nM)**	**n-fold**	**IC_{50} (nM)**	**n-fold**
WT	84.09 ± 13.84	1	7.69 ± 0.31	1	0.61 ± 0.13	1	0.69 ± 0.15	1
T20-resistants								
I37T	659.92 ± 79.83	7.85	62.64 ± 0.49	8.15	1.35 ± 0.16	2.21	1.22 ± 0.25	1.77
V38A	1514.55 ± 246.72	18.01	56.45 ± 10.52	7.34	1.07 ± 0.04	1.75	0.89 ± 0.23	1.29
V38M	689.42 ± 162.86	8.2	34.03 ± 5.27	4.43	0.99 ± 0.14	1.62	1.21 ± 0.14	1.75
Q40H	2207.22 ± 519.43	26.25	107.01 ± 21.72	13.92	1.06 ± 0.04	1.74	1 ± 0.27	1.45
N43K	681.7 ± 161.14	8.11	812.6 ± 67.36	105.67	0.79 ± 0.1	1.3	1.13 ± 0.23	1.64
D36S/V38M	471.88 ± 84.14	5.61	16.67 ± 1.55	2.17	1.39 ± 0.32	2.28	1.48 ± 0.22	2.14
I37T/N43K	6075 ± 1572.61	72.24	>2000	>260.08	1.42 ± 0.13	2.33	1.45 ± 0.32	2.1
V38A/N42T	3785.94 ± 1268.36	45.02	86.21 ± 3.43	11.21	0.57 ± 0.13	0.93	0.44 ± 0.1	0.64
HP23-resistants								
E49K	165.4 ± 19.6	1.97	87.38 ± 8.88	11.36	4.45 ± 0.71	7.3	5.2 ± 0.14	7.54
L57R	86.78 ± 4.41	1.03	38.96 ± 1.79	5.07	133.68 ± 5.84	219.15	39.49 ± 0.19	57.23
N126K	182.98 ± 38.03	2.18	12.08 ± 1.28	1.57	1.76 ± 0.04	2.89	1.59 ± 0.77	2.3
E136G	211.4 ± 18.71	2.51	22.72 ± 0.11	2.95	4.73 ± 1.1	7.75	4.66 ± 0.91	6.75
E49K/N126K	203.1 ± 18.48	2.42	134.04 ± 9.33	17.43	5.01 ± 0.45	8.21	4.38 ± 0.8	6.35
L57R/E136G	43.13 ± 14.64	0.51	65.67 ± 2.69	8.54	429.62 ± 93.64	704.3	175.12 ± 46.72	253.8

[a]The assay was performed in triplicate and repeated 3 times. Data are expressed as means ± standard deviations.

2P23 is a potent fusion inhibitor of diverse primary HIV-2 isolates. One of the main purposes of this study was to create a short-peptide fusion inhibitor that is active for both HIV-1 and HIV-2 isolates. Our above-described data demonstrated that 2P23 had potent activities against a large panel of HIV-1 isolates, one HIV-2 isolate (ROD), and two SIV isolates (SIV_{pbj} and SIV_{239}).

In order to demonstrate whether 2P23 had a broad-spectrum anti-HIV-2 activity, we further measured its inhibition on a panel of primary HIV-2 isolates and a panel of ROD-based mutants which utilize different coreceptors [14, 24, 45]. Apart from P3 and HP23, the previously reported third-generation peptide inhibitors SFT and T2635 were also included as controls. As shown in Table 5, 2P23 was able to efficiently inhibit infection of distinct primary HIV-2 isolates and ROD mutants, with mean IC_{50}s of 20.17 and 15.38 nM, respectively.

T2635 also exhibited similar inhibitory activity on two panels of viruses, showing mean IC_{50}s of 17.21 and 34.83 nM, respectively. In contrast, SFT, P3, and HP23 showed significantly decreased anti-HIV-2 activity, as they inhibited primary HIV-2 isolates with mean IC_{50}s of 69.96, 64.76, and 62.39 nM, respectively, and inhibited ROD mutants with mean IC_{50}s of 226.12, 191.09, and 94.69 nM, respectively. We conclude therefore that 2P23 is an ideal inhibitor of diverse HIV-2 isolates.

Table 5. Inhibitory activity of 2P23 and control peptides on diverse HIV-2 isolates [a].

	IC_{50} (nM)				
HIV-2	SFT	T2635	P3	HP23	2P23
Primary isolates					
00PTHDECT (R5)	30.71 ± 8.86	7.35 ± 3.2	48.89 ± 4.28	99.82 ± 8.29	21.96 ± 5.05
03PTHCC6 (R5)	63.69 ± 0.08	30.11 ± 9.11	114.65 ± 13.45	44.47 ± 14.13	14.51 ± 1.12
03PTHCC19 (R5)	25.7 ± 15.76	43.38 ± 37.01	121.0 ± 11.9	9.14 ± 8.72	7.35 ± 1.57
03PTHCC1 (R5)	16.49 ± 1.47	4.79 ± 1.15	19.36 ± 8.37	2.22 ± 2.08	2.99 ± 0.76
00PTHCC20 (X4)	15.02 ± 13.61	6.79 ± 1.41	13.28 ± 0.65	27.19 ± 5.14	8.74 ± 2
10PTHSMNC (R5)	55.87 ± 2.58	5.33 ± 0.21	86.7 ± 19.3	32.85 ± 8.14	16.4 ±0.31
03PTHCC12 (R5)	58.85 ± 2.71	16.51 ± 0.12	72.85 ± 19.42	29.3 ± 2.08	24.84 ± 0.53
03PTHSM2 (R5)	98.91 ± 12.99	22.33 ± 0.41	44.02 ± 2.98	98.39 ± 11.22	25.72 ± 2.15
03PTHSM9 (X4)	264.45 ± 32.15	18.29 ± 3.3	62.12 ± 2.07	218.15 ± 37.45	59.04 ± 0.83
Mean IC_{50} (range)	69.96 (15.02 ~ 264.45)	17.21 (4.79 ~ 43.38)	64.76 (13.28 ~ 114.65)	62.39 (2.22 ~ 218.15)	20.17 (2.99 ~ 59.04)
ROD and its mutants					
ROD10 WT (X4)	188 ± 13.5	24.77 ± 14.48	80.59 ± 1.86	57.08 ± 18.31	13.22 ± 2.67
H18L (R5/X4)	312.3 ± 9	49.44 ± 2.54	228.35 ± 8.75	178.8 ± 63.9	16.99 ± 0.56
d23d24 (R5/X4)	174.7 ± 0.3	19.49 ± 0.36	172.65 ± 30.05	82.21 ± 6.35	10.35 ± 0.23
K29T (X4)	179.5 ± 12.7	42.24 ± 0.82	197.15 ± 15.65	52.87 ± 3.88	11.8 ± 1.04
H18L+d23d24 (R5)	274.05 ± 3.35	39.22 ± 1.28	262.3 ± 29.8	113.65 ± 14.66	21.69 ± 1.41
H18L+K29T (R5/X4)	219.55 ± 5.75	24.65 ± 0.52	163.95 ± 1.05	78.41 ± 6.47	15.14 ± 0.47
H18L+d23d24+K29T (R5)	234.75 ± 14.55	44 ± 0.19	232.65 ± 33.35	99.84 ± 5.27	18.46 ± 0.2
Mean IC_{50} (range)	226.12 (174.7 ~ 312.3)	34.83 (19.49 ~ 49.44)	191.09 (80.59 ~ 262.3)	94.69 (52.87 ~ 178.8)	15.38 (10.35 ~ 21.69)

Structural properties of 2P23 in itself. To get more insights into the mechanism underlying the binding and antiviral activities of 2P23 peptide, we determined its own secondary structure and stability by CD spectroscopy. The peptide inhibitors HP23, T20, C34, SFT, T1249, T2635, and P3 were also analyzed for comparison. As shown in Figures 6A and B, 2P23 alone exhibited high α-helicity at different peptide concentrations, and its thermal unfolding transition (T_m) was dependent on the peptide concentration, which indicated its helical and oligomeric features were similar to those of HP23 (Figures 6C and D); however, both the helical contents and T_m values of 2P23 at each concentration were much higher than those of HP23.

In sharp contrast, T20, C34, and SFT had little or no α-helicity, suggesting their random conformation, while T1249 and P3 displayed much lower levels of helical structures.

Although the helical content of the electronically constrained peptide T2635 was comparable to that of 2P23, it had a significantly lower $T_m$value (40.3 versus 48.1°C), as demonstrated by the data shown in Figures 6E and F. These results suggest that 2P23 is a helical, oligomeric short-peptide fusion inhibitor having high stability.

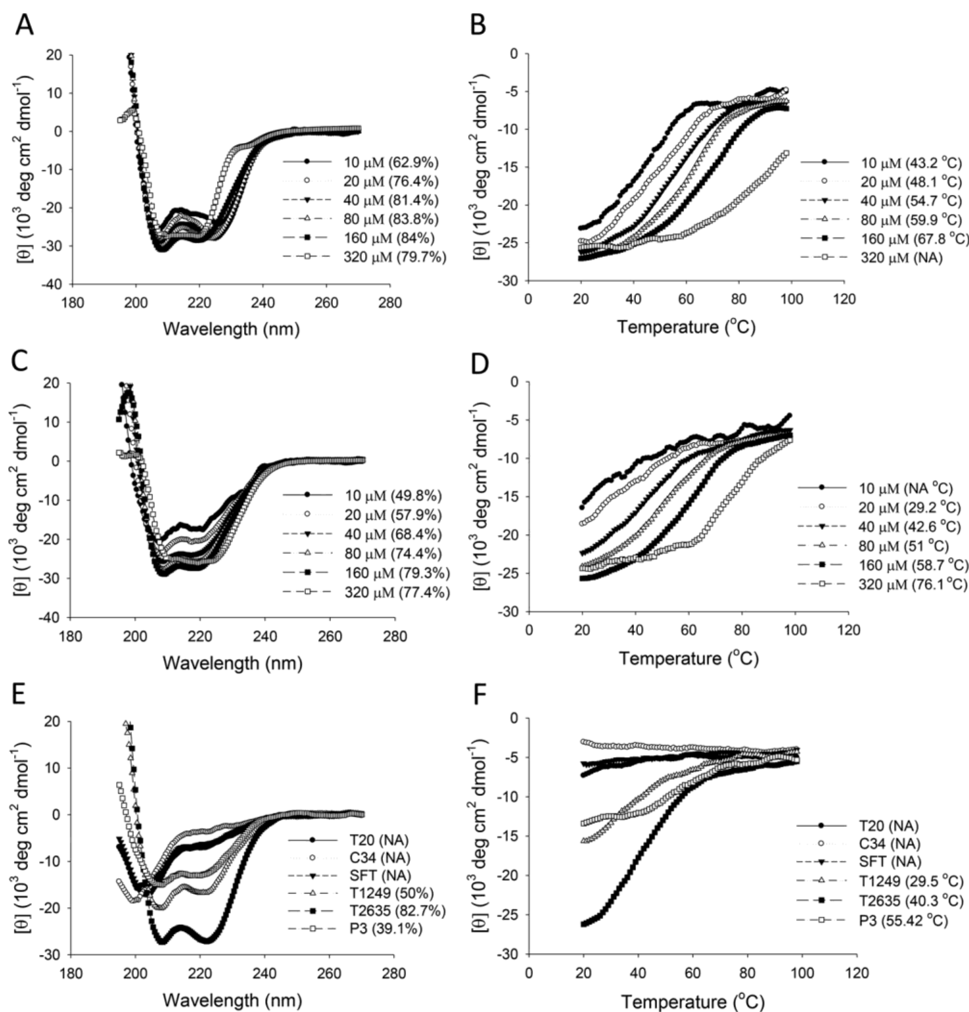

Figure 6. Secondary structure and stability of 2P23 and control peptides determined by CD spectroscopy. The α-helicity (A) and thermostability (B) of 2P23 in itself and the α-helicity (C) and thermostability (D) of HP23 in itself were measured at different concentrations in PBS. The α-helicity (E) and thermostability (F) of control peptides (T20, C34, SFT, T1249, T2635, and P3) were measured at a final concentration of 20 M in PBS. The helical contents and T_m values are shown in parentheses. NA means not applicable for precise calculation. The experiments were repeated at least two times, and representative data are shown.

Discussion

In the present study, we have dedicated our efforts to developing a short-peptide fusion inhibitor that is effective on both HIV-1 and HIV-2 isolates. First, we verified that the M-T hook structure strongly boosts the binding and inhibitory activities of CHR-based peptides to the NHR target of HIV-2 isolates, as it does for HIV-1 isolates. We then successfully designed a 23-mer helical peptide, termed 2P23, by adding the M-T hook structure and HIV-2 sequences, which can enhance the inhibitor binding to its target, and introducing the salt bridges that can stabilize the helical structure of the peptide *per se*. Promisingly, 2P23 does show a very potent and broad-spectrum antiviral activity that includes HIV-1, HIV-2, and SIV.

Human (HIV-1/2) and simian (SIV) immunodeficiency viruses infect host cells by fusion of the viral and cellular membranes, which is mediated by viral Env glycoprotein consisting of the surface subunit, gp120, and the transmembrane subunit, gp41. Binding of gp120 to the cellular receptor CD4 and a chemokine coreceptor initiates the fusogenic activity of gp41, resulting in a prehairpin intermediate state in which the fusion peptide of gp41 is inserted into the target membrane. Ultimately, three C-terminal helices (CHR) pack in an antiparallel orientation onto the trimeric coiled coil of N-terminal helices (NHR) to form a six-helix bundle (6-HB) structure, which drives the apposition of the viral and cell membranes, resulting in concomitant cell fusion [18, 46, 47]. Peptide fusion inhibitors can bind to the exposed NHR or CHR during the prehairpin stage, thereby blocking the formation of 6-HB in a dominant-negative manner [18, 19, 21]. However, it was found that the only clinically available HIV-1 fusion inhibitor peptide, T20, and most of the newly developed next-generation peptides had significantly decreased activity in inhibiting HIV-2 isolates (Table 1), thus limiting their potential use for the treatment of HIV-2-infected patients. As noted, the second-generation inhibitor T1249 and the third-generation inhibitor T2635 did exhibit improved potency over HIV-2, but their large sizes (39-mer and 38-mer, respectively) would hamper their formulation and production cost. In an advance stage, the third-generation inhibitor SFT (sifuvirtide) has been approved for clinical phase III trials in China and will hopefully become the next HIV-1 fusion inhibitor in clinical use [33, 35, 48]. Nonetheless, our data here indicate that SFT has dramatically decreased inhibitory activity on HIV-2 (Table 1).

Additionally, SFT has a similar low genetic barrier to the development of resistance, and the selected HIV-1 variants display high cross-resistance to T20 [35, 49]. These data emphasize the importance of developing new fusion inhibitors with significantly improved pharmaceutical profiles.

The structures of both HIV- and SIV-derived 6-HBs revealed the atomic interactions between the NHR and CHR sequences and identified a deep hydrophobic pocket on the NHR helices, which is penetrated by the pocket-binding domain (PBD) of the CHR helix [18, 19, 21, 50-52]. Many studies demonstrated that the deep pocket critically determines the NHR-CHR interaction as well as inhibitor binding [18, 19, 21]. Our previous studies demonstrated that the M-T hook residues (Met115 and Thr116) preceding the PBD of a CHR peptide can mediate extensive hydrophobic interactions with the pocket, thus dramatically fortifying the binding affinity and antiviral activity of inhibitors [35, 37-39]. The results shown here demonstrate that the M-T hook structure also functions well for inhibiting HIV-2 and SIV isolates and suggest that the pocket site is highly conserved among HIV-1/2 and SIV. Importantly, the results also suggest that the M-T hook structure is a general strategy for designing fusion inhibitors with broad-spectrum activity. Obviously, the M-T hook structure is not the only factor for the excellent performance of 2P23. The second design strategy is introducing the residues that are critical for binding HIV-2 NHR, such as valine (V), leucine (L), and glutamic acid (E) (Table 1). This is clear when comparing 2P23 and HP23, since both have the M-T hook residues, but 2P23 exhibited greatly improved binding and inhibitory activities to HIV-2 and SIV isolates. The third player for 2P23 is a group of introduced salt bridges, which can facilitate the helical conformation of inhibitor and also stabilize its binding to the NHR target.

In summary, 2P23 has prominent advantages over many other peptide HIV fusion inhibitors. First, it is highly effective on both HIV-1 and HIV-2 isolates. Second, it is only 23 amino acids in length, which will significantly benefit its production. Third, 2P23 binds to the targets with high stability, which can confer a high genetic barrier to resistance. Therefore, we conclude that 2P23 has high potential for clinical development. Also, it provides a novel tool for exploring the mechanisms of HIV and SIV Env-mediated cell fusion.

Materials and methods

Cells and reagents. HEK293T cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD). TZM-bl indicator cells stably expressing large amounts of CD4 and CCR5, along with endogenously expressed CXCR4, plasmids for HIV-1 Env panels (subtypes A, B, B′, C, G, A/C, A/E, and B/C), and molecular clones of HIV reference strains (HIV-1_{NL4-3} and HIV-2_{ROD}) were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH.

Two plasmids encoding SIV Env (pSIVpbj-Env and pSIV239) were kindly provided by Jianqing Xu at the Shanghai Public Health Clinical Center & Institutes of Biomedical Sciences, Fudan University, China. Cells were cultured in complete growth medium that consisted of Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1× MEM nonessential amino acids (Gibco/Invitrogen, USA) and were maintained at 37°C in 5% CO_2.

Peptide synthesis. A total of 29 CHR- or NHR-derived peptides were synthesized using a standard solid-phase 9-flurorenylmethoxycarbonyl (FMOC) method as described previously [39]. All peptides were acetylated at the N terminus and amidated at the C terminus. Peptide concentrations were determined using UV absorbance and a theoretically calculated molar extinction coefficient, ε, at 280 nm of 5,500 and 1,490 mol/liter per cm based on the number of tryptophan and tyrosine residues, respectively [53].

Single-cycle infection assay. A single-cycle infection assay was performed as described previously [48]. Briefly, HIV-1 or SIV pseudoviruses were generated via cotransfection of HEK293T cells with an Env-expressing plasmid and a backbone plasmid, pSG3Δenv, that encodes Env-defective, luciferase-expressing HIV-1 genome. Culture supernatants were harvested 48 h after transfection, and 50% tissue culture infectious doses ($TCID_{50}$) were determined in TZM-bl cells. To measure the antiviral activity of inhibitors, peptides were prepared in 3-fold dilutions, mixed with 100 $TCID_{50}$ of viruses, and then incubated for 1 h at room temperature. The mixture was added to TZM-bl cells (10^4/well) and incubated for 48 h at 37°C. Luciferase activity was measured using luciferase assay reagents and a luminescence counter (Promega, Madison, WI).

The percent inhibition of viral entry by the peptides and 50% inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Cell-cell fusion assay.A dual split protein (DSP)-based assay was performed to determine HIV or SIV Env-mediated cell-cell fusion as described previously [54, 55]. Briefly, a total of 1.5×10^4 293T cells (effector cells) were seeded on a 96-well plate, and a total of 8×10^4 U87-CXCR4 cells (target cells) were seeded on a 24-well plate.

On the following day, effector cells were transfected with a mixture of an Env-expressing plasmid and a DSP_{1-7} plasmid, and target cells were transfected with a DSP_{8-11} plasmid. Forty-eight hours posttransfection, the target cells were resuspended in 300 µl prewarmed culture medium, and to each well 0.05 µl EnduRen live cell substrate (Promega) was added. Aliquots of 75 µl of the target cell suspension then were transferred over each well of the effector cells in the presence or absence of serially 3-fold-diluted peptide fusion inhibitors. The cells were then spun down to maximize cell-cell contact and incubated for 1 h at 37°C. Luciferase activity was measured by a luminescence counter (Promega).

Inhibition of infectious HIV-1$_{NL4-3}$ and HIV-2$_{ROD}$ isolates. The anti-HIV activity of peptide inhibitors was initially assessed by using molecular clones of wild-type HIV-1$_{NL4-3}$ and HIV-2$_{ROD}$ as two indicator viruses. Briefly, viral stocks were prepared by transfecting a plasmid (pNL4-3 or pROD) into HEK293T cells. Culture supernatants were harvested 48 h posttransfection and quantified for $TCID_{50}$ in TZM-bl cells. Viruses were used at 100 $TCID_{50}$ to infect TZM-bl cells in the presence or absence of serially 3-fold-diluted peptides. Cells were harvested 2 days postinfection and lysed in reporter lysis buffer, and luciferase activity was measured as described above.

Inhibition of HIV-2 primary isolates. A total of 9 HIV-2 primary isolates were obtained from Portuguese patients by cocultivation with peripheral blood mononuclear cells (PBMCs) from seronegative subjects [14, 24]. The antiviral activity of fusion inhibitor peptides was evaluated in TZM-bl cells. First, 10,000 TZM-bl cells were seeded in 96-well tissue culture plates and incubated overnight. The next day, the growth medium was removed and replaced by 200 µl of fresh growth medium supplemented with 19.7 µg/ml of DEAE-dextran. Cells were infected with 200 $TCID_{50}$ of each virus in the presence of 3-fold dilutions of peptides.

After 48 h of infection, luciferase expression was quantified with the Pierce firefly luciferase glow assay kit (Thermo Fisher, USA) according to the manufacturer's instructions. The cytotoxicity of the compounds was evaluated using control wells in the absence of the virus. At least two independent experiments were performed for each analysis, and each assay was set up in duplicate wells.
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 GraphPad Prism software.

Inhibition of HIV-2$_{ROD}$ mutants. A panel of HIV-2$_{ROD}$ mutants carrying mutations in amino acid positions of the envelope V3 loop that determine CCR5 and/or CXCR4 usage was used for evaluating the inhibitory activity of the peptides as described above. These mutants were generated in the pROD10 plasmid using the QuikChange II XL site-directed mutagenesis kit (Stratagene) as described previously [45]. Mutant viruses were obtained by transient transfection of HEK293T cells using the jetPrime transfection reagent (Polyplus) according to the manufacturer's instructions. Transfections were performed with 10 μg of DNA in a 100-mm tissue culture dish. Cell culture supernatants were collected 48 h posttransfection, filtered, and stored at −80°C until use.

CD spectroscopy. Circular dichroism (CD) spectroscopy was performed according to our previously described protocols [39]. Briefly, a CHR peptide was incubated with an equal molar concentration of the NHR peptide N36 at 37°C for 30 min in PBS (pH 7.2). CD spectra were acquired on a Jasco spectropolarimeter (model J-815) using a 1-nm bandwidth with a 1-nm step resolution from 195 to 270 nm at room temperature. Spectra were corrected by subtraction of a solvent blank. The α-helical content was calculated from the CD signal by dividing the mean residue ellipticity (θ) at 222 nm by the value expected for 100% helix formation ($-33{,}000$ degree · cm^2 · $dmol^{-1}$). Thermal denaturation was performed by monitoring the ellipticity change at 222 nm from 20°C to 98°C at a rate of 2°C/min, and T_m (melting temperature) was defined as the midpoint of the thermal unfolding transition.

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CHAPTER VI

Resistance mutations to protease inhibitors in proviral DNA of HIV-2 infected patients predict response to treatment

Resistance mutations to protease inhibitors in proviral DNA of HIV-2 infected patients predict response to treatment

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Research article

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Abstract

Protease inhibitors (PIs) constitute an essential component in the treatment of HIV-2 infected patients. However, compared to HIV-1, data on the diversity of the HIV-2 protease (PR) gene and evolution of resistance to PIs is limited. Herein, we make the characterization of PR diversity and resistance to PIs in proviral DNA of HIV-2 infected individuals using clonal sequencing.

Blood samples were collected from 27 HIV-2 infected patients attending a central hospital in Lisbon, Portugal. Fifteen were on treatment and 12 were untreated. The protease gene was amplified from proviral DNA present in PBMCs, cloned and sequenced. Protease diversity was analyzed by phylogenetic and entropy analysis. PIs resistance mutations were identified using EU HIV-2 internet tool-HIV Grade (http://www.hiv-grade.de). The treatment outcomes and resistance mutations of all patients were analysed eight years after enrolment.

In total, 91 clonal sequences were generated from PI treated patients and 96 from untreated patients. PR mutations associated with resistance to the most potent PIs, DRV, LPV, SQV, were detected in 42.8% treated patients. The most common resistance mutations in this subgroup of patients were L90M (n=3, 21.4%) and I84V (n= 2, 14.2%). Other resistance mutations were I54M (n=2, 14.2%) and I82F (n=2, 14.2%). Importantly, we found well-known resistance mutations to PIs in 15.4% untreated individuals, indicating two potential cases of transmitted drug resistance.

Eight years after study entry, patients were screened for PI resistance mutations in proviral DNA, to investigate the impact of archived resistant variants in treatment response. This follow up assessment allowed us to identify the following cases: 1) loss of resistance mutations, that were initially detected at baseline, presumably as a consequence of treatment interruption or poor adherence; 2) long term persistence of resistance mutations, which may pose the patients at risk of failure and might raise concern about transmission of drug resistance in the future and 3) development of new resistance mutations due to previous treatment failures. Additionally, we found that 80% (4/5) of treated patients who presented at least one of these PI resistance mutations (I54M, I82F, L90M, I84V) at baseline experienced virologic failure during the study period.

After eight years of follow up, the analysis of genetic diversity in PR showed an increase in this parameter in two treated patients, with undetectable viral loads and higher CD4+ T counts, indicating persistent viral replication during long-term HAART, regardless of plasma viral load.

Entropy analyses of PR identified three PI resistance associated positions (84, 90, 99) with significant higher entropy levels in treated group of patients compared with untreated group (0.51 *vs* 0.063; 0.44 *vs* 0.063 and 0.347 *vs* 0.0), respectively). Furthermore, we found that 65% of the amino acid positions in PR that vary significantly between treated and untreated groups were located within some of the best-characterized CTL epitopes described for HIV-1, suggesting a potential interaction between PI treatment and CTL immune response in HIV-2, similar to that described for HIV-1.

Our results show that proviral DNA is a good alternative to genomic RNA for testing for drug resistance mutations in HIV-2 infected patients and indicate that early resistance analysis of the viruses archived in PBMCs predict treatment response particularly at low or undetectable viral loads.

Introduction

HIV-2 has recently been ranked as the third most important human pathogen in Europe based on the H-index [1]. The impact of HIV-2 infection is especially noted in Portugal where this virus accounts for 3.3% of all HIV cases [2]. Treatment of HIV-2 infection is challenging as few antiretroviral drugs are fully active on this virus [3, 4]. The protease inhibitors (PIs) saquinavir (SQV), lopinavir (LPV) and darunavir (DRV) are the most potent inhibitors against HIV-2 and are commonly used in combination therapy with nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) or integrase strand transfer inhibitors (INSTIs) to treat HIV-2 infected patients in Portugal and worldwide [5, 6]. HIV-2 patients treated with boosted PI based regimens have higher CD4+ T cell count responses relative to patients treated with triple NRTI or unboosted PI based treatments [7].

Conversely, HIV-2 isolates display decreased susceptibility to the remaining PIs and, in general, have a lower genetic barrier to resistance to this drug class [8-13].

HIV-2 resistance testing is suggested in case of treatment failure when a regimen needs to be changed, although no commercial standardized assay is available [14, 15].

The in-house resistance assays used in clinical practice are based on plasma viruses and may fail at low or undetectable viral loads which is the case for most HIV-2 infected patients [16-23]. In these cases, resistance genotyping in proviral DNA should be a good alternative to plasma testing [16, 19, 24-29]. Several earlier studies reported a variety of protease changes associated to HIV-2 resistance to PIs in treated and/or untreated individuals based on proviral DNA testing using conventional population sequencing [30-34] or more sensitive methods such as clonal genotyping analysis [35-37]. However, at that time, HIV-2 resistance pathways to PIs were not well characterized, and most of the HIV-2 genotypic resistance analyses were based on PI resistance data available for HIV-1.

Recently new data on HIV-2 phenotypic resistance to PIs has been reported [13, 36] and an automated tool for HIV-2 drug resistance interpretation was developed and implemented in several studies [38-42]. To our knowledge, resistance to PIs based on HIV-2 proviral DNA and clonal sequencing, has not yet been investigated using this new approach. Moreover, the impact of PI resistance mutations in the HIV-2 provirus on treatment outcomes of HIV-2 infected individuals is still unclear [34, 36]. Hence, the main objectives of this study were to: 1) make the first characterization of protease diversity and PI resistance mutations in HIV-2 proviral DNA archived in peripheral blood mononuclear cells (PBMCs) of PI treated and untreated HIV-2 infected individuals living in Portugal over a period of eight years; 2) evaluate the impact of resistance mutations in treatment outcome eight years post-therapy.

Material and methods

Study population

Blood samples were collected at study entry from 27 HIV-2 infected patients attending Hospital de Santa Maria in Lisbon, Portugal. Twelve patients were untreated and fifteen were on antiretroviral treatment (ART), the majority with AZT + 3TC + LPV/r, and one subject (patient 9) was on non-PI-based regimen.

Ethics Statement

Written informed consent for blood collection and participation in the study was obtained from all participants. The study was approved by the Ethical Board of the Hospital de Santa Maria, Lisbon, Portugal.

DNA amplification and cloning

At study entry proviral DNA was extracted from 26 patients PBMCs and RNA was obtained from 1 patient plasma sample (patient 27). At 2015, proviral DNA was extracted from 10 patients PBMCs.

A 363 bp DNA fragment corresponding to the PR was amplified using a nested PCR method. First PCR was performed with primers CRPR1 (5´-CCTAGAAGACAGGGM-TGCTGGAA-3´, position in HIV-2_{ALI}: 2314-2336) and CRPR2 (5´-AGCATYCTCCATTTGTTYTTGTC-3´, position: 3148-3126). Second PCR was performed with primers CRPR3 (5´-TGCTGCACCTCAATTCTCTCTTTGGA-3´; position: 2624-2649) and CRPR4 (5´-TTGGTCCATCTTTYCCWGGCTT-3´, position: 2985-2964). The following cycling conditions were used: denaturation at 95ºC for 5 min followed by 40 amplification cycles of 94ºC for 1 min, 59ºC for 1 min and 72ºC for 1 min, and a final elongation step at 72ºC for 15 min. Amplified products were cloned into pCR4-TOPO® (Invitrogen) and a median of 8 clones per patient were sequenced.
HIV-2 PR clonal sequences were submitted to GenBank and were given the accession numbers KT588925-KT589104.

Sequence analysis

PIs resistance mutations were identified using EU HIV-2 internet tool-HIV Grade (http://www.hiv-grade.de) [38, 39]. The HIV-2 group and within patient evolutionary relatedness were determined by phylogenetic analysis. Sequences were aligned with reference sequences collected from the Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov) and maximum likelihood (ML) phylogenetic analyses was performed using the MEGA6 software [43]. Modeltest v3.7 was used to determine the best model of molecular evolution under the Akaike information criteria [44].

To find the ML tree, an iterative heuristic method combining two different tree rearrangement methods was used: nearest neighbor interchange and subtree pruning and regrafting. The reliability of the obtained topology was estimated by bootstrap test (1,000 replicates). Sequences were also subtyped using the new Rega subtyping tool v3 [45]. Editing of the ML tree was performed with FigTree v1.4.3 (available at http://tree.bio.ed.ac.uk/software/figtree/).

The entropy at each position in PR alignment was measured with Shannon´s entropy (http://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy.html). Protease LOGO plots were generated using Analyze Align (http://www.hiv.lanl.gov/content/sequence/ANALYZEALIGN/analyze_align.html). Prediction of CTL epitope location in HIV-2 PR was based on HIV protein epitope maps available at HIV molecular immunology database (http://www.hiv.lanl.gov/content/immunology) [46].

Statistical analyses

Statistical analyses were performed with GraphPad Prism version 5 with a level of significance of 5%. Fisher test was used to compare differences in gender, country of origin, ethnicity and viral load between treated and untreated groups of patients. Mann-Whitney test was used to compare differences in age, CD4+ T cell counts, and genetic distance between both groups of patients. The average CD4+ T cell count per patient during the follow up was estimated with a fixed-effects regression model using the CD4+ T cell count as the dependent variable and viral load status as the independent variable.

Results

Characteristics of the patients at study entry

Epidemiological characteristics of the HIV-2 infected patients genotyped in this study are described in Table 1. At study entry, median T CD4+ cell count was significantly lower in the treated patients compared to untreated patients (264 cells/mm^3±197 vs 553 cells/mm^3±442; P=0.0019). Of the 22 patients with viral load data, 5 (19%) had detectable viral load (range: 8841-100.000 copies/ml) and 17 had low or undetectable levels (<200 copies/ml). Eight years after enrolment, immunologic and virologic data were available for 17 individuals, 1 untreated and 16 treated patients (Figures S1; S2 and Table S1).

Median total T CD4+ cell count was 625 cells/mm^3 ±316 (range: 163-1369). Of the 13 patients with viral load data, 9 (69.2%) had undetectable viral load (<40 copies/ml) and the remaining 4 (30.7%) had detectable viral levels (range: 67- 6637 copies/ml).

Table 1. Epidemiological characteristics of the HIV-2 infected patients included in this study.

Variable	Total (%)	Treated patients (%)	Untreated patients (%)	*P* value[a]
No. of subjects (%)	27 (100)	15 (56)	12 (44)	
Gender [N (%)]				0.6957*
Female	19 (70)	10 (67)	9 (75)	
Male	8 (30)	5 (33)	3 (25)	
Mean age, years (SD; range)	48 (11.5;27-64)	48 (9.7;29-63)	47 (14;27-64)	0.8261#
Country of origin [N(%)]				
Portugal	8 (30)	5 (33)	3 (25)	0.6957*
Guinea-Bissau	12 (45)	5 (33)	7 (58)	0.2576*
Cape-Verde	3 (11)	3 (20)	0 (0)	0.2308*
Mozambique	2 (7)	2 (14)	0 (0)	0.4872*
Unknown	2 (7)	0 (0)	2 (17)	0.1880*
Ethnicity [N(%)]				
Caucasian	10 (37)	5 (33)	5 (42)	0.7063*
Black	15 (55)	9 (60)	6 (50)	0.7068*
Indian	1 (4)	1(7)	0 (0)	1.0000*
Unknown	1 (4)	0 (0)	1 (8)	0.4444*
Median CD4, cells/mm3 (SD; range)	448 (400;72-1594)	264 (197;72-731)	553 (442;305-1594)	**0.0019#**
Viral load, cp/ml [N(%)]				
<200	17 (62)	10 (67)	7 (58)	0,7063 *
>200 [N(%; range)]	5 (19; 8841-100.000)	3 (20; 8841-100.000)	2 (17; 10425-13627)	1.0000*
Unknown	5 (19)	2 (13)	3 (25)	0,6280 *

[a]*P* values are based on comparison of treated patients and untreated patients. Values in bold indicates a statistically significant difference (P<0.05);

*Fisher´s exact test;

Mann Whitney test;

SD-standard deviation.

Genetic distance is similar between treated and untreated patients

In total, 91 clonal sequences were generated from treated patients and 96 from untreated patients (median: 8 clones/patient; min-max: 1-11). Phylogenetic analysis indicates that all patients were infected with group A viruses. Within patient sequences formed monophyletic clusters supported by high bootstrap values (Figure 1). Mean genetic distance within patients was 0.020±0.012 substitutions per site and did not differ significantly between treated and untreated patients (0.02379 *vs* 0.01658, p= 0.1611, respectively). There was no evidence for epidemiologic linkage between patients with the exception of patients 07PTHSM8 and 07PTHSM23 who were a couple.

Significant entropy variation between PR sequences from treated and untreated patients

Entropy difference between amino acid sequences from treated and untreated patients was calculated and sites where significant variation occurred were determined. Amino acids with the higher entropy (values above 0.5) were located at positions 7, 14, 15, 40, 41, 65, 68, 70 and 89 in untreated group and at positions 14, 40, 65, 68, 70 and 84 in treated group (Figs. 2A and B). Twenty (20.2%) amino acids presented significant variation in entropy between both groups (positions 7,10, 15, 21, 29, 37, 40, 43, 46, 56, 57, 64, 68, 70, 72, 75, 77, 84, 90, 99) ($P<0.05$) (Fig. 2C and Table S2). Of these, only amino acids at positions 84 and 90 are well associated with HIV-2 resistance to PIs while substitutions at position 99 may act as accessory mutations associated to PI resistance [36]. These three positions presented higher entropy in treated group compared with untreated group (0.51 vs 0.063; 0.44 vs 0.063 and 0.347 vs 0.0), respectively).

Interestingly, 13 out of the 20 (65%) amino acids showing high entropy were located in CTL epitopes described for HIV-1 PR (positions: 7, 37, 40, 57, 64, 68, 70, 72, 75, 77, 84, 90 and 99) (Fig. 3). The I84V resistance mutation is found within the putative epitopes B81 and A*0201 while L90M is located at epitope B81 [47].

Figure 1. Phylogenetic relationship between protease sequences from HIV-2 infected patients at study entry and after 8 years of follow-up.

The phylogenetic tree was constructed under ML with reference sequences from HIV-2 groups from different locations. Clonal sequences from HIV-2 patients are shown with the patients numbers highlighted next to the respective cluster. Only bootstrap values above 70% are shown. Filled markers correspond to 2015 protease sequences; non-filled markers correspond to protease sequences at study entry.

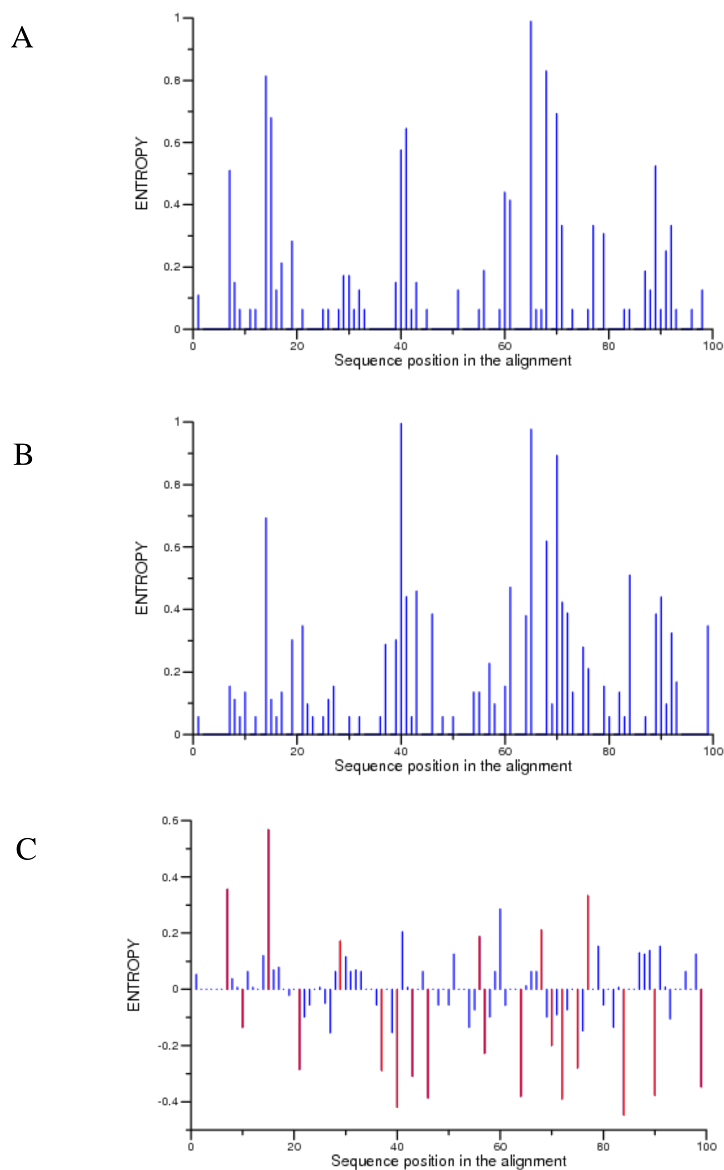

Figure 2. Diversity of protease amino acid sequences at study entry as assessed by Shannon's entropy. (A) Untreated patients; (B) Treated patients; (C) Entropy difference between protease sequences from treated and untreated patients. Sites with significant differences in entropy (with P≤0.05) are shown in red in the plot. The entropy of each position in the sequence set was calculated with Shannon Entropy-Two (http://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy.html).

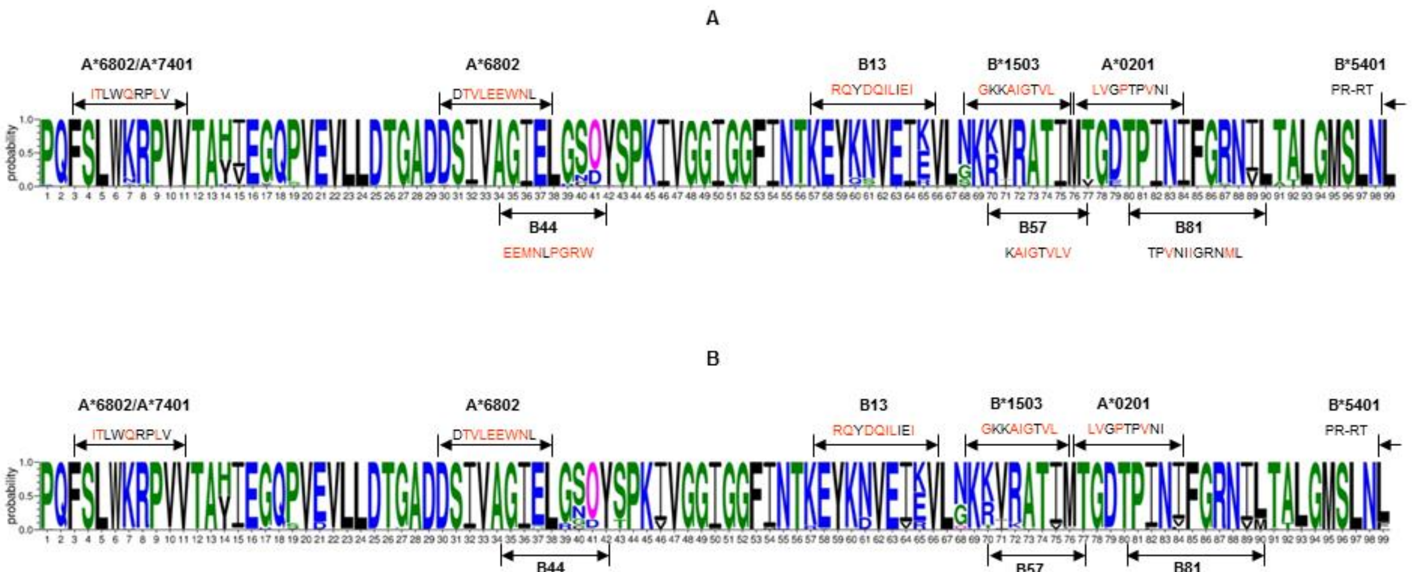

Figure 3. Diversity of protease amino acid sequences at study entry as shown in LOGO plots. (A) untreated patients; (B) treated patients. The colors of amino acids correspond to their hydrophobicity: hydrophilic amino acids (RKDENQ) are blue, neutral (SGHTAP) are green and hydrophobic (YVMCLFIW) are black. N-linked glycosylation sites are marked as "O" in pink color. The overall height of each letter or a stack of letters indicates the sequence conservation at that position (measured in probability) and bases are listed in decreasing order of frequency from top to bottom. LOGO plots were generated using AnalyzeAlign. The location and sequence of HLA restriction elements of CTL epitopes present in HIV-1 protease are indicated on the amino acid sequence. The CTL epitopes were obtained from http://www.hiv.lanl.gov/content/immunology. Red letters indicate residues that differ from HIV-1 sequence for which the epitope was defined.

Baseline resistance profiles

As expected, the frequency of PR mutations associated with PI resistance were higher in PIs treated than in untreated patients (6/14 (42.8%) *vs* 3/13 (23%) (Table 2). Likewise, more clones contained resistance mutations in PI treated patients than in untreated patients [19/91 (20.9%) from 6 treated patients vs 4/96 clones (4.2%) from 3 untreated patients, $P<0.05$].

The most common resistance mutations in PIs treated patients were L90M (n=3, 21.4%) and I84V (n=2, 14.2%). Although considered by HIV-Grade as a mutation that causes intermediate resistance to DRV, LPV and SQV, I84V alone does not confer significant phenotypic resistance to these inhibitors *in vitro* [36]. However, when combined with L90M or I54M it confers resistance to the PIs that are most effective on HIV-2 (SQV, LPV and DRV) [36]. Other resistance mutations identified were I54M (n=2, 14.2%) and I82F (n=2, 14.2%). The I54M mutation confers phenotypic resistance to LPV and DRV while I82F may confer resistance to LPV [36]. Mutations I84L and G48R were found in only 1 (7.1%) treated patients each. These are rare substitutions at these positions and their impact in drug resistance is still unknown.

As for the untreated patients, three out of the thirteen patients harbored mutations associated to PIs resistance, particularly I84V (patient 23), L90M (patient 25) and I50T (patient 9) (Table 3). The role of I50T on the resistance to PIs is still unclear.

Of note, resistance mutations (I84V or L90M) were present in a minority of clones (9% or 10%) in both patients.

Table 2. PI resistance mutations of HIV-2 infected patients.

Patient #	At enrollment (2007)		2015 (8 yrs after)	
	Resistance mutations (mutated clones/sequenced clones)	Resistance Profile[a]	Resistance mutations (mutated sequences/ sequenced samples)	Resistance Profile[a]
1	I84V (1/6)	DRV, LPV, SQV [IR]	None (0/7)	-
2	-	-	-	-
3	I84V (10/10), L90M (10/10)	DRV, LPV,SQV	-	-
4	I54M (2/2), I82F (2/2), L90M (2/2)	DRV, LPV, SQV	-	-
5	G48R* (1/9)	?	-	-
6	-	-	None (0/1)	-
7	-	-	-	-
8	-	-	I84V(1/1); L90M (1/1)	DRV, LPV, SQV
9	I50T* (2/9)	?	-	-
10	-	-	-	-
11	I54M (1/2), I82F (1/2)	DRV, LPV	V47A (1/3); I54M (2/3);I82F (3/3); I84L* (2/3); L90M /2/3)	DRV, LPV, SQV
12	-	-	I54M (1/3); I84V (1/3)	DRV, LPV; SQV[IR]
13	-	-	None (0/9)	-
14	I84L* (4/11), L90M (4/11)	DRV, LPV [IR]; SQV	None (0/3)	-
15	-	-	-	-
16	-	-	-	-
17	-	-	-	-
18	-	-	None (0/1)	-
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-
22	-	-	-	-
23	I84V (1/10)	DRV, LPV, SQV [IR]	-	-
24	-	-	-	-
25	L90M (1/9)	DRV,LPV [IR]; SQV	-	-
26	-	-	None (0/3)	-
27	-	-	None (0/3)	-

[#]Treated patients (1-8; 10-14, and 27); untreated patients (15-26 and 9); Patient 9 was on non-PI-based ART;
[a] According to HIV-Grade [38,39]; DRV-darunavir; LPV-lopinavir; SQV-saquinavir;
[IR]Intermediate Resistance;
*G48R, I50T and I84L are rare mutations not yet scored by HIV-Grade [38,39].

Patient outcomes

These patients were followed up for a period of 8 years allowing for the investigation of clinical and treatment outcomes (Table S1). Genotypic characterization of resistance mutations was done at year 8 for 10/16 treated patients (Table 2). Of these, two had resistance mutations at baseline that were not detected eight years later (patients 1 and 14), one presented more resistance mutations than those initially detected (patient 11); two presented resistance mutations only at the last genotypic analysis (patients 8 and 12) and two had no detectable resistance mutations at baseline or eight years after study entry (patients 6 and 13). Patient 14, had a major PI resistance mutation (L90M) at baseline. At the time of blood sampling this patient interrupted treatment due to toxicity issues. Despite the normal and stable CD4+ T cell counts, viral load become detectable in 2009 and progressively increased until 2012, when the patient restarted treatment with other PI (DRV/r). In 2015, this patient was on immunologic recovery, with undetectable viral load and high level CD4+ T cell count (1369 cells/mm^3) and with no detectable resistance mutations.

Subject 11 was on a failing LPV/r based regimen at baseline and, consistently showed I54M and I82F resistance mutations. Due to high viral load (9309 copies/ml) in 2008 he started a SQV/r regimen which was switched to DRV/r in 2010. In 2015, he was virologically suppressed but showed two additional resistance mutations that were not present at baseline (L90M and V47A): L90M confers resistance to SQV whereas V47A is frequent among patients failing LPV/r-based treatment [36].

Patient 8, who was being treated with IDV/r at baseline, had low levels of CD4+ T cell count (84 cells/mm3), undetectable viral load (< 200 copies/ml) and no resistance mutations. One year after study entry (in 2008), this patient switched to a SQV/r based regimen. In 2015, the patient was virologically suppressed with increased CD4+ T cell count (413 cells/mm^3) despite the presence of the resistance mutations I84V and L90M.

Patient 12 was on a SQV/r based regimen, with low CD4+ T cell count at baseline. During the follow up, the levels of virologic and immunologic parameters fluctuated, particularly viral load, and the patient switched to LPV/r (2009) and then to DRV/r regimen (2010-present). In the last genotypic analysis performed in 2015 two mutations were detected (I54M and I84V) that confer resistance to the current regimen (DRV/r).

Patients 6 and 13 had no resistance mutations associated to PIs in the first and last genotypic resistance analysis.

The reasons for switching PI treatment during this period were immunologic and/or virologic failure, despite the absence of PI resistance. Of the two remaining patients who were still receiving the same PI, one had a major resistance mutation at study entry that was not present at the latter genotypic analysis and the other had no mutations at both genotypic resistance assays (patient 1 and 27, respectively).

The impact of resistance mutations in treatment was also observed in patients 3 and 4 whose follow up is incomplete because they deceased during this study. These patients had a strong evidence of virologic failure at study entry with viral loads >100,000 and 75,571 copies/ml, respectively (Table S1). Consistent with this, both patients had resistance mutations in proviral DNA for all clonal sequences at study entry (Table 2). Patient 3 was on a SQV/r based regimen and displayed the I84V and L90M mutations which confer moderate level resistance to SQV, DRV and LPV [36]. One year after study entry viral load was still detectable with low CD4+ T count despite the change to a DRV/r based regimen and the patient ultimately died. Patient 4 displayed the I54M, I82F and L90M mutations in proviral DNA at study entry in all clonal sequences. These mutations confer resistance to LPV, DRV and SQV [36]. Resistance profile of plasma viruses was also available for this patient at study entry. I54M and I82F were present in 100% (8/8) of the clonal sequences from plasma viruses but L90M was absent. Despite the change to a SQV/r based regimen this patient died two years after study entry with clear signs of treatment failure (very low $CD4^{+}$ T cell count).

The analysis of genotypic resistance mutations eight years after enrolment was also performed in two untreated patients. These subjects (18 and 26) had no detectable PI resistance mutations in the initial and in the last genotypic analyses. Although they started treatment (with a PI) during this study due to virologic and/or immunologic failure, both patients were still experiencing treatment failure at the end of the study.

The analysis of genetic diversity in PR showed an increase in this parameter in two treated patients (1 and 13), with undetectable viral loads and higher CD4+ T counts, comparing with the baseline.

On the other hand, a reduction in PR genetic diversity was exhibited in one untreated and two PI treated patients (26, 27 and 14, respectively), who presented detectable viral loads in at least one time point during the follow up (Figures S3A,B).

Discussion

We made the first characterization of primary and secondary HIV-2 resistance to PIs using clonal sequences obtained from proviral DNA from HIV-2 infected patients from Portugal. Patients were followed for a period of 8 years to characterize treatment outcomes. At study entry 42.8% of the patients treated with PIs harbored at least one proviral DNA clone with resistance mutations. This is a lower rate compared to Raugi *et al*. [36] that, using the same methodology of clonal sequencing, found resistance mutations in 93% of treated patients from Senegal. Other studies reported similar or much lower rates of PI resistance using direct PCR sequencing of proviral DNA (France, 45.5% (5/11) [32]; Senegal, 30% (7/23) [34]; Portugal, 25% (1/4) [30]; Ivory Coast, 12.5% (1/8 patients) [31]). This variation in resistance rates may be related with differences in treatment regimens and adherence rates but it may also be related with methodological issues since clonal sequencing of proviral DNA, as used in our study and that of Raugi *et al*. [36], increase the likelihood of detecting minority resistant variants relative to population sequencing [16].

Crucial to this type of studies is to investigate the evolution of PR genetic diversity over time and the impact of archived drug resistance mutations in patient response to therapy.

Due to small sample size it was not possible to investigate a potential relationship between PR genetic diversity and CD4+ T cell counts, presence of resistance mutations or/and treatment status. However, the increase in PR genetic diversity in two treated patients with long term virologic suppression, during the eight years follow up, seems to indicate the existence of a persistent viral replication under HAART, regardless of plasma viral load. Indeed, HIV evolution on effective HAART has been demonstrated in some studies performed in context of HIV-1 [48-50] and HIV-2 infection [51]. Collectively, these findings suggest that maintenance of viral replication might act as a possible source of new proviral quasispecies, resulting in the gradual substitution of the ancestral variants over time.

The follow up assessment of genotypic resistance to PIs eight years after the beginning of the study allowed us to identify the following cases: 1) loss of resistance mutations that were initially detected at baseline in two patients (2; 20%); 2) long term persistence of resistance mutations (1;10%), and 3) development of new resistance mutations (3; 30%).

The first case was observed in two patients that were virologically suppressed and had high CD4+ T cells counts at the end of the study. The disappearance of the resistant isolates during the follow up period might be related to treatment interruption or poor adherence. As previously described for HIV-1, the absence of continuously drug pressure leads to the re-emergence of wild type virus decreasing the load of drug resistant variants in proviral DNA to undetectable levels [17, 19, 52]. The second case was observed in one patient in which the mutations detected at baseline were present, along with other mutations, at the end of the study. This subject had failed previous PI regimens and was subsequently suppressed under a new PI treatment at the last genotypic analysis. The persistence of resistance mutations in proviral DNA for a long period of time may pose this subject at risk of imminent failure and might raise concern about transmission of drug resistance in the future, especially in the setting of virologic failure, as it was shown for HIV-1 [16, 19, 28, 29, 53]. Finally, three patients developed resistance mutations that were not present at baseline (case 3). The subjects changed PI therapy during the study, and the development of the resistance mutations may result from previous treatment failures as observed by the presence of detectable viral load and/or decreased CD4+ T cell count during the follow-up period.

Overall we have shown that a high proportion of HIV-2 patients treated with PIs archives resistance isolates as proviral DNA for a long period of time. When drug pressure is maintained most of these resistant isolates reemerge to compromise treatment response. These findings raise special concerns in the HIV-2 infected population, for whom therapeutic options are scarce compared with HIV-1. On the other hand, these results confirm and extend previous studies suggesting that the early detection of resistance mutations in viruses archived in PBMCs may predict treatment response in HIV-2 infected patients, particularly in those with low or undetectable levels of plasma viral load [31, 34, 36]. Similar findings have been made previously for HIV-1 [16, 19, 27, 28, 54].

In this study two out of the thirteen untreated patients (15.4%) contained provirus bearing the PI resistance mutations I84V and L90M. This is consistent with transmitted resistance since these mutations were also the more prevalent in the treated population.

HIV-2 transmitted drug resistance in PBMCs has been scarcely reported in the literature.

In 2006, Parreira *et al*. [35] found the I50V resistance mutation in 3% (n=30) of clonal DNA sequences from untreated patients from Portugal while Gottlieb *et al*. [37] did not find resistance mutations in proviral DNA from ART-naïve women in Senegal in 2008.

Using population sequencing from plasma isolates, Charpentier et al. identified TDR at a prevalence of 5% (V47A in two cases and I82F in one) [42]. Other two studies performed by Silva et al. in 2010 and Damond et al. in 2005, reported lower prevalence of TDR (1.7% and 3.1%, respectively) observed in two PR samples (I54M and I64V) and in three PR samples (I54M in two cases and L90M in one), respectively [55,56].

Recently, Duarte *et al*. 2016 [40] reported an L90M mutation in a drug-naïve patient followed in a Portuguese hospital, whereas Pieniazek *et* al. [33] did not find resistance mutations in drug-naïve patients from Ivory Coast and other countries, in 2004.

Overall the results were consistent with the recent implementation of PR-based HAART in those countries. On the other hand the higher prevalence of transmitted PI resistance in our study is consistent with the high prevalence of resistant isolates found in this and other studies performed in Portugal [5]. Nonetheless, caution is needed in the interpretation of our results due the small sample size. Additional studies with a higher number of patients will be needed to determine if primary drug resistance is a major problem in HIV-2 infected patients in Portugal.

In the current study we found that 65% of the amino acid positions in PR that vary significantly between treated and untreated groups were located within some of the best-characterized CTL epitopes described for HIV-1. CTL pressure on the PR can lead to the emergence of CTL escape mutations associated with HIV-1 resistance to PIs; conversely, PI pressure can lead to the selection of drug resistance mutations that also lead to escape from the CTL response [57-59]. Our studies suggest that a similar interaction between PI treatment and CTL immune response may occur in HIV-2 infected patients.

Conclusion

In summary, a high proportion of treated and untreated patients contained PI resistance mutations in proviral DNA. During the eight years follow-up period, treatment failure was observed by the presence of detectable viral load or decrease in CD4+ T cells count in the majority of patients who presented resistance mutations in archived viruses at study entry.

Our studies suggest that drug resistance testing in proviral DNA may be useful to guide and predict treatment response of HIV-2 infected patients.

Diversity hotspots in the HIV-2 PR are mostly located within putative CTL epitopes suggesting a relationship between PI treatment and cellular immune responses in HIV-2 patients.

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Table S1. Virologic, immunologic and treatment data of HIV-2 infected patients.

Patient	**2007**			**2008**		
	Plasma Viral load (copies/ml)	**CD4 count (cells/mm3)**	**ART[a]**	**Plasma Viral load (copies/ml)**	**CD4 count (cells/mm3)**	**ART**
1	<200	600	AZT,3TC,LPV/r	<40	666	AZT,3TC,LPV/r
2	<200	313	3TC,AZT,SQV	<40	390	3TC,AZT,SQV
3	<100000	72	3TC,d4T,SQV/r	5352	96	TDF,RAL,DRV/r
4	75571	159	AZT,3TC,LPV/r	294	86	AZT,3TC,SQV/r
5	<200	403	AZT,3TC,LPV/r	<40	727	AZT,3TC,LPV/r
6	<200	484	AZT,3TC,SQV/r	<40	467	AZT,3TC,SQV/r
7	<200	409	AZT,3TC,LPV/r	NA	793	AZT,3TC,LPV/r
8	<200	87	AZT,3TC,IDV/r	<40	127	AZT,3TC,SQV/r
9	<200	448	AZT,3TC	NA	NA	NA
10	<200	264	TDF/FTC,LPV/r	NA	NA	NA
11	NA	161	TDF/FTC,LPV/r	9309	124	TDF/FTC,SQV/r
12	NA	190	ABC,3TC,SQV/r	3344	193	AZT,3TC,SQV/r
13	<200	99	AZT/3TC,LPV/r	NA	405	AZT/3TC,SQV/r
14	<200	731	3TC,d4T,SQV/r	<40	573	Untreated
15	NA	1182	Untreated	NA	1266	Untreated
16	<200	1202	Untreated	NA	NA	NA
17	<200	305	Untreated	<40	355	Untreated
18	<200	548	Untreated	<40	652	AZT,3TC,SQV/r
19	NA	1594	Untreated	NA	NA	NA
20	NA	557	Untreated	NA	NA	NA
21	10425	453	Untreated	5300	448	Untreated
22	<200	722	Untreated	NA	595	Untreated
23	<200	462	Untreated	<40	443	Untreated
24	<200	546	Untreated	<40	813	Untreated
25	<200	1409	Untreated	<40	1670	Untreated
26	13627	385	Untreated	<40	570	AZT,3TC,SQV/r
27	8841	254	ABC, 3TC,SQV/r	<40	381	ABC/3TC,SQV/r

[a] Although included in HIV-Grade, IDV exhibits at least partial resistance *in vivo* and/or *in vitro* to HIV-2 and is not recommended for clinical use in HIV-2 infected patients;
NA - not available;
LTFU - lost to follow up.

Table S1. Cont.

Patient	2009			2010		
	Plasma Viral load (copies/ml)	**CD4 count (cells/mm3)**	**ART**	**Plasma Viral load (copies/ml)**	**CD4 count (cells/mm3)**	**ART**
1	<40	796	AZT,3TC,LPV/r	<40	940	AZT,3TC,LPV/r
2	<40	380	3TC,AZT,SQV	<40	536	3TC,AZT,SQV
3	NA	359	TDF,RAL,DRV/r	NA	NA	NA
4	NA	26	AZT,3TC,SQV/r	NA	NA	NA
5	NA	883	AZT,3TC,LPV/r	NA	926	AZT,3TC,LPV/r
6	<40	747	AZT,3TC,SQV/r TDF	<40	636	AZT,TDF,SQV/r
7	NA	925	AZT,3TC,LPV/r	<40	596	AZT,3TC,LPV/r
8	<40	220	TDF/FTC,SQV/r	231	147	TDF/FTC,SQV/r
9	NA	NA	NA	NA	NA	NA
10	NA	NA	NA	NA	NA	NA
11	1580	209	TDF/FTC,SQV/r	<40	277	TDF/FTC,RAL,DRV/r
12	3220	98	TDF/FTC,LPV/r	<40	249	ABC,RAL,DRV/r
13	<40	429	AZT/3TC,SQV/r	<40	578	AZT/3TC,SQV/r
14	2850	716	Untreated	4394	550	Untreated
15	<40	985	Untreated	<40	1629	Untreated
16	NA	NA	NA	SD	969	Untreated
17	<40	335	Untreated	<40	423	Untreated
18	89	540	Untreated	<40	360	Untreated
19	NA	NA	NA	NA	NA	NA
20	4230	429	TDF/FTC,SQV/r	NA	NA	NA
21	NA	NA	NA	NA	456	Untreated
22	NA	NA	NA	NA	NA	NA
23	SD	579	Untreated	<40	638	Untreated
24	<40	794	Untreated	<40	662	Untreated
25	<40	1613	Untreated	<40	1756	Untreated
26	<40	662	AZT,3TC,SQV/r	243	383	ABC,3TC,RAL
27	<40	441	ABC/3TC,SQV/r	NA	519	ABC/3TC,SQV/r

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NA - not available;
LTFU - lost to follow up.

Table S1. Cont.

Patient	2011			2012		
	Plasma Viral load (copies/ml)	**CD4 count (cells/mm3)**	**ART**	**Plasma Viral load (copies/ml)**	**CD4 count (cells/mm3)**	**ART**
1	<40	842	AZT,3TC,LPV/r	<40	737	AZT/3TC,LPV/r
2	NA	444	3TC/AZT,RAL	<40	344	3TC/AZT,RAL
3	NA	NA	NA	NA	NA	NA
4	NA	NA	NA	NA	NA	NA
5	NA	922	TDF/FTC,DRV/r	NA	891	TDF/FTC,DRV/r
6	<40	638	TDF,3TC,SQV/r	NA	564	TDF,3TC,SQV/r
7	<40	632	ABC/3TC,DRV/r	<40	598	ABC/3TC,DRV/r
8	<40	238	TDF/FTC,SQV/r	<40	194	TDF/FTC,SQV/r
9	<40	823	NA	NA	NA	NA
10	NA	NA	NA	NA	NA	NA
11	<40	219	TDF/FTC,RAL, DRV/r	NA	307	TDF/FTC,RAL, DRV/r
12	1813	229	ABC,RAL,DRV/r	NA	276	ABC,RAL,DRV/r
13	NA	971	AZT/3TC,SQV/r	<40	761	AZT/3TC,SQV/r
14	NA	735	Untreated	2144	469	RAL,DRV/r
15	SD	1484	Untreated	<40	1268	Untreated
16	NA	NA	NA	<40	734	Untreated
17	<40	390	Untreated	<40	338	Untreated
18	<40	432	Untreated	<40	451	Untreated
19	NA	NA	NA	NA	NA	NA
20	131	382	TDF/FTC,SQV/r	<40	300	TDF/FTC,SQV/r
21	NA	NA	NA	NA	NA	NA
22	NA	NA	NA	NA	NA	NA
23	<40	673	TDF,FTC,SQV/r	<40	530	TDF,FTC,SQV/r
24	<40	607	Untreated	<40	811	Untreated
25	<40	1775	Untreated	<40	1794	Untreated
26	1392	384	AZT/3TC,RAL	<40	286	AZT/3TC,RAL
27	<40	495	ABC/3TC,SQV/r	74	562	ABC/3TC,SQV/r

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Table S1. Cont.

Patient	2013			2014		
	Plasma Viral load (copies/ml)	**CD4 count (cells/mm3)**	**ART**	**Plasma Viral load (copies/ml)**	**CD4 count (cells/mm3)**	**ART**
1	<40	827	AZT/3TC,LPV/r	<40	675	AZT/3TC,LPV/r
2	NA	405	3TC/AZT,RAL	NA	439	3TC/AZT,RAL
3	NA	NA	NA	NA	NA	NA
4	NA	NA	NA	NA	NA	NA
5	NA	975	TDF/FTC,DRV/r	NA	843	TDF/FTC,DRV/r
6	<40	260	TDF,3TC,SQV/r	NA	NA	TDF,3TC,SQV/r
7	NA	784	ABC/3TC,DRV/r	NA	NA	ABC/3TC,DRV/r
8	NA	235	TDF/FTC,SQV/r	NA	360	TDF/FTC,SQV/r
9	NA	NA	NA	NA	NA	NA
10	NA	NA	NA	NA	NA	NA
11	<40	336	TDF/FTC,RAL, DRV/r	<40	364	TDF/FTC,RAL,D RVr
12	315	261	ABC,RAL,DRV/r	<40	195	ABC,RAL,DRV/r
13	<40	760	AZT/3TC,SQV/r	<40	1089	AZT/3TC,SQV/r
14	<40	809	DRV/r, RAL	<40	1113	DRV/r, RAL
15	<40	1196	Untreated	<40	1810	Untreated
16	<40	903	Untreated	NA	NA	NA
17	<40	362	Untreated	<40	376	Untreated
18	<40	681	Untreated	SD	557	Untreated
19	NA	NA	NA	NA	NA	NA
20	20684	173	TDF/FTC,SQV/r	NA	NA	NA
21	<40	398	Untreated	NA	NA	NA
22	NA	NA	NA	NA	473	Untreated
23	<40	493	ABC/3TC,SQV/r	NA	666	ABC/3TC,SQV/r
24	NA	NA	NA	NA	NA	NA
25	<40	1654	Untreated	<40	728	Untreated
26	1191	367	AZT/3TC,RAL	2492	260	AZT/3TC,RAL
27	82	514	ABC/3TC,SQV/r	172	919	ABC/3TC,SQV/r

[a] Although included in HIV-Grade, IDV exhibits at least partial resistance *in vivo* and/or *in vitro* to HIV-2 and is not recommended for clinical use in HIV-2 infected patients;
NA - not available;
LTFU - lost to follow up.

Table S1. Cont.

Patient	2015			
	Plasma Viral load (copies/ml)	**CD4 count (cells/mm3)**	**ART**	**Observation**
1	<40	813	AZT/3TC,LPV/r	
2	NA	385	3TC/AZT,RAL	
3	NA	NA	NA	Deceased 2009
4	NA	NA	NA	Deceased 2009
5	NA	946	TDF/FTC,DRV/r	
6	125	295	TDF,3TC,DRV/r	
7	NA	969	ABC/3TC,DRV/r	
8	NA	413	TDF/FTC,SQV/r	
9	NA	NA	NA	LTFU
10	NA	NA	NA	Deceased 2008
11	<40	420	RAL,DRV/r	
12	<40	163	ABC,RAL,DRV/r	
13	<40	866	AZT/3TC,SQV/r	
14	<40	1369	DRV/r, RAL	
15	NA	NA	NA	
16	NA	NA	NA	LTFU
17	<40	433	Untreated	
18	67	428	TDF/FTC,RAL	
19	NA	NA	NA	LTFU
20	NA	NA	NA	
21	NA	NA	NA	
22	100	507	TDF/FTC, DRV/r	
23	<40	766	ABC/3TC,SQV/r	
24	NA	NA	NA	LTFU
25	<40	754	TDF/FTC, LPV/r	
26	6637	299	AZT/3TC,RAL	
27	<40	803	ABC/3TC,SQV/r	

[a] Although included in HIV-Grade, IDV exhibits at least partial resistance *in vivo* and/or *in vitro* to HIV-2 and is not recommended for clinical use in HIV-2 infected patients;
NA - not available;
LTFU - lost to follow up.

Table S2. Determination of PR variability between clonal sequences from untreated and treated HIV-2 infected patients based on entropy values generated for each amino acid position.

Position	Query consensus	Entropy diff between 2 sets (Hdiff = Hb-Hq)	Random entropy # > Hdiff	Highest random entropy	P-value at this position
1	P	0,053	57	0,243	0,57
2	Q	0	100	0	1
3	F	0	100	0	1
4	S	0	100	0	1
5	L	0	100	0	1
6	W	0	100	0	1
7	**K**	**0,356**	**2**	**0,397**	**0,02**
8	R	0,038	64	0,315	0,64
9	P	0,007	82	0,212	0,82
10	**V**	**-0,135**	**4**	**0,168**	**0,04**
11	V	0,063	31	0,135	0,31
12	T	0,007	83	0,224	0,83
13	A	0	100	0	1
14	Y	0,12	8	0,233	0,08
15	**I**	**0,568**	**0**	**0,36**	**0**
16	E	0,069	37	0,259	0,37
17	G	0,078	43	0,328	0,43
18	Q	0	100	0	1
19	P	-0,021	85	0,276	0,85
20	V	0	100	0	1
21	**E**	**-0,284**	**1**	**0,303**	**0,01**
22	V	-0,098	22	0,186	0,22
23	L	-0,056	52	0,135	0,52
24	L	0	100	0	1
25	D	0,007	84	0,172	0,84
26	T	-0,049	69	0,218	0,69
27	G	-0,154	12	0,224	0,12
28	A	0,063	34	0,15	0,34
29	**D**	**0,172**	**3**	**0,232**	**0,03**
30	D	0,116	21	0,299	0,21
31	S	0,063	25	0,109	0,25

H- Entropy
Hb- Entropy for clonal sequences from untreated group
Hq- Entropy for clonal sequences from treated group

Table S2. Cont.

Position	Query consensus	Entropy diff between 2 sets (Hdiff = Hb-Hq)	Random entropy # > Hdiff	Highest random entropy	P-value at this position
32	I	0,069	45	0,251	0,45
33	V	0,063	26	0,135	0,26
34	A	0	100	0	1
35	G	0	100	0	1
36	I	-0,056	53	0,135	0,53
37	**E**	**-0,288**	**1**	**0,327**	**0,01**
38	L	0	100	0	1
39	G	-0,153	11	0,262	0,11
40	**S**	**-0,419**	**0**	**0,32**	**0**
41	N	0,204	8	0,324	0,08
42	Y	0,007	84	0,172	0,84
43	**S**	**-0,309**	**1**	**0,388**	**0,01**
44	P	0	100	0	1
45	K	0,063	31	0,135	0,31
46	**I**	**-0,386**	**0**	**0,216**	**0**
47	V	0	100	0	1
48	G	-0,056	49	0,135	0,49
49	G	0	100	0	1
50	I	-0,056	52	0,109	0,52
51	G	0,125	7	0,196	0,07
52	G	0	100	0	1
53	F	0	100	0	1
54	I	-0,135	8	0,186	0,08
55	N	-0,072	45	0,254	0,45
56	**T**	**0,188**	**4**	**0,232**	**0,04**
57	**K**	**-0,227**	**2**	**0,279**	**0,02**
58	E	-0,098	24	0,199	0,24
59	Y	0,063	28	0,15	0,28
60	K	0,286	6	0,43	0,06
61	N	-0,057	63	0,393	0,63
62	V	0	100	0	1
63	E	0	100	0	1
64	**I**	**-0,38**	**0**	**0,246**	**0**

H- Entropy
Hb- Entropy for clonal sequences from untreated group
Hq- Entropy for clonal sequences from treated group

Table S2. Cont.

Position	Query consensus	Entropy diff between 2 sets (Hdiff = Hb-Hq)	Random entropy # > Hdiff	Highest random entropy	P-value at this position
65	K	0,012	84	0,202	0,84
66	V	0,063	23	0,135	0,23
67	L	0,063	28	0,15	0,28
68	**N**	**0,211**	**3**	**0,231**	**0,03**
69	K	-0,098	25	0,15	0,25
70	**K**	**-0,2**	**1**	**0,239**	**0,01**
71	V	-0,09	33	0,255	0,33
72	**R**	**-0,389**	**1**	**0,403**	**0,01**
73	A	-0,072	49	0,312	0,49
74	T	0	100	0	1
75	**I**	**-0,279**	**0**	**0,224**	**0**
76	M	-0,147	18	0,311	0,18
77	**T**	**0,333**	**0**	**0,279**	**0**
78	G	0	100	0	1
79	D	0,153	21	0,312	0,21
80	T	-0,056	43	0,135	0,43
81	P	0	100	0	1
82	I	-0,135	8	0,186	0,08
83	N	0,007	82	0,224	0,82
84	**I**	**-0,447**	**0**	**0,235**	**0**
85	F	0	100	0	1
86	G	0	100	0	1
87	R	0,13	23	0,301	0,23
88	N	0,125	12	0,232	0,12
89	I	0,138	15	0,26	0,15
90	**L**	**-0,377**	**0**	**0,206**	**0**
91	T	0,153	8	0,222	0,08
92	A	0,008	97	0,22	0,97
93	L	-0,105	26	0,23	0,26
94	G	0	100	0	1
95	M	0	100	0	1
96	S	0,063	29	0,15	0,29
97	L	0	100	0	1
98	N	0,125	11	0,212	0,11
99	**L**	**-0,347**	**0**	**0,193**	**0**

H- Entropy
Hb- Entropy for clonal sequences from untreated group
Hq- Entropy for clonal sequences from treated group

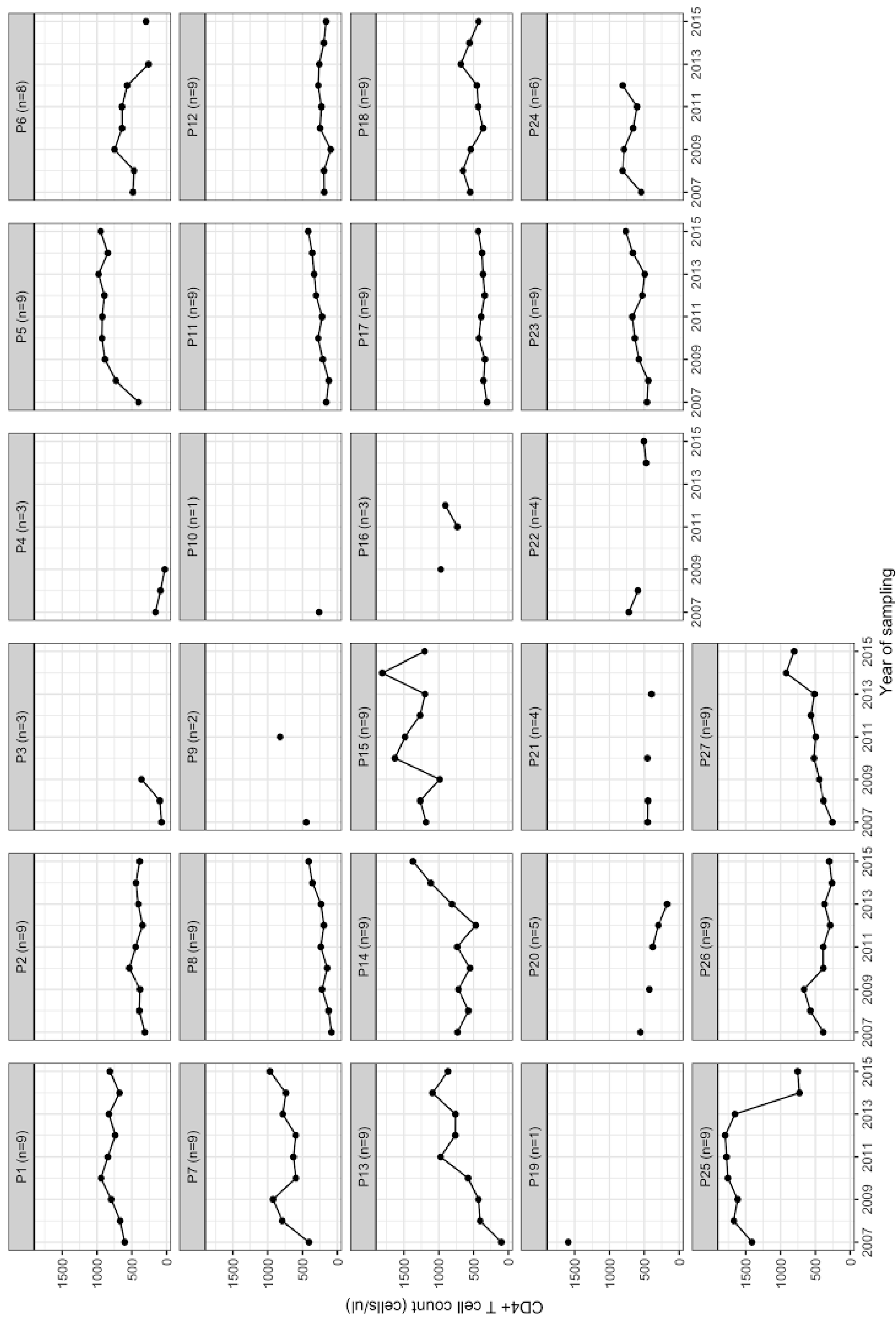

Figure S1. Evolution of CD4+ T cell count in HIV-2 infected patients during eight years of follow up. The number of clonal sequences analysed per patient is indicated in parentheses.

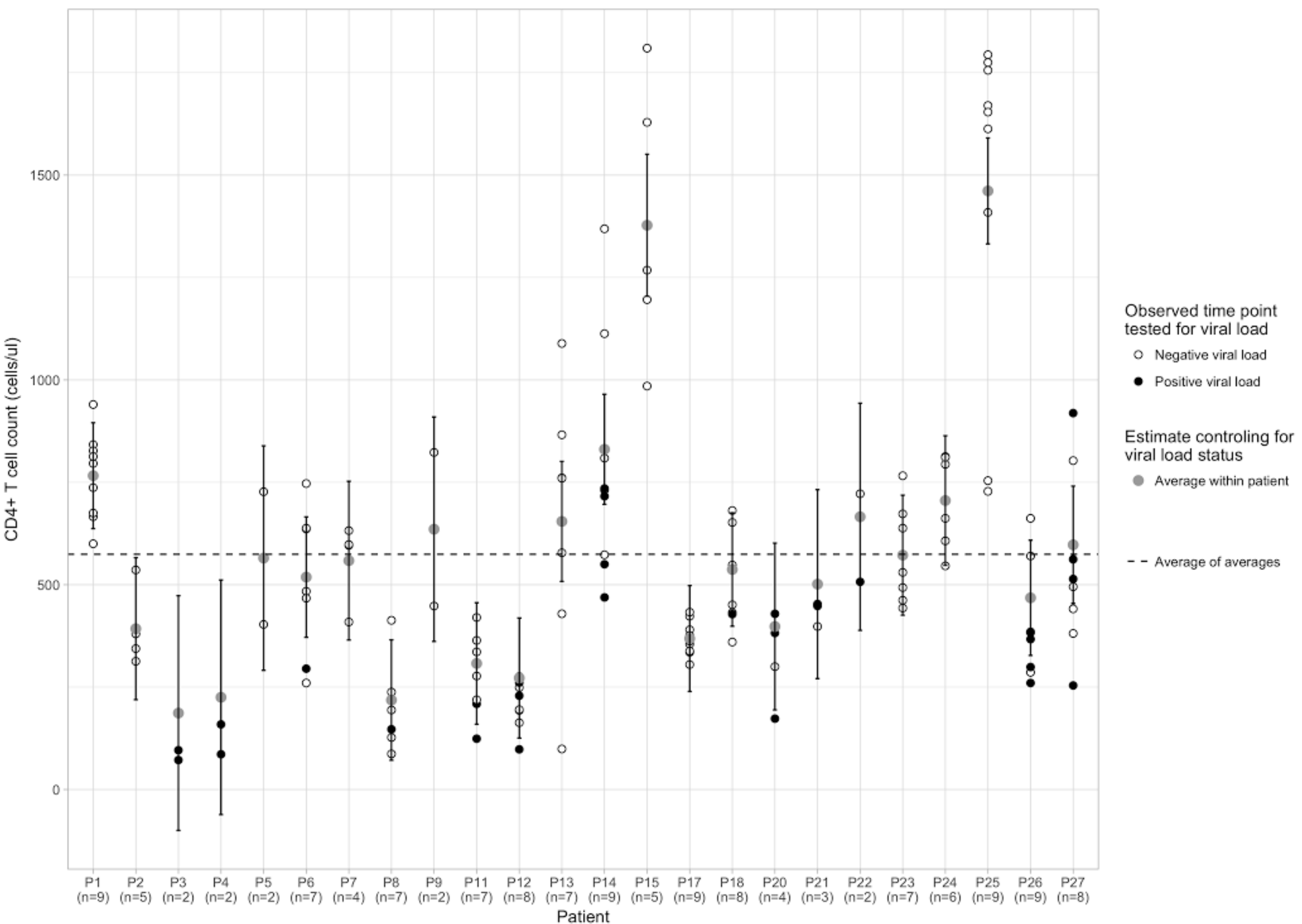

Figure S2. CD4+ T cell count in each HIV-2 infected patient at time points tested for viral load. Time points with negative viral load are represented in open dots and time points with positive viral load are represented with closed dots. The average CD4+ T cell counts within patients, while controlling for the viral load status, are shown as grey circles. The average of the averages within patient is represented in the dashed line.

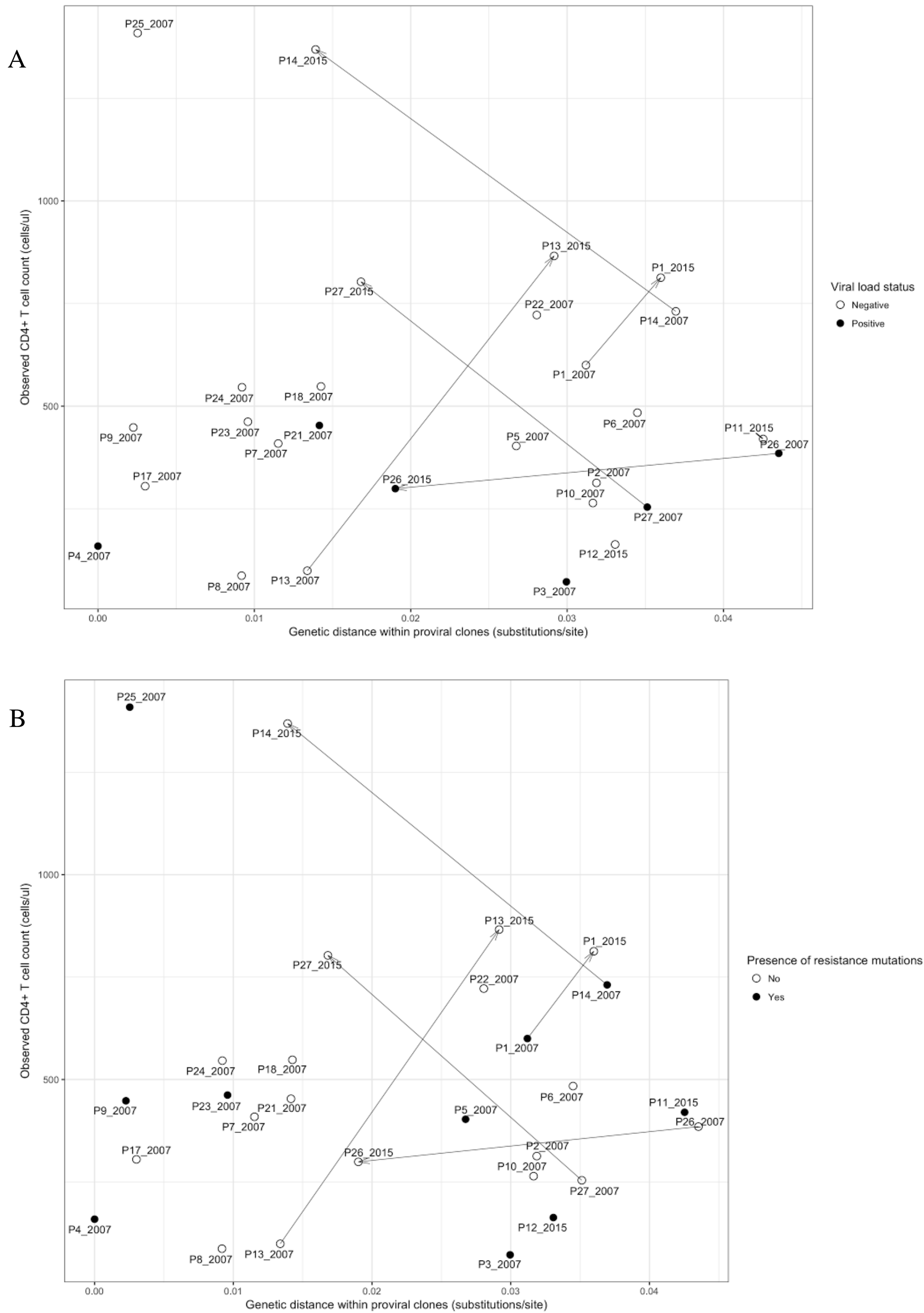

Figure S3. Association between the observed CD4+ T cell counts and the protease genetic distance between proviral clones at the same time point in each patient. The viral load status at each time point is presented in (A), while the presence of resistance mutations is presented in (B).The arrows connect two samples observed in the same patient; the direction of the arrows represents the chronological sequence between the time points.

CHAPTER VII

General discussion and conclusions

HIV-2 shares many similarities with HIV-1, including the genomic and structural organization, transmission routes and the life cycle. However, both viruses exhibit distinct characteristics in terms of pathogenesis, epidemiology, treatment algorithms and evolutionary histories.
Comparing with HIV-1, the majority of HIV-2 infected patients present undetectable viral load and normal CD4+ T cell counts and the ratio of patients with disease progression is much lower. On the other hand, there are much less treatment options for HIV-2 than for HIV-1 infected patients.

Some studies have suggested a potential association between specific genetic determinants in V3 with HIV-2 CXCR4 usage, such as any substitution at position 18, insertions at position 24 and the presence of positively charged amino acids at positions 19 and 27 [1-4]. However, with one exception [4], site-directed mutagenesis has not been used to investigate the specific role of selected V3 amino acids on HIV-2 coreceptor usage. The study of Isaka et al. was the first to show the role of V3 in HIV-2 coreceptor use, as exchanging the C terminal half of the V3 loop between HIV-2 strains ROD (X4) and GH-1 (R5) changed the coreceptor use reciprocally.
To determine the key interacting residues in V3 associated to coreceptor tropism we produced, by site-directed mutagenesis, six new V3 mutants in pROD10, an infectious molecular clone of HIV-$2_{ROD,}$ which is CXCR4 tropic, replicates well in CD4+ T lymphocytes but not in macrophages and is resistant to antibody neutralization. We demonstrated that minor changes in V3 sequence were sufficient to induce major changes in V3 structure, coreceptor use, cell tropism and susceptibility to antibody neutralization. Full X4-to-R5 switch of HIV-2_{ROD10} required the H18L substitution and deletions (H23,Y24) in V3. In terms of structure, these tree mutations lead to a major loss of positive charge and aromatic moiety which is important for the establishment of interactions with other amino acids within and outside (e.g. with coreceptors) the V3 environment [5]. These results provide definitive evidence for the role of these residues and V3 in general in coreceptor use [2-4]. In addition, this is, to our knowledge, the first demonstration of the functional cross-dependence of residues at position 18 and 23/24 in V3.

Like many other X4 isolates, we confirmed that HIV-2_{ROD10} is naturally resistant to antibody neutralization. We found in addition that this is exacerbated with the mutation K29T that reduces the charge of V3 and lead to loss of the interactions with isoleucine at position 27. Higher sensitivity to plasma neutralization in HIV-2_{ROD10}, on the other hand, was associated with dual CCR5 + CXCR4 use caused by mutations H18L and H23Δ + Y24Δ. These results provide definitive evidence for a direct association between V3 loop sequence, size and structure, co-receptor use and susceptibility to antibody neutralization [6-8].

To further evaluate if resistance to antibody neutralization is an inherent characteristic of primary HIV-2 isolates that use the CXCR4 co-receptor, the neutralization phenotype of an extended set of primary X4 isolates obtained from HIV-2 patients in diverse disease stages was determined. The data demonstrated that X4 viruses were significantly more resistant to Nabs than R5 tropic viruses independently of disease stage, confirming that neutralization resistance is an intrinsic characteristic of X4 tropic strains [6-8]. Additionally, we found that resistance to antibody neutralization seems to evolve over time in primary X4 isolates of HIV-2, with viruses from early infection being less resistant to Nabs compared to viruses from late infection.

In line with the results obtained with HIV-2_{ROD10} mutants a charged mutation at position 18 or 19, a 1-3 amino acid insertion in position 24 and a global positive net charge ≥7 was found in the V3 region of all X4 strains.

Cellular tropism and coreceptor usage are important determinants of HIV pathogenicity.

HIV infects both macrophages and activated $CD4^+$ T cells, however, it was suggested by Marchant et al 2006, that HIV-1 and HIV-2 have a different ability to replicate in macrophages [9].

Macrophages constitute a persistent viral reservoir for HIV-1, irrespective of effective treatment, as recently demonstrated in humanized myeloid-only mice (MoM) infected with macrophage-tropic HIV-1 viruses [10]. This may pose a major obstacle to the complete eradication of HIV. To date, no similar data exists for HIV-2.

Information about determinants of macrophage tropism is still limited for HIV-1 and inexistent for HIV-2. In HIV-1, some studies have identified molecular determinants in V3, such as S306R or M326I, which conferred macrophage tropism to X4 using viruses [11].

Similarly, we showed that selected changes in the V3 loop also impact HIV-2 replication capacity in CD4+ T lymphocytes and/or macrophages. This was the case of the K29T variant (X4) that acquired macrophage tropism without compromising its ability to replicate in CD4+ T lymphocytes and H18L + Δ23Δ24 (R5) and Δ23Δ24 (R5/X4) mutants that gained macrophage tropism albeit at some cost on replication capacity in CD4+T lymphocytes. Moreover, we showed that higher levels of replication of HIV-2_{ROD10} occurred earlier in macrophages (3 days post infection) than in $CD4^+$ T cells (6 days post infection). This is in agreement with Marchant et al. [9] that demonstrated that infection in macrophages by HIV-2 primary isolates occurred with lower replication levels (compared to HIV-1) and with a transient peak of virus production 2 days postinfection, followed by an apparent state of latency.

To our knowledge this is the first report showing that a single amino acid in Env can determine HIV-2 cell tropism and that this may be partially independent of co-receptor use. These results imply that other features in Env besides coreceptor use are involved in the entry into macrophages, such as CD4 binding or accessory proteins as shown for HIV-1 [11].

Lower susceptibility to antibody neutralization and acquisition of macrophage tropism were associated with a threonine at position 29 in V3 instead of lysine. This mutation can therefore be a marker of the late virus reservoir in HIV-2 infected patients and eradication of these types of viruses should be important to achieve a cure.

Overall, our studies provide important new information about the molecular and structural determinants in the V3 region of HIV-2 linking coreceptor usage, cellular tropism and escape to antibody neutralization. We propose a new model of HIV-2 evolution and pathogenesis based on the changes that occur in the V3 region over the course of infection (Figure 1). In the early stages of infection, R5 tropic viruses predominate and are characterized by the absence of a regular secondary structure in V3, low charge and amino acids L at position 18 and deletions of an H and Y at positions 23 and 24. These viruses are highly susceptible to neutralizing antibodies [6, 7].

As disease progress, CD4+ T cells decline, viral load increases and the *env* gene, in particular the C2, V3 and C3 regions [7, 12], is under a strong antibody pressure. In response, alterations in the V3 sequence emerge that favour a coreceptor switch (R5 to X4) and the adoption of a β-hairpin conformation in the V3 structure of X4 variants.

Resistance to Nabs increase with the tropism switch with X4 tropic viruses from late infection being more resistance to neutralization compared to variants from the early stages of infection. Loss of tropism for macrophages seems also to be a consequence of tropism switch and acquisition of a charged residue at position 29 in V3.

According to our study L18H and insertions at residues 23H and 24Y might be the first genetic alterations to occur in V3. Other changes might develop in this region, particularly V19K/R which increases global positive net charge [3, 6, 7]. The acquisition of a lysine at residue 29 might take place at more advanced stages of disease as the presence of L or a K in this position did not change the X4 phenotype while induced loss of macrophage tropism and increased lymphocyte tropism. Moreover, as lysine is a positively charged amino acid, it increases the global charge of V3, which is associated to an X4 phenotype [3, 6, 7].

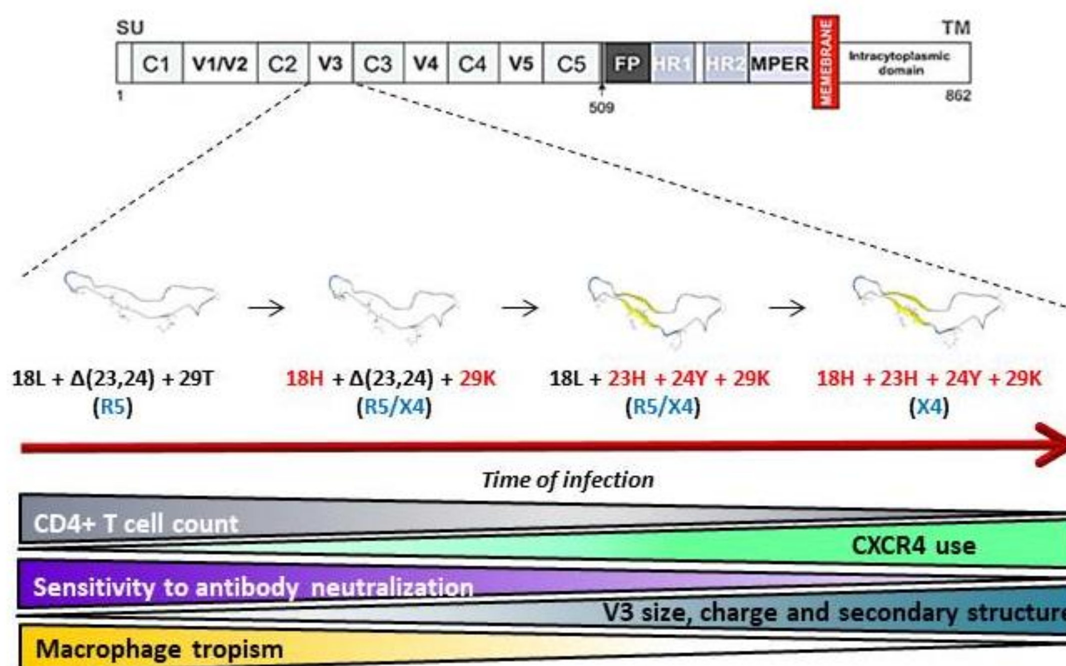

Figure 1. Model of HIV-2 evolution and pathogenesis over the course of infection. This evolution is marked by changes in V3 sequence and structure that lead to R5-X4 coreceptor switch, escape from antibody neutralization and changes in cellular tropism. Structural and functional domains of HIV-2 envelope glycoproteins (gp125-SU and gp36-TM) are shown. The 3D structures of V3 mutants and ROD wild type (wt) show the differences in V3 structure during R5 to X4 tropism switch in HIV-2_{ROD10}.

CCR5 antagonists perform their antiviral activity against HIV by binding to CCR5 coreceptor and blocking viral entry. To date, maraviroc is the first and the only CCR5 antagonist approved for treatment of HIV infection. It can be used to treat HIV-2 infected patients [13-15], although coreceptor determination is mandatory before initiating the treatment.

Viral coreceptor usage can be determined either phenotypically or genotypically, however, no standardized phenotypic or online genotypic assay is available to determine HIV-2 coreceptor, in contrast to HIV-1.

In the second study (Chapter 4) we helped to develop and validate the first online tool - geno2pheno[coreceptor-hiv2] - to predict HIV-2 coreceptor usage based on V3 loop sequence.

The predictive performance of the geno2pheno was based on an independent data set which included the V3 mutants of pROD10 with the corresponding phenotypic measurements of coreceptor usage determined in the previous chapter. All six mutants were classified correctly by geno2pheno, under the recommended FPR cutoff of 5%. Therefore, it was demonstrated a higher accuracy of tropism prediction for geno2pheno comparing with the existing approach developed by Visseaux et al [3] (100% *versus* 77.7%, respectively).

Together with our previous findings from site-directed mutagenesis study, these results showed that individual amino acids are highly predictive of coreceptor usage in HIV-2 and that discriminatory features of HIV-2 tropism occur at the end of V3 loop, rather than on interdependent substitutions of amino acids occurring along the full extent of the V3 as seen in HIV-1.

The geno2pheno [coreceptor-hiv2] can help to guide clinicians in the management of HIV-2 infection by supporting treatment decisions and in the management of HIV-2 infected patients who are eligible for treatment with MVC.

Besides MVC, T-20 is the other entry inhibitor approved for clinical use in HIV-1 infection. This drug inhibits HIV-1 entry by competitive binding to the complementary HR-1 of gp41, thereby blocking the formation of 6-HB that is essential for fusion of the viral and cellular membranes. However, due to its reduced activity against HIV-2, T-20 is not recommended as part of an antiretroviral regimen for HIV-2 infected patients [16, 17].

In the third study (Chapter 5), we demonstrated that the new short-peptide 2P23 containing a "M-T hook" structure had a potent activity against HIV-2 in addition to HIV-1 and SIV and that this was independent of coreceptor use.
Comparing with other fusion inhibitor peptides (e.g. P3) 2P23 exhibits some advantages, including potent inhibition of HIV-2 and SIV isolates, high structural stability and strong activity against T-20 resistant mutants. Recently, it has been demonstrated that 2P23 has antiviral activity *in vivo* in SIV-infected rhesus monkeys [18].
Thus, 2P23 seems to be a good candidate for further clinical development and in the future it may be useful to treat HIV-2 infected patients.

Protease inhibitors (PIs) are one important drug class used in the treatment of HIV-2 infection. Saquinavir (SQV), darunavir (DRV) and lopinavir (LPV) are the most potent PIs against HIV-2, and therefore, are currently recommended by Portuguese guidelines to use in the treatment of HIV-2 patients in association with nucleos(t)ide reverse transcriptase inhibitors (NRTIs) or integrase inhibitors.
HIV-2 resistance testing is recommended as a prerequisite before TARV initiation, and also when changing ART regimens, because resistance in HIV-2 develops much faster and there are less treatment options than for HIV-1 [19-21]. RNA plasma genotyping is the standard procedure in the clinical practice to determine the presence of drug resistance mutations, although proviral DNA from PBMCs might represent the reservoir of further drug resistant viruses.
Though the clinical role of archived resistance on future treatment outcomes is not fully defined, archived resistant variants may appear under proper selective pressure potentially contributing to treatment failure and transmitted drug resistance thus limiting future treatment options [22-24]. Furthermore, the standard assays based on plasma viruses may fail at low or undetectable viral loads, which is the case for most HIV-2 infected patients.
In the fourth study (Chapter 6) we characterized PR diversity and genotypic resistance to PIs in proviral DNA of 27 HIV-2 infected individuals living in Portugal, 15 on treatment and 12 untreated. Patients were followed for a period of eight years to characterize treatment outcomes and PR evolution.
PR mutations associated with resistance to the most potent PIs, DRV, LPV and SQV were identified in 42.8% of treated patients. This rate was significantly lower when comparing to other study which applied the same methodology of clonal sequencing (90%) [25].

In contrast, similar and much lower rates of PI resistance were found when considering other studies which used direct PCR sequencing of proviral DNA (12.5%-45.5%) [26-29]. Clonal sequencing allows the detection of minority variants comparing to bulk population sequencing. In this work this method allowed the detection of at least one mutated clone in the virus population present in the patients. The differences in the resistance rates detected in this and other studies might be explained by several factors as adherence to treatment, treatment regimens or/and the methodology used to perform the resistance testing.

The follow-up evaluation of genotypic resistance to PIs eight years after study entry demonstrated the loss of resistance mutations detected at study entry (2; 20%); the persistence of resistance mutations during the follow-up period (1; 10%), and, the development of new resistance mutations (3; 30%).

The first case might derive from treatment interruptions or poor adherence to antiretroviral therapy leading to the re-emergence of wild-type virus and decreasing of resistant variants to undetectable levels in proviral DNA as it was shown for HIV-1 [30-32]. On the other hand, the persistence of resistance mutations for long periods of time and the development of new resistance mutations might lead to treatment failure in the future and pose the risk of transmitted drug resistance.

These results underscore the importance of early detection of resistance mutations in viruses archived in PBMCs, as it may predict treatment response in HIV-2 infected patients and help to guide future treatment decisions, which is particularly important in HIV-2 infected population, for whom therapeutic options are limited compared with HIV-1. However to confirm these results more studies based on paired plasma HIV-2 RNA and proviral DNA are required during HAART and in HAART failure to investigate if archived mutations could limit future treatment options and if the additional information about resistance in reservoirs improve the selection of potentially active drugs.

In this study, we also found well-known resistance mutations to PIs among the untreated patients (15.4%). The resistance mutations found in these patients were the same found with highest prevalence in the treated group, L90M and I84V. Archived drug resistance mutations in proviral DNA raises concern about the potential for TDR. HIV-2 drug naïve patients that begin TARV with TDR mutations present a lower genetic barrier to initial regimens and consequently have an increased risk of treatment failure [33, 34].

This raises more concern in HIV-2, given the less fully active drugs available for this virus, comparing with HIV-1.
Data on HIV-2 TDR based on proviral DNA is scarce. One Portuguese study performed by Parreira et al. in 2006 [35] found the I50V resistance mutation in 3% (n=30) of clonal DNA sequences from untreated patients from Portugal. The lower rate of TDR found by Parreira et al. may be related to the higher number of patients examined (n=30) compared to our study (n=12).
Using population sequencing from plasma isolates, Charpentier et al. in France identified TDR at a prevalence of 5% (PR mutation V47A in two cases and I82F in one) [36]. Lower prevalence of TDR have been reported in two studies performed in France (3.1%; PR mutation I54M in two cases and L90M in one) and Portugal (1.7%; I54M and I64V) [37, 38]. Recently, other study [39] reported one case of TDR (PR mutation L90M) in a drug naïve patient followed in a Portuguese hospital.
The presence of these resistance mutations in the naïve population suggest that resistant variants can be transmitted in HIV-2 infection prior to ART exposure. However, a small sample size was analysed in this and all other studies. Additional studies with a higher number of patients are required to determine if primary drug resistance is a major problem in HIV-2 infected patients in Portugal.

Studies have shown in HIV-1 that resistance mutations selected by PI treatment can alter CTL epitopes repertoire in PR, allowing the virus to escape immune recognition by PR-specific cytotoxic T- lymphocytes [40].
In this study we showed that 65% of the amino acid positions in PR that vary significantly between treated and untreated groups were located within some of the best-characterized CTL epitopes described for HIV-1 [41]. Our findings suggest that a similar interaction between PI treatment and CTL immune response may occur in HIV-2 infected patients. The presence of these mutated and immune-shaped viruses might contribute to lower efficacy of PI treatment and consequently constitute an obstacle to effective control of HIV replication.

Conclusions

There are still many questions to be answered about HIV-2 infection, including the best optimal treatment strategy to treat this population and the pathways involved in drug resistance.

As fewer antiretroviral drugs are effective for this virus, and because genotypic assays are not simply understood in this context, therapeutic decisions are challenging when treating HIV-2 infected patients.

In this scenario, the development of novel drugs with high potency against HIV-2 is urgently needed. Moreover, tools to monitoring HIV-2 drug resistance need to be strengthened and constitute an important priority.

In this work efforts have been made to help to create a novel antiretroviral drug effective against HIV-2 and to provide new tools for tropism and resistance testing in HIV-2 infection, in order to aid clinicians in disease management and patient care.

Our findings provided new insights into the molecular determinants of coreceptor usage, cell tropism and susceptibility to neutralizing antibodies in HIV-2 envelope allowing the development of a new model of HIV-2 evolution and pathogenesis. Our findings also contributed significantly for the development and validation of the first genotypic tool to predict HIV-2 coreceptor usage. This online tool, geno2pheno[coreceptor-hiv-2], can help to guide clinicians in the management of HIV-2 infected patients who are eligible for treatment with maraviroc.

On the other hand, this work demonstrated that DNA proviral in PBMCs can be an attractive choice for monitoring HIV-2 drug resistance and consequently support decision making in the setting of low or undetectable plasma viral loads. Additionally, our results showed that resistance testing based on DNA proviral may predict long term treatment response as its reflects the history of therapeutic failures. This may help to guide future therapeutic decisions, which is crucial in HIV-2 infected individuals for whom therapeutic options are limited compared with HIV-1.

Finally, our data showed that the new short peptide fusion inhibitor 2P23 has a potent viral activity against HIV-2, irrespective of coreceptor tropism, as well against HIV-1 and SIV. Therefore, 2P23 has potential to further development for clinical use, thus expanding the therapeutic armamentarium for the treatment of HIV-2 infection.

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