UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

NEW PREVENTION AND TREATMENT STRATEGIES FOR HIV INFECTION

Ana Rita Ramos Diniz de Quadros e Costa

Orientadores:
Prof. Doutor Nuno Eduardo Moura dos Santos Taveira
Prof. Doutor José António Frazão Moniz Pereira

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

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2018

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Ana Rita Ramos Diniz de Quadros e Costa teve o apoio financeiro da Fundação para a Ciência e Tecnologia através de uma bolsa de doutoramento (SFRH/BD/89140/2012).

“An inefficient virus kills its host. A clever virus stays with it”

– James Lovelock

Ao Flávio e ao Francisco

AGRADECIMENTOS

Quanto iniciei o meu doutoramento, tinha acabado de concluir o Mestrado Integrado em Ciências Farmacêuticas e não sabia ao certo o que me esperava. Ao longo destes anos aprendi muito. Aprendi que fazer investigação é gerir tempo, recursos e expectativas. É ir ao laboratório à meia noite ou ao sábado revelar um ensaio, porque são as células e os vírus que mandam e ditam os *time-points*. É repetir 10 vezes a mesma experiência, para um dia ela dar certo e ficarmos a pensar “Valeu a pena! Vamos comemorar!”. É ter orgulho em ver um artigo publicado com o nosso nome, fruto do nosso trabalho. E acima de tudo é ficar com a sensação de que se sabe muito pouco, pois ainda há tanta coisa para aprender sobre o tema que estudamos.

Ao longo deste percurso, muitas pessoas contribuiram para a realização desta tese e deixo aqui os meus sinceros agradecimentos.

Em primeiro lugar agradeço ao meu orientador, o Professor Nuno Taveira pela oportunidade de fazer este doutoramento no seu grupo, pelos valiosos conselhos, incentivo e paciência em todas as fases deste longo percurso. Obrigada por tudo o que me ensinou, pelos constantes desafios e por nunca ter desistido de mim.

Agradeço também ao Professor José Moniz Pereira, Coordenador do Departamento de Microbiologia da Faculdade de Farmácia por me ter permitido desenvolver o meu trabalho cientifico na sua unidade e pela sua co-orientação.

Aos meus colegas de laboratório Francisco Martin, Rita Calado, Marta Calado, Inês Moranguinho, João Perdigão, Carla Silva, Andreia Martins, Claudia Palladino, Marta Giria, Inês Figueiredo, Joana Duarte, Rita Mateus, Joana Vital e Cheila Rocha obrigada pela boa-disposição, amizade, entreajuda, picnics e jantares que partilhámos. O excelente ambiente do nosso grupo deve-se a todos vocês!

À Inês Bártolo, obrigada pela oportunidade de trabalhar contigo no nosso projeto e por todo o teu apoio. Contigo aprendi muitas coisas. És uma grande mulher, mãe e profissional.

Obrigada Pedro Borrego por estares sempre disponivel para me ajudar a ultrapassar os obstáculos que surgiram e sempre com a tua boa-disposição contagiante! És uma referência para mim.

À Fundação para a Ciência e Tecnologia agradeço o apoio financeiro que me permitiu desenvolver o meu trabalho.

Aos meus pais, obrigada por todo o apoio, segurança e motivação que sempre me deram. Tudo aquilo que consegui alcançar foi graças a vocês.

Ao meu filho Francisco, que com um sorriso apaga todas as minhas preocupações. Tu vieste dar luz aos meus dias e mostrar-me que o melhor do mundo é ser tua mãe.

Ao meu marido Flávio, o meu melhor amigo e companheiro nos bons e maus momentos, obrigada pela tua compreensão, amor e dedicação. Tu deste-me a força que precisei e tornaste o caminho mais fácil. Obrigada por tudo, do fundo do coração.

PREFACE

The research described in this thesis was performed from January of 2012 to April of 2017 at Research Institute for Medicines (iMed ULisboa), Faculty of Pharmacy, Lisbon University, under the supervision of Prof. Doctor Nuno Taveira and the co-supervision of Prof. Doctor José Moniz Pereira.

The major findings were communicated in the form of research papers published in peer reviewed international journals or in preparation, oral communications and poster communications in national and international conferences.

This thesis is based on the following publications:

Manuscripts in international journals

Original manuscripts

Diniz AR, Borrego P, Martin F, Figueiredo I, Gomes P, Gonçalves F, Caixas U, Vaz Pinto I, Barahona, I, Bártolo I, Taveira N. (2018) Differences in susceptibility patterns of isolates from HIV-2 infected patients to raltegravir and dolutegravir. (submitted to Scientific Reports, currently under revision)

Briz V, Sepúlveda-Crespo D, **Diniz AR**, Borrego P, Rodes B, de la Mata FJ, Gómez R, Taveira N, Muñoz-Fernández MA. (2015) Development of water-soluble polyanionic carbosilane dendrimers as novel and highly potent topical anti-HIV-2 microbicides. *Nanoscale*. 7, p.14669-83

Bártolo I*, **Diniz AR***, Borrego P, Ferreira JP, Bronze MR, Barroso H, Pinto R, Cardoso C, Pinto JF, Diaz RC, Broncano PG, Muñoz-Fernández MA, Taveira N. Evaluation of the fusion inhibitor P3 peptide as a potential microbicide to prevent HIV transmission in women. (submitted to Plos-One)

*These authors contributed equally to this work.

Reviews

Diniz AR, Martin F, Martins AR, Bártolo I, Taveira N. Antiretroviral treatment of HIV-2 infection. (submitted)

Manuscripts in portuguese journals

Reviews

Diniz AR, Canhões R, Taveira N. (2015) Pre-exposure prophylaxis for prevention of HIV infection. *Revista Portuguesa de Farmacoterapia*, vol. 7, p. 15-33.

Oral communications in international conferences

Taveira N, **Diniz AR**, Borrego P, Martin F, Gomes P, Gonçalves F, Caixas U, Vaz Pinto I, Barahona, I, Bártolo I. Dolutegravir has a highly potent activity against most primary HIV-2 isolates including those that have acquired resistance to Raltegravir. – June 1-5, 2017 – ASM Microbe – New Orleans, USA.

Diniz AR, Borrego P, Bártolo I, Taveira N. Entry inhibitors are less effective at preventing cell-associated HIV-2 infection than HIV-1. – May 23-24, 2016 – ICHA 2016: 18th International Conference on HIV and AIDS – London, United Kingdom;

Bártolo I, **Diniz AR**, Borrego P, Taveira N. Pre-clinical evaluation of a new vaginal microbicide gel for prevention of HIV infection – March 20-24, 2016 – Keystone Symposia Conference, HIV Vaccines – Resort at Squaw Creek, Olympic Valley, Califórnia, EUA;

Oral communications in portuguese conferences

Diniz AR, Borrego P, Martin F, Gomes P, Gonçalves F, Caixas U, Vaz Pinto I, Barahona, I, Bártolo I, Taveira N. Dolutegravir has a highly potent activity against most primary HIV-2 isolates including those that are resistant to raltegravir. – 3-9 May, 2017 – redeSAÚDE's 4th Annual Conference, ULisboa Innovation Week – Lisbon, Portugal.

Calado R, Duarte J, **Diniz AR**, Borrego P, Marcelino JM, Bártolo I, Clemente S, Taveira N. Expression and antigenecity of gp120 and C2-V3-C3 polypeptides from different HIV-1 genetic forms – July 2nd 2014 – 6th iMed.ULisboa Postgraduate Students Meeting – Faculty of Pharmacy, Lisbon University – Lisbon, Portugal;

Diniz AR, Briz V, Borrego P, Palladino C, Rodés C, de la Mata FJ, Gómez R, Muñoz-Fernández MA, Taveira N. Inhibition of cell-free and cell-associated HIV-2 infection: new compounds and new assays. – April 5, 2014 – HIV-2, the forgotten virus. – Molecular Medicine Institute, Lisbon, Portugal.

Poster communications in international conferences

Bártolo I, **Diniz AR**, Borrego P, Taveira N. Pre-clinical evaluation of a new vaginal microbicide gel for prevention of HIV infection – March, 20-24 – 2016 Keystone Symposia Conference, HIV Vaccines – Resort at Squaw Creek, Olympic Valley, Califórnia, EUA;

Diniz AR, Briz V, Borrego P, Palladino C, Rodés C, de la Mata FJ, Gómez R, Muñoz-Fernández MA, Taveira N. Entry Inhibitors and Carbosilane Dendrimers are Potent Inhibitors of Cell-free and Cell-Associated HIV-2 Infection – March, 22-27, 2015 – Keystone Symposia Conference, HIV Vaccines: Adaptive Immunity and Beyond – Fairmont Banff Springs, Banff, Alberta, Canada;

Calado R, Duarte J, **Diniz AR**, Borrego P, Marcelino JM, Bártolo I, Clemente S, J Wilton, Taveira N. Envelope C2-V3-C3-specific antibodies correlate with neutralization activity in plasma from HIV-1 infected patients from Angola – March 22-27, 2015 – Keystone Symposia Conference, HIV Vaccines: Adaptive Immunity and Beyond – Fairmont Banff Springs, Banff, Alberta, Canada;

Briz V, Borrego P, Palladino C, **Diniz AR**, Rodés B, de la Mata FJ, Gómez R, Taveira N, Muñoz-Fernández MA. Carbosilane dendrimers as topical microbicides to prevent HIV-2 infection. – June, 23-27, 2013 – 8th International Dendrimer Symposium. Madrid, Spain.

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Martin F, Palladino C, Mateus R, **Diniz AR**, Calado R, Clemente S, Taveira N. Neutralizing antibody response in HIV-1 infected patients from Angola – July, 13-14 2017 – 9th iMed.ULisboa Postgraduate Students Meeting – Faculty of Pharmacy, Lisbon University – Lisbon, Portugal

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Calado R, Duarte J, **Diniz AR**, Borrego P, Marcelino JM, Bártolo I, Clemente S, Taveira N. Expression and antigenecity of gp120 and C2-V3-C3 polypeptides from different HIV-1 genetic forms – July 2nd 2014 – 6th iMed.ULisboa Postgraduate Students Meeting – Faculty of Pharmacy, Lisbon University – Lisbon, Portugal;

Resumo

Desde o início da epidemia do HIV, 78 milhões de pessoas foram infetadas e 35 milhões morreram por complicações relacionadas com a SIDA. O trabalho desenvolvido nesta tese vem reforçar o benefício de aliar estratégias de prevenção e tratamento, para controlar de forma eficaz a propagação global da infeção por HIV. Para além disso, este trabalho pretende realçar a importância do HIV-2, um vírus frequentemente negligenciado e para o qual as opções de tratamento são limitadas.

Os principais objetivos desta tese foram: a) caracterizar a atividade do inibidor da transferência da cadeia da integrase (INSTI) dolutegravir (DTG) num painel de isolados primários de HIV-2 obtidos de pacientes INSTI-naïves e pacientes em falha terapêutica com raltegravir (RAL) e identificar polimorfismos e mutações de resistência aos INSTIs; b) avaliar a actividade de dendrimeros polianiónicos carbosilados como novos microbicidas anti-HIV-2; c) avaliar o péptido inibidor da fusão P3 como potencial candidato a microbicida para prevenir a transmissão de HIV nas mulheres.

O Capítulo 1 consiste numa introdução aos temas abordados neste trabalho. A epidemia de HIV/SIDA tem grande impacto a nível mundial, mas em particular na África sub-Sariana. De grande importância é o facto de, em algumas regiões de África, o número de mulheres infetadas pelo HIV constituir mais de metade do número total de pessoas infetadas, adquirindo o vírus maioritariamente por via heterossexual. Na Secção II da Introdução, foi compilada informação sobre o tratamento da infeção por HIV-2, resultando num artigo de revisão. Globalmente, 1 a 2 milhões de pessoas estão infetadas pelo HIV-2, a grande maioria na África ocidental, mas também em países que mantêm ligações socioeconómicas com estas regiões, como é o caso de Portugal e França. Em comparação com o HIV-1, a progressão da doença é mais lenta, com maior longevidade (mais 10-20 anos que na infeção por HIV-1) e as taxas de mortalidade são mais reduzidas. No entanto, se a infeção por HIV-2 não for tratada, evolui para SIDA e morte em 20-25% dos pacientes infetados. Os antirretrovirais disponíveis foram desenvolvidos para HIV-1, pelo que alguns têm reduzida ou nula eficácia contra HIV-2. Para além disso, o HIV-2 pode desenvolver resistência aos antirretrovirais por mecanismos diferentes do HIV-1, sendo que existem poucos estudos clínicos que indiquem quais as melhores opções para terapia de primeira e segunda linha para estes doentes. Na Secção III, apresentamos um artigo de revisão que consiste num estudo exaustivo das estratégias de Profiláxia de pre-Exposição (PreP) existentes para prevenir a infeção por HIV. A PrEP consiste na administração de um ou vários antirretrovirais a um individuo HIV-negativo com comportamentos de alto risco. Esta profilaxia pode ser feita com a toma oral de antirretrovirais, como é o caso do uso já aprovado de tenofovir (TDF) /emtricitabina, ou pela administração tópica vaginal ou retal de microbicidas. Os microbicidas são formulações (ex: géis, cápsulas, cremes, supositórios, anéis vaginais) usadas para prevenir a infeção por HIV durante o ato sexual. O objetivo dos microbicidas é atuar nos primeiros passos da infeção, inibindo ou bloqueando a entrada do vírus na mucosa vaginal ou retal. Um microbicida eficaz terá a capacidade de dar grande poder às mulheres uma vez que não necessita de cooperação, consentimento e até conhecimento do parceiro para ser usado.

No Capítulo 2, caracterizámos a atividade do DTG num painel de 16 isolados primários de HIV-2 obtidos de pacientes INSTI-naïves e pacientes em falha terapêutica com raltegravir (RAL). Verificámos que o RAL e o DTG são muito eficazes contra os isolados HIV-2 obtidos de pacientes RAL-naïve. No entanto, os isolados virais obtidos de pacientes em falha terapêutica com RAL

demonstraram ser muito resistentes ao RAL, confirmando que terapêuticas com RAL em pacientes HIV-2 levam à seleção de vírus extremamente resistentes a este fármaco. De grande importância foi o facto de o DTG ter conseguido inibir todos os isolados resistentes ao RAL, o que indica que o DTG é muito útil como fármaco de segunda linha para pacientes em falha terapêutica com RAL. Foram encontradas as mutações de resistência E92Q e T97A num isolado primário resistente ao RAL, confirmando que a combinação destas mutações confere de facto resistência a este antirretroviral. A mutação de resistência major Q148K foi encontrada em combinação com a mutação minor E92A num segundo isolado resistente ao RAL. Apesar da mutação Q148K estar presente, este isolado demonstrou elevada suscetibilidade ao DTG, o que prova que a presença desta mutação não é suficiente para conferir resistência ao DTG, ao contrário do que se encontra descrito noutros trabalhos. Neste trabalho, descrevemos pela primeira vez resistência primária ao DTG, mas não ao RAL, em dois vírus de uma criança infetada à nascença pela mãe. Curiosamente, o isolado da mãe, era totalmente sensível ao RAL e ao DTG. A análise da sequência de aminoácidos da integrase dos vírus destes doentes, permitiu identificar mutações nos codões 221 e 222 no domínio C-terminal da integrase do HIV-2 como prováveis determinantes de resistência natural ao DTG. Alguns estudos indicam que mutações no domínio C-terminal da integrase têm forte influência na actividade da transcriptase reversa, aumentando a força da ligação integrase-transcriptase reversa e assim intensificando a retrotranscrição. Deste modo, a replicação viral aumenta, resultando num mecanismo indireto de resistência ao DTG. Os resultados obtidos neste trabalho permitem concluir que o DTG tem potente atividade contra isolados primários de HIV-2, incluindo isolados resistentes ao RAL. No entanto, resistência primária a este fármaco pode ocorrer através de um novo mecanismo de resistência proposto. Globalmente, os nossos resultados indicam que o DTG é uma importante opção terapêutica para pacientes infetados com HIV-2.

No Capítulo 3, analisámos a actividade de três novos dendrimeros polianiónicos carbosilados (G2-S16, G2-NS16 e G3-Sh16) como microbicidas anti-HIV-2. Estes dendrimeros inibiram potentemente a infeção por HIV-2 *in vitro* (infecção vírus célula e célula-célula). Os dendrimeros destabilizam a membrana do HIV-2 e impedem a ligação do virus aos receptores da superfície celular, deste modo bloqueando a infecção viral. Demonstrámos ainda que os dendrímeros atuam de forma sinérgica quando usados em combinação com o TDF e o RAL. Para além disso, demonstraram não ter atividade antimicrobiana e espermicida, o que significa que não afetam a flora vaginal nem o esperma, características desejadas num potencial microbicida. Finalmente, o gel 3% HEC-G2-S16 demonstrou ter um bom perfil de segurança e não causou alterações no epitélio vaginal de ratos BALB/c. Em resumo, os resultados obtidos neste capítulo permitiram demonstrar que os dendrimeros polianiónicos carbosilados G2-S16, G2-NS16, e G3-Sh16 são bons candidatos a microbicidas vaginais. De acordo com resultados de outros estudos, o melhor dendrimero para inibir o HIV-1 é o G2-S16, no entanto, os nossos resultados indicam que o dendrimero com melhores resultados para HIV-2 é o G2-NS16.

No Capítulo 4, avaliámos a utilização do péptido de fusão P3 como potencial microbicida para a prevenção da transmissão vaginal do HIV. O P3 foi desenvolvido com base nas sequências ancestrais das glicoproteínas transmembranares do HIV-2 e SIV e tem potente atividade contra HIV-1 e HIV-2. O P3 demonstrou ser estável e ativo na presença de fluidos corporais (fluido vaginal e plasma seminal), o que é importante para ter atividade na mucosa vaginal. Para além disso, o P3 é estável não só à temperatura corporal, mas também a 65ºC e a 4ºC, temperaturas às quais este péptido pode ser sujeito durante a sua produção e armazenamento, respetiva-

mente. O P3 manteve a atividade antiviral em ambiente ácido, o que indica que terá atividade no pH ácido normal da vagina e demonstrou também ser ativo na presença do H_2O_2, que é produzido no ambiente vaginal pelos *Lactobacillus*. O Péptido P3 não tem atividade espermicida e apenas causou lesões no epitélio vaginal em concentrações ~8300 vezes superiores ao seu IC_{90}, mantendo o efeito protetor do ambiente vaginal normal. Finalmente, a formulação do P3 num gel de 1,5%-HEC, demonstrou ter o mesmo efeito antiviral do que o P3 livre, provando que, pelo menos *in vitro*, o P3 é eficazmente libertado do gel e inibe a infeção por HIV-1. Em resumo, este trabalho demonstrou que o P3 é um candidato promissor a microbicida vaginal.

Em conclusão, neste trabalho demonstramos que o DTG é um potente inibidor da infeção por HIV-2, embora o aparecimento de resistência primária seja uma preocupação, ao contrário do RAL para o qual este tipo de resistência não se desenvolveu. Para além disso, contribuímos para um conhecimento mais aprofundado sobre as mutações na integrase que levam à resistência aos INSTIs. Neste trabalho, concluímos também que os dendrimeros polianiónicos carbosilados G2-S16, G2-NS16, e G3-Sh16 são activos contra HIV-2, em particular o dendrimero G2-NS16, sendo potenciais candidatos a microbicidas vaginais. Os resultados dos ensaios feitos com o péptido inibidor da fusão P3 permitem concluir que este péptido tem também muitas qualidades que recomendam a sua formulação num gel microbicida contra HIV.

Palavras-chave: HIV; raltegravir; dolutegravir; microbicidas; dendrimeros; P3.

Abstract

To successfully control the global spread of HIV, it is crucial to combine effective prevention and treatment strategies. The aims of this thesis are: a) to characterize the antiviral activity of dolutegravir (DTG) against HIV-2 primary isolates; b) to develop dendrimers as topical anti-HIV-2 microbicides; c) to evaluate P3 peptide as a potential microbicide to prevent HIV transmission in women.

In Chapter 2, we observed that DTG had potent activity against all raltegravir (RAL)-resistant HIV-2 isolates, indicating its usefulness as second line therapy for patients failing RAL. Mutation Q148K was responsible for high-level of resistance to RAL, however, it did not affect the activity of DTG. Importantly, we described for the first time primary resistance to DTG and we propose a new mechanism of resistance: mutations K221Q and D222K increase the activity of reverse transcriptase, thus increasing viral replication, leading to indirect resistance to DTG.

In Chapter 3, we showed that dendrimers G2-S16, G2-NS16 and G3-Sh16 inhibited HIV-2 infection acting in the early steps of the HIV-2 lifecycle. They blocked HIV-2 cell-free and cell-to-cell fusion and had synergistic effects with tenofovir and RAL. Vaginal application of 3% HEC-G2-S16 gel formulation in mice did not affect mucosa integrity, further confirming that these dendrimers are promising candidates for future topical microbicides.

In Chapter 4, we proved that the fusion inhibitor peptide P3 was stable and active in the presence of body fluids and at different temperatures. P3 was also active in the acidic environment of the vagina, was not be affected by the H_2O_2 produced by *Lactobacillus* and did not have spermicidal activity. Importantly, we showed that a P3-1.5% HEC gel was very effective at blocking HIV-1 infection.

These findings suggest that dendrimers and P3 are good microbicide candidates to prevent vaginal HIV transmission in humans.

Keywords: HIV; raltegravir; dolutegravir; microbicides; dendrimers; P3.

List of acronyms

3' PPT	3' polypurine tract
3TC	lamivudine
AAP	American Academy of Pediatrics
ABC	abacavir
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral therapy
ARV	Antiretroviral
ATZ	atazanavir
AZT	zidovudine
BHIVA	British HIV Association
CC_{50}	50% cytotoxic concentration
CCD	catalytic core domain
CCR5	C-X-C chemokine receptor type 5
CDC	Center for Disease Control
CTC	Cell-to-cell fusion
CTD	C-terminal domain
CVC	Cenicriviroc
CXCR4	C-X-C chemokine receptor type 4
d4T	estaduvine
DC	Dendritic Cell
ddI	didanosine
DHHS	Department of Health and Human Services
DLV	delavirdine
DMEM	Dulbecco's minimal essential medium
dNTP	Deoxyribonucleotide triphosphate
DRV	darunavir
DTG	Dolutegravir
EC_{50}	Half maximal effective concentration
ECL	extracellular loop
EFV	efavirenz
Env	envelope
ETV	etravirine

EVG	Elvitegravir
FNC	Azvudine
FPV	fosamprenavir
FTC	emtricitabine
GM	Growth medium
GRADE	Genotypic Resistance-Algorithm Deutschland
h-BLT	humanized bone marrow–liver– thymus
HEC	Hydroxyethyl Cellulose
HIV-1	Human Immunodeficiency Virus type-1
HIV-2	Human Immunodeficiency Virus type 2
HSV-2	Herpes simplex virus type 2
IC_{50}	50% inhibitory concentration
IC_{90}	90% inhibitory concentration
ICAM-1	Intercellular Adhesion Molecule 1
IDV	indinavir
IN	Integrase
INI	integrase inhibitor
INSTI	Integrase Strand Transfer Inhibitor
IVR	intra-vaginal rings
LA	long acting
LC	Langerhans cell
LC-MS/MS	Liquid chromatography and mass spectrometry
LFA-1	Lymphocyte function-associated antigen 1
LPV	lopinavir
LTR	long terminal repeat
MC	Male circumcision
MIC	Minimum Inhibitory Concentration
MPI	Maximum Percentage of Inhibition
MSM	men who have sex with men
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide
MVC	Maraviroc
NFV	nelfinavir
NNRTI	non-nucleoside reverse transcriptase inhibitors

NRTI	nucleoside/nucleotide reverse transcriptase inhibitors
NTD	N-terminal domain
NVP	nevirapine
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffer saline
PEP	post-exposure prophylaxis
PI	Protease Inhibitors
PR	Protease
PrEP	Pre-Exposure Prophilaxy
RAL	Raltegravir
RPV	rilpivirine
RT	reverse transcriptase
RTV	ritonavir
rVV	recombinant vaccinia virus
SIV	Simian Immunodeficiency Virus
SP	Seminal Plasma
SQV	saquinavir
STD	sexually transmitted disease
TAF	Tenofovir alafenamide
TAM	timidine analog-associated mutation
TCID50	50% Tissue Culture Infective Dose
TDF	fumarato de tenofovir disoproxil
TFV	tenofovir
TI	therapeutic index
TPV	tipranavir
UNAIDS	Joint United Nations Program on HIV/AIDS
VFS	Vaginal Fluid Simulant
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

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CHAPTER 1
GENERAL INTRODUCTION

Section I
The HIV/AIDS epidemic

The Acquired Immunodeficiency Syndrome (AIDS) was first described in 1981 [1] and is a complex disease of the human immune system caused by the human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) [2]. The HIV epidemic arose after zoonotic infections with simian immunodeficiency viruses (SIV) from African primates: HIV-1 is thought to have arisen from cross-species transmission of a chimpanzee virus to humans [3, 4] and HIV-2 from cross-species transmission of a Sooty mangabey virus [5]. HIV-1 is responsible for the global pandemic while HIV-2 is largely confined to West Africa [6] and countries with historical links to that area, like Portugal [7].

Four groups of HIV-1 have been described and represent three separate transmission events from chimpanzees (M, N, and O), and one from gorillas (P) [8]. Most HIV-1 infections are caused by group M viruses, which are the cause of the global HIV pandemic and these are divided into 9 subtypes known as clades (A–D, F–H, J, and K) [8]. The most common clade in the Americas, Europe, and Australia is clade B, whereas clade C predominates in the most heavily affected part of the world, southern Africa [8]. Groups N, O, and P are restricted to west Africa [8].

HIV-2 was first isolated in 1986 and in comparison with HIV-1, infection with HIV-2 is associated with a slower progression to immune deficiency and lower transmissibility [9, 10]. HIV-2 has 9 groups (A-I), of which A and B are the most common, and only one recombinant form (CRF01_AB) [9, 11-13]. Recently, it was reported that group A may be divided in two distinct subtypes [14].

Two broad epidemiologic patterns in the global distribution of HIV-1 infection have emerged. Most countries in the world have concentrated HIV-1 epidemics, in which HIV-1 infection is detected in specific groups at risk (men who have sex with men (MSM), injecting drug users, sex workers, and the regular partners of such persons) but is not well established in the general population [15, 16]. In these cases, HIV prevention, treatment, care, and support efforts can be focused on the most affected subpopulations. In many countries of sub-Saharan Africa, however, HIV-1 epidemics are generalized. A generalized HIV epidemic is an epidemic that is self-sustaining in the population through heterosexual transmission [16]. However, even in settings of generalized epidemics, the risk of infection with HIV-1 is not equal across societies but is increased in persons with higher rates of partner change or who acquire classical sexually transmitted diseases (STDs) and in persons who experience other significant potential exposures to HIV-1, such as injection drug use [15].

Since the start of the epidemic, 78 million people have become infected with HIV and 35 million people have died from AIDS-related illnesses [17]. In 2015, there were 36.7 million people globally living with HIV [17]. The number of people living with HIV continues to increase, in large part because more people globally are accessing antiretroviral therapy and as a result are living longer, healthier lives [18]. In 2015, around 46% of all people living with HIV had access to treatment, including 77% of pregnant women intending to prevent transmission of HIV to their babies [17]. AIDS-related deaths have fallen by 45% since the peak in 2005 [19], however, 2.1 million people became newly infected with HIV in 2015, with eastern and southern Africa

accounting for 46% of the global total of new HIV infections (960 000 new HIV infections, in 2015) [17]. Women account for more than half the total number of people living with HIV in eastern and southern Africa [17]. Since 2005, mortality due to HIV declined probably as a result of the implementation of ART programs and interventions focused on the prevention of mother-to-child transmission [19]. However, HIV/AIDS is still the leading cause of Years of Life Lost (YLL) in Southern sub-Saharan Africa (Botswana, Lesotho, Namibia, South Africa, Swaziland and Zimbabwe) [19].

The estimated per-act HIV transmission risk (all expressed as per 10 000 exposures) is greatest for blood transfusion [9250 (95% CI8900–9610)], followed by mother-to-child transmission [2260 (95% CI 1700–2900)], receptive anal intercourse [138 (95% CI 102–186)], needle-sharing injection drug use [63 (95%CI 41–92)], and percutaneous needle stick injuries [23 (95% CI 0–46)] [20]. Risk for other sexual exposures is 4 (95% CI 1–14) for insertive penile–vaginal intercourse, 8 (95% CI 6–11) for receptive penile–vaginal intercourse, and 11 (95% CI 4–28) for insertive anal intercourse [20]. The transmission risk for receptive and insertive oral sex is quite low (95% CI 0–4) [20].

There is evidence that HIV-1 transmission is directly correlated with the level of virus in circulation [21, 22]. Notably, up to 50% of new HIV-1 infections are acquired from recently infected patients [23]. Early (acute) and later stages of infection (advanced disease) are the periods with higher viral loads and consequently, higher rates of HIV-1 transmission [15, 24, 25].

Although HIV-1 strains are responsible for most of the global infections, HIV-2 strains are an important cause of disease in West African nations, Portugal, France, and in the United States [26]. Moreover, co-infection with both HIV-1 and HIV-2 occurs in some countries of West Africa where the viruses co-circulate [26]. Sexual transmission is responsible for the majority of HIV-2 infections due to infected semen or cervicovaginal secretions containing infected lymphocytes [26].

The impact of HIV on women

In contrast to the first two decades of the HIV pandemic, today girls and women make up more than half of the 36.7 million people living with HIV, but with large regional differences—in western and central Africa, nearly 60% of all people living with HIV are women [18] who acquire the virus largely by heterosexual exposure [27]. Worldwide, 18.6 million women and girls are living with HIV and more than 100 adolescents died of AIDS every day in 2015 [28]. Every year, nearly 1 million new HIV infections occur in young women and girls and every 4 minutes, 3 young women become infected with HIV [28].

In sub-Saharan Africa, three in four new infections in 15–19 year olds are among girls [28]. Globally, young women are twice as likely to acquire HIV as their male counterparts [28]. This age and sex differential in HIV distribution is considered to be a key driver of the generalized epidemics described in this region [29].

HIV is the leading cause of death among women aged 30–49 globally and is the third leading cause of death among young women aged 15–29 globally [30], killing more women than men in this age group (Figure 1) [31]. Approximately 75% of young women aged 15–19 report they do not have a final say in decisions about their own health [28].

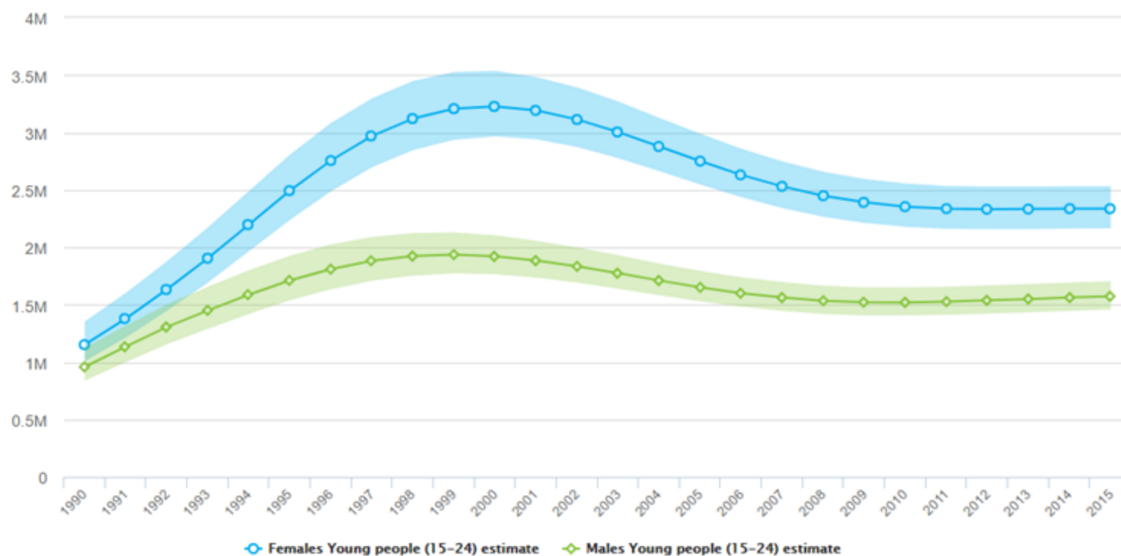

Figure 1 – *HIV prevalence among young people (15-24 years of age).*
Source: UNAIDS Estimates 2016.

Regarding maternal and newborn health, every day 200 children are born with HIV and another 200 children are infected through breastfeeding, although 98% of new HIV infections among children could be prevented [28]. While the exact contribution of HIV/AIDS to maternal mortality rates is unknown, there is growing evidence which shows that HIV/AIDS is a leading cause of pregnancy-related death [32].

The epidemic imposes a particular burden on women and girls, who are at the center of the AIDS response and face multiple inequalities and challenges during their life. In addition to their greater physiological susceptibility to HIV acquisition, the pervasive social, legal and economic disadvantages faced by women reduce their ability to protect themselves from HIV infection [33]. Gender inequalities, lower access to education, lower levels of economic independence and intimate partner violence erode the ability of young women to negotiate safer sex and retain control of their bodies [28]. Men, meanwhile, tend to seek services infrequently, are diagnosed with HIV and initiate treatment very late, often with deadly consequences [28].

To better address the impact of HIV on women, particularly on young women and adolescent girls, strategies are needed that consciously adopt a women-centered approach. A deeper understanding of the female genital tract and mucosal immunity is key to guiding the development of strategic and targeted products most suitable for combating HIV infection in women.

Mechanisms of HIV infection

Cell binding and entry of HIV

HIV can spread by cell-free virus or, alternatively, by cell-to-cell contact [34, 35]. HIV entry, the first phase of the replication cycle, begins with the adhesion of cell-free virus to the host cell and ends with the fusion of the cell and viral membranes with subsequent delivery of the capsid into the cytoplasm (Figure 2) [36]. Cell-to-cell infection occurs when an infected cell transfers infectious virus particles to a bystander target cell, through syncytia formation, virological synapses or membrane nanotubes [37-39].

An important step of virus entry entails binding of Env to its primary receptor, the host protein CD4 [40]. Env is a heavily glycosylated trimer of gp120 and gp41 heterodimers. The gp120 subunit is composed by five hypervariable regions, V1 to V5, separated by five more conserved regions, C1 to C5 and is responsible for CD4 binding [36, 41].

The CD4 receptor is a transmembrane protein that exists on the surface of T cells, monocytes, macrophages and dendritic cells [41] and normally functions to enhance T-cell receptor-mediated signaling [36]. Four domains compose the extracellular region of CD4, D1 to D4. Env gp120 binds to CD4 leading to the formation of the bridging sheet and increases the exposure of V1, V2, V3 and C4, resulting in the approximation of the viral envelope and the cellular membrane and the subsequent interaction of V3 with the coreceptor [41-43].

In vivo, the major coreceptors for HIV entry are the CCR5 and CXCR4 G-protein coupled receptors that function as the natural receptors for α and β chemokines [41, 44]. These receptors are integral membrane proteins with seven transmembrane helices, an extracellular N-terminus and three extracellular loops that form a small pocket [44]. HIV strains can be broadly classified based on their coreceptor usage. Viruses that use the chemokine receptor CCR5 are termed R5 HIV, those that use CXCR4 are termed X4 HIV, and viruses that can use both coreceptors are called R5X4 HIV [41]. CCR5 is predominantly expressed on the surface of memory T lymphocytes, activated T lymphocytes and macrophages, while CXCR4 is mainly found in T lymphocytes, monocytes, dendritic cells and B lymphocytes [45]. After CD4 binding, the V3 region is exposed and interacts with the coreceptor. Coreceptor binding, activates the membrane fusion potential of Env [36] inducing exposure of the hydrophobic gp41 fusion peptide, which inserts into the host cell membrane resulting in a six-helix bundle formation, that is the driving force that brings the opposing membranes into close proximity, resulting in the formation of a fusion pore through which the viral capsid enters the target cell (Figure 2) [46-48].

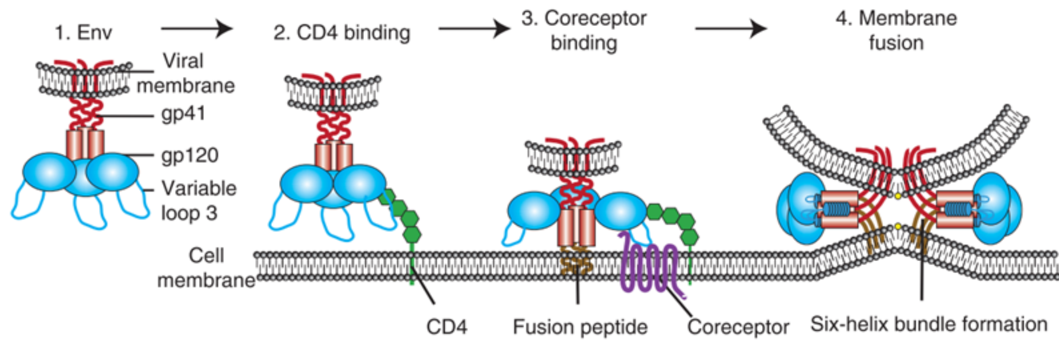

Figure 2 – *HIV fusion to a host cell. HIV Env (1) binds to CD4 receptor of the host cell (2) causing conformational changes in Env, allowing coreceptor binding (3), development of the six-helix bundle formation and membrane fusion (4). [36]*

Cell-associated HIV infection

Cell-to-cell infection can be a fast and direct path of virus transmission and overcome several barriers, like distance between infected and target cells, presence of neutralizing antibodies and low viral infectivity [49]. Syncytia formation, virological synapses and membrane nanotubes are three different ways of HIV cell-to-cell transmission.

HIV infected cells, expressing Env proteins on cell surface, can engage receptors on adjacent cells and trigger cell-to-cell fusion, resulting in the formation of multinucleated giant cells, known as syncytia, that can harbor large amounts of virus (Figure 3) [37, 50].

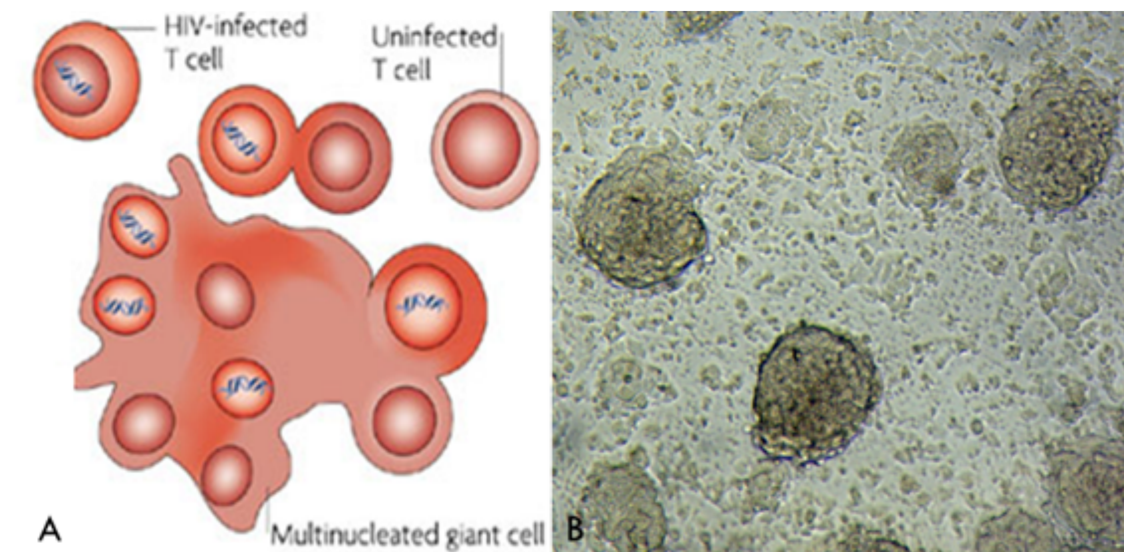

Figure 3 – *Syncytia formation. (A) An HIV-infected cell can fuse with an uninfected cell leading to the formation of a multinucleated giant cell (Adapted from [51]). (B) Microscopic observation of syncytia.*

Formation of virological synapses requires the interaction between HIV envelope glycoproteins expressed by infected cells and CD4-CCR5/CXCR4 receptors of uninfected cells, as well as several cellular adhesion molecules, such as LFA-1 and ICAM-1 [38, 52, 53] and cytoskeletal rearrangements [54, 55]. Once the virological synapse is established, the infected cell assembles and releases a large number of viruses into the synaptic cleft that then infect the target cell [38, 56]. This strategy seems to be important for viral amplification, particularly for initial viral spread from mucosa tissues to DCs and T cells [57], as it promotes efficient cell-to-cell transfer of HIV, without requiring cell fusion.

Alternatively, HIV can induce the formation of membrane nanotubes connecting infected and uninfected cells. HIV can be transferred between cells within the nanotubes or can move along the outside of these structures until attachment and binding to the receptors of uninfected cell occurs [39]. Formation of membrane nanotubes is considered particularly efficient in secondary lymphoid tissue, due to the abundance of target T cells [39].

It is considered that HIV spreads more efficiently by cell-to-cell infection than by cell-free infection [38, 58, 59], which strongly suggests that cell-to-cell infection may have a critical role in efficient virus dissemination in infected individuals. Cell-to-cell infection allows high efficiency of viral delivery, exposing uninfected cells to a large number of virions [60-62]. The efficacy of coreceptor antagonists, fusion inhibitors and broadly neutralizing antibodies (bNAbs) against cell-to-cell infection is inconsistent [61, 63-67]. Virological synapses have been shown to confer some level of protection from NAbs [61]. Studies have proved that cell-to-cell infection can be blocked by bNAbs, however higher concentrations or combinations of multiple bNAbs are required [54, 55, 60, 68, 69]. Furthermore, presence of bNAbs prior to the virological synapse formation, specific epitopes, and type of bNAbs used are all factors that influence the level of protection (revised in [61]). In addition, cell-to-cell infection minimizes the distance that virions need to cross [35, 38], leading to earlier onset of viral gene expression, and multiple cell infection increases the frequency of recombination and escape adaptations [69], overcoming low infectivity and cellular restriction factors [66, 70].

HIV infection of the female reproductive tract

The vast majority of HIV infections in adults result from vaginal and rectal transmission [71].

The vaginal environment is very dynamic, and is defended by innate mucosal barriers and immune mechanisms. Cervicovaginal mucus, which has different consistency and volume throughout the menstrual cycle, can act as a first-line defense of the vaginal mucosa by impairing the mobility of HIV [72]. Neutralizing antibodies and defensive proteins (e.g. complement, lysozyme and lactoferrin) present in the genital tract are also barriers against HIV entry [73-75].

HIV infection of the female reproductive tract is strongly associated with the concentration of virus in semen [21, 25, 76]. Seminal viral load depends on the stage of the infection: in the acute phase of HIV infection, levels of virus in semen are higher and the risk of HIV transmission is therefore increased compared to the chronic phase [21, 77, 78]. HIV penetration and infection have been demonstrated in the vaginal, ectocervical, endocervical, and endometrial mucosa [79].

Another important infection site is the rectal epithelium. Unprotected anal intercourse represents a 10-20 times greater risk of HIV transmission than unprotected vaginal intercourse [80] and rectum and vagina are two very different compartments (Table 1). Rectum has simple columnar epithelium (only one cell layer thick) being more vulnerable to tearing during intercourse, while the vagina and ectocervix are lined with stratified squamous epithelium that is constantly sloughing off the superficial layers (Figure 4) – this process is considered a good mechanical protection against HIV infection [79, 81]. Also, rectal mucosal lining is covered with mucus, that presents a slightly alkaline pH (7-8), with minimal buffering capacity [82], while the vagina has acidic pH (4 – 4.5) that has a protective effect against HIV infection (Table 1) [83].

The rectum is very susceptible to HIV infection, because macrophages present in the rectal mucosa show high expression of CCR5 co-receptors [84], and there is also high density of lymphoid nodules in this area [85]. One study has shown that intestinal CD4+ T lymphocytes are in a physiological state of activation with marked expression of HIV-1 co-receptors, thus being naturally susceptible to HIV-1 infection [86].

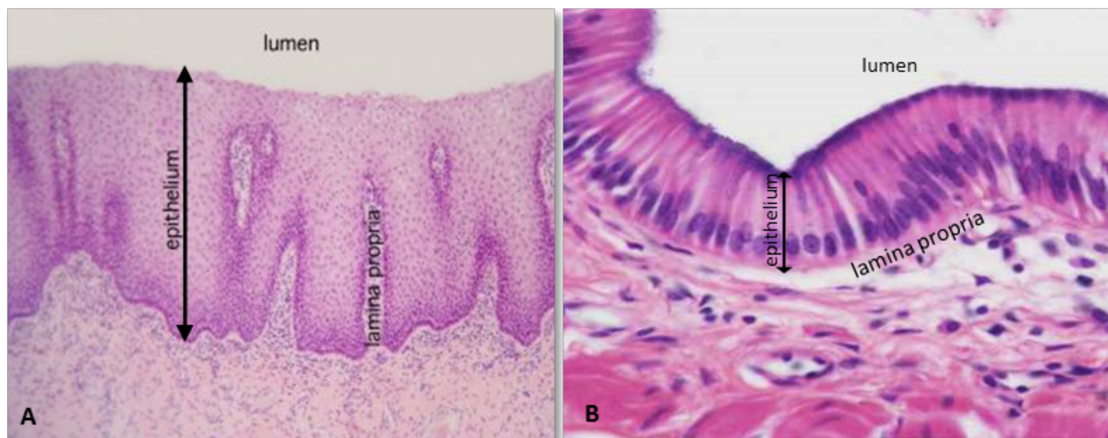

Figure 4 – *(A) Multilayer stratified squamous epithelium of the vagina. (B) Simple columnar epithelium of the rectum.*

Table 1 – Main differences between vaginal and rectum compartments and biological challenges for HIV infection. (Adapted from [87])

Vagina	Rectum
Stratified squamous epithelium that is constantly sloughing off the superficial layers (40-cell layer thick)	Simple columnar epithelium (one cell layer thick)
Acidic pH (4 – 4.5)	Alkaline pH (7 – 8)
Fewer CD4 cells than rectum	More inflammatory cells under surface (Macrophages and T lymphocytes with high expression of CCR5 co-receptors) High density of lymphoid nodules
Enclosed pouch	Open-ended tube

Throughout the menstrual cycle, the thickness of the vaginal epithelium varies. During the luteal phase, the thinning of the epithelium brings Langherans Cells (LCs) and CD4+ T lymphocytes closer to the mucosa surface, facilitating contact with HIV, thus increasing the risk of infection [88, 89]. During the follicular or ovulatory phase of the cycle, vaginal epithelium is thickest and the production of mucus increases, conferring a better barrier to transmission [90, 91].

Cell-free and cell-associated viruses are present in semen and deposited in the vagina during intercourse. As seen before, both forms can establish HIV infection (Figure 4) [92].

Physical abrasions on the vaginal and ectocervical epithelium may allow the penetration of cell-free virus or HIV-infected CD4+ T cells and dendritic cells [81]. Cell-free virus may also penetrate through thin gaps between epithelial cells in the intact squamous epithelium of the vagina and interact with two important cell types: intraepithelial LCs and CD4+ T lymphocytes of the lower genital tract (Figure 5) [79, 81].

LCs, a subtype of dendritic cells, are abundant in the cervix and vaginal epithelium of women forming a tight network in the mucosal squamous epithelium [93-95]. LCs express CD4, CCR5 and CXCR4 that function as highly efficient viral attachment factors for HIV [96, 97]. Moreover, LCs have dendritic processes that they extend through the epithelium to the lumen of the vagina, where they sample lumenal antigens [98, 99]. HIV present in the vaginal mucosa can bind to LCs and become internalized into cytoplasmic vesicles [100]. After HIV is captured, LCs migrate to the deeper submucosa and present the virus to CD4+ and CCR5+ T cells, macrophages, and other cells that became productively infected and begin viral amplification (Figure 5) [98, 101, 102]. This process can be performed in just 30 min, with maximal migration to the lymph nodes occurring after 24h [103, 104].

CD4+ T cells are abundant and largely spatially dispersed in the normal vaginal, ecto and endocervical mucosa, lying mostly beneath the epithelium, but also in the deeper submucosa. These cells, that outnumber macrophages and dendritic cells, are one of the early targets of infection at the portal of entry [105, 106]. CD4+ T cells can migrate to the superficial vaginal epithelium due to some factors such as breaks in the epithelial mucosa, inflammation caused by infections and also due to hormone-induced physiologic epithelial thinning that increases exposure of CD4+ T cells and LCs, naturally present in the vaginal mucosa, facilitating contact with luminal HIV and causing viral infection [105, 107].

Initial focally infected population consists mainly of resting CD4+ T cells. There is a rapid increase in HIV replication along with strong induction of cytokines and chemokines [108], with recruitment and activation of additional CD4+ target cells to the site [109, 110]. HIV expands locally, infected cells exit the mucosa and are then taken up by lymphatic or venous microvessels, that lead to dissemination to draining lymph nodes or into the blood circulation, reaching secondary lymphoid organs and finally creating a systemic infection [79, 110].

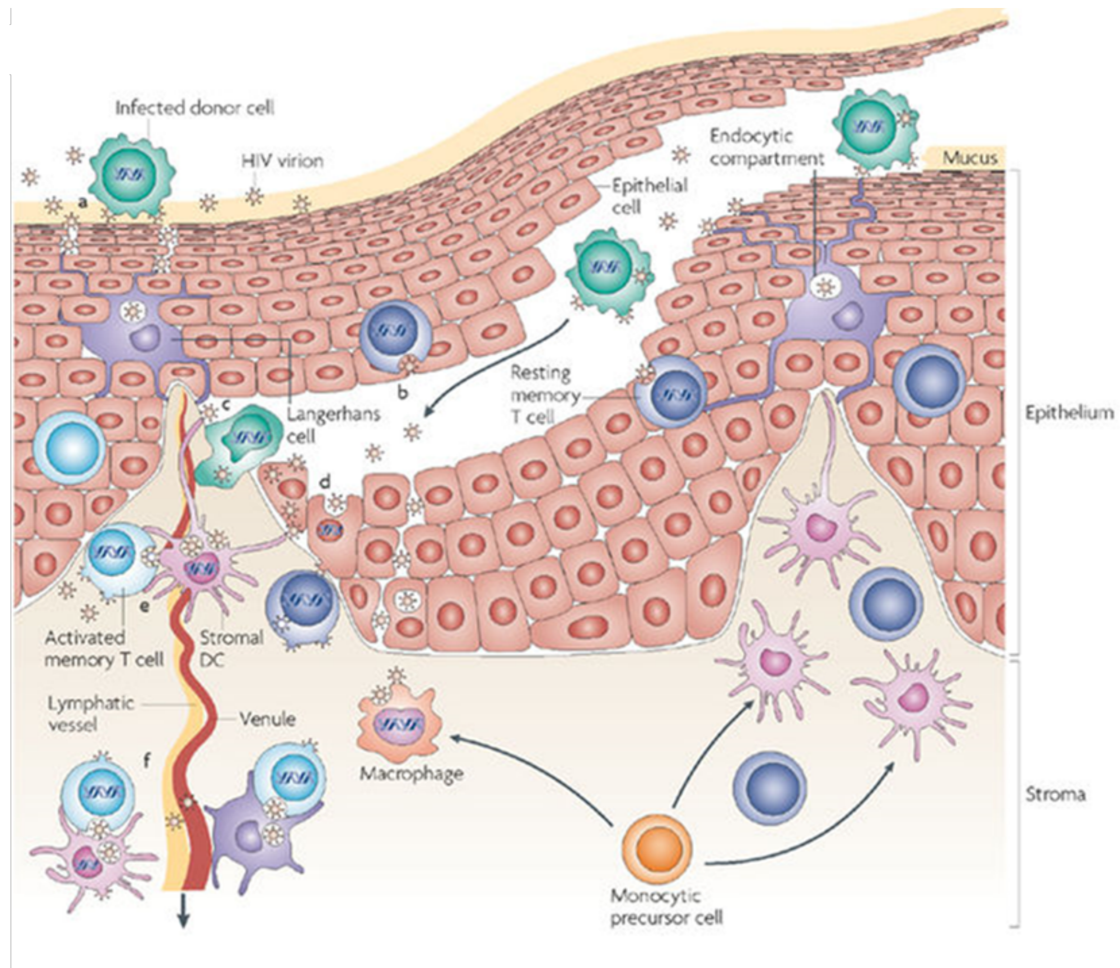

Figure 5 – *Sexual transmission of HIV-1. Microabrasions can be a port of entry for both cell-free and cell-infected viruses. Cell-free virus may also penetrate into thin gaps between squamous epithelial cells of the lower genital tract. Epithelial Langherhans cells sample and internalize virus, present them to CD4+ T cells, macrophages, and other cells that became productively infected, travel to lymph nodes and start a systemic infection (adapted from [111]).*

Other causes of lower genital tract inflammation, like bacterial vaginosis, syphilis, Herpes simplex type 2 (HSV-2) infection and trichomoniasis can promote HIV infection by thinning and disrupting tight junctions between epithelial cells of the multilayered squamous epithelium and recruiting HIV target cells to the site of initial viral proliferation [112-115], resulting in rapid viral dissemination to draining lymph nodes and the systemic circulation.

Prevention and treatment of HIV infection

Since the beginning of HIV epidemics, a large number of therapeutic options became available to treat HIV-infected patients. HIV-1 treatment regimens consist of combinations of multiple ARVs, following the recommendations of available guidelines: two nucleoside reverse transcriptase inhibitors (NRTIs) in combination with a third active ARV drug from one of three drug classes: an integrase strand transfer inhibitor (INSTI), a non-nucleoside reverse transcriptase inhibitor (NNRTI), or a protease inhibitor (PI) with a pharmacokinetic enhancer (booster) (cobicistat or ritonavir) [116]. Examples of therapeutic regimens active against HIV-1 are: dolute-

gravir/abacavir/lamivudine; dolutegravir plus tenofovir/emtricitabine; elvitegravir/cobicistat/ tenofovir/emtricitabine; and raltegravir plus tenofovir/emtricitabine [116].

However, available ARVs were specifically designed for HIV-1 and some do not work as well against HIV-2, which is intrinsically resistant to many antiretroviral drugs in clinical use namely NNRTIs, enfuvirtide (T-20) and some PIs [117]. There is an important lack of randomized clinical trials that could provide clinical orientations concerning the right time to start treatment and the choice of first and second line regimens for HIV-2 patients. In addition, HIV-2 has a lower genetic barrier to resistance to current drugs compared to HIV-1 and resistance pathways in HIV-2 may differ from HIV-1 [118].

In Section II, the therapeutic options available for HIV-2 treatment are reviewed, as well as promising drugs in development, drug resistance mutations and resistance pathways selected in HIV-2-infected patients.

Pre-Exposure Prophylaxis (PrEP) includes several approaches intended to prevent HIV transmission. The prophylactic use of oral ARVs (tenofovir/emtricitabine) by HIV-negative individuals with high risk behaviors is already recommended [119], and great efforts have been made to formulate topical microbicides able to prevent vaginal and rectal HIV infection [120, 121]. In Section III, oral and topical PrEP clinical trials are compared, and new approaches including vaginal rings, long-acting formulations and intermittent PrEP are analyzed.

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Section II
Antiretroviral treatment of HIV-2 infection

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Review article

(submitted)

Abstract

To date, no antiretroviral drugs have been specifically developed or licensed for the treatment of HIV-2 infection and as a result clinical decisions concerning the use of antiretroviral therapy in HIV-2 patients rely entirely on drugs that were optimized for the treatment of HIV-1 infection and on the results of small and limited *in vivo* and *in vitro* HIV-2 studies. Therefore, information about the clinical efficacy of current antiretroviral drugs in HIV-2 infected patients and development of resistance is still incomplete.

HIV-2 shows natural resistance to a number of antiretroviral drugs designed to suppress HIV-1 replication. HIV-2 is intrinsically resistant to NNRTIs, the fusion inhibitor enfuvirtide (T-20), and some PIs used for HIV-1 ART. However, HIV-2 is sensitive to NRTIs, INSTIs and maraviroc, a CCR5 antagonist.

Boosted PI-containing regimens have resulted in more favorable virologic and immunologic responses than two or three-NRTI-based regimens. Regimens that combine two NRTIs (typically AZT and 3TC) and one boosted PI (LPV/r) are also highly recommended. When resistance emerges, second line options are DRV/r-, SQV/r- or INSTIs-based regimens. MVC should also be considered for patients infected with HIV-2 strains with R5 tropism. However, cross-resistance dramatically reduces second-line treatment options.

The aims of this work are not only to review current knowledge on antirretroviral drugs presently available to treat HIV-2 infected patients but also to explore novel studies of promising drugs in development for HIV-2 infection, such as TAF, a prodrug of tenofovir, that has strong activity against HIV-2 *in vitro*; fusion inhibitor peptides like P3, that potently inhibits both HIV-1 and HIV-2 cell entry and replication and the short-peptide fusion inhibitor 2P23, with strong activity against HIV-1 and HIV-2 isolates, including T20-resistant HIV-1 mutants. In this work, we also highlight novel resistance pathways, for example new resistance mutations recently associated with resistance to the potent integrase inhibitor dolutegravir and also, concerning the use of maraviroc, we underline that higher concentrations of this drug may be necessary to fully suppress HIV-2 infection, especially late-stage infections.

Considering the several challenges in HIV-2 infection, it is a priority to develop novel drugs specific for HIV-2, as well as to conduct randomized controlled trials with available antiretrovirals among HIV-2 infected patients.

Keywords: HIV-2 infection; HIV-2 treatment; reverse transcriptase inhibitors; protease inhibitors; integrase inhibitors; CCR5 antagonists; resistance mutations; resistance pathways.

Introduction

Acquired immunodeficiency syndrome (AIDS) is caused by human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2). Whereas HIV-1 is disseminated worldwide, HIV-2 is largely confined to West Africa and countries with socioeconomic ties to the region, like Portugal and France [1]. Currently, it is estimated that approximately 1–2 million people worldwide are infected with HIV-2 accounting for 3-5% of the global burden of HIV [2, 3].

HIV-2 is composed of 9 groups termed A to I of which group A is by far the most disseminated (reviewed in [4]). HIV-1 and HIV-2 share the same routes of transmission, although HIV-2 shows remarkable differences in the course of the infection. It differs from HIV-1 by having lower perinatal and sexual transmission rates, lower viral loads and T CD4+ cells suppression and longer asymptomatic phase with slower progression to AIDS (Table 1) (reviewed in [5]). Although the course of HIV-2 infection is extended compared to that of HIV-1, without effective antiretroviral therapy a substantial proportion of infected individuals will progress to AIDS [6].

Table 1 – Major differences between HIV-1 and HIV-2 [7-13].

	HIV-1	HIV-2
Prevalence	36.7 million people	1-2 million people
Geographic Distribution	Worldwide	West Africa and countries with historical links to that region
Viral load	High	Low and often undetectable
Heterosexual transmission	High	~ 3.5 times lower than HIV-1
Perinatal transmission	15-45%	0-5%
Time to AIDS	7-10 years	10-20 years
Natural resistance to ARVs	-	NNRTIs, T-20, fAPV, TPV
Development of resistance to ARVs	Slow	Fast

However, available antiretroviral drugs (ARVs) were specifically designed for HIV-1 and some do not work as well against HIV-2 since these viruses differ in genetic content by almost 50% and there are important structural differences between the targets of these drugs in HIV-2 and HIV-

1 [14]. HIV-2 is intrinsically resistant to many antiretroviral drugs in clinical use namely non-nucleoside reverse transcriptase inhibitors, enfuvirtide and some protease inhibitors [11]. In addition, unlike for HIV-1, to date there are no randomized clinical trials to provide clinical orientations for the treatment of HIV-2 infection, particularly concerning the right time to start treatment and the choice of first and second line regimens for HIV-2 patients. All clinical decisions rely on results from small cohort studies and case series. HIV-1 and HIV-2 infections lead to similar clinical features and in many places rapid antibody tests used to differentiate the two viruses may not be available. This may lead to treatment initiation with ART regimens that are indicated for HIV-1 but are ineffective for HIV-2 (e.g. NNRTI-based regimens) [15, 16]. The management of ART in these patients relies in in-house viral load assays with varying clinical performances and the results cannot be directly compared. This problem may have been solved by the recent validation of a commercial viral load assay for HIV-2 [17]. Finally, HIV-2 seems to have a lower genetic barrier to resistance to current drugs compared to HIV-1 and resistance pathways in HIV-2 may differ from HIV-1 [12]. New antiretroviral drugs are needed for HIV-2 and a better use of the currently available ones is imperative if we want them to remain effective. Here we review the therapeutic armamentarium presently available to treat HIV-2 infected patients as well as promising drugs in development. Additionally, we identify drug resistance mutations and resistance pathways developed in HIV-2 infected patients under treatment.

Reverse Transcriptase Inhibitors

The reverse transcriptase (RT) enzyme is responsible for the synthesis of proviral DNA using viral RNA as template. RT has two distinct functions: DNA polymerase that mediates DNA polymerization from DNA and RNA templates and RNase H that RNA degradation [18]. Like HIV-1, HIV-2 RT is a heterodimer composed of two subunits, p68 and p54 [14]. The larger subunit p68 contains polymerase and RNase H active sites and is divided in four domains: palm, fingers, thumb, connection and RNase H domains. The smaller subunit, p54, is derived from proteolytic cleavage of p68 by RNase H and lacks the RNase H active site [19].

Although RT shows significant amino acid sequence homology in HIV-2 and HIV-1 the ATP binding pocket lacks physical depth and is less defined in HIV-2 relative to HIV-1 and this may influence the ability to bind ATP [14, 18, 20]. Furthermore, RT from HIV-2 seems to have reduced processivity, particularly in the presence of low dNTP concentrations [21-23]. In fact, Lenzi et al. have shown that RTs of several HIV-2 strains require higher dNTP concentrations for efficient DNA synthesis, and this can be relevant when considering viral replication kinetics in monocytes, macrophages, dendritic cells, and resting CD4 T cells that harbor very low dNTP concentrations [24]. Interestingly, the Vpx protein that only exists in HIV-2 and related SIV lineages, promotes a major increase of HIV-2 RT intermediates and viral cDNA in infected resting CD4 T cells and may therefore help to overcome the lower processivity of HIV-2 RT [25].

Since proviral DNA synthesis is essential in HIV replicative cycle, RT has been one of the main therapeutic targets for HIV treatment. Presently, there are two RT inhibitor classes: nucleoside/ nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs).

Activity of NNRTIs against HIV-2

All NNRTIs act by binding in a non-competitive way to HIV-1 RT. The binding occurs in an allosteric site approximately 10 Å from the polymerase active site in the palm domain of the enzyme p68 subunit. Their binding can induce conformational changes in RT or interfere with the mobility of the enzyme [26, 27].

NNRTIs comprise delavirdine (DLV), nevirapine (NVP) and efavirenz (EFV), called first-generation inhibitors and etravirine (ETV) and rilpivirine (RPV), the second-generation inhibitors. The first three compounds inhibit HIV-2ROD and HIV-2EHO at concentrations 50-fold higher than those required to inhibit HIV-1IIIB [11] and therefore its use is not recommended for HIV-2 treatment [28]. The natural resistance of HIV-2 RT to these inhibitors is due to sequence and structural differences within the NNRTIs pocket binding site at positions 101, 106, 138, 181, 188 and 190 that may lead to unfavorable contacts with NNRTIs or pocket binding site destabilization [26, 29].

One study showed that in a group of HIV-2-infected patients, 17% of patients were initially treated with regimens containing NNRTI, resulting in poor immunological response [16]. This was caused by the late confirmation of HIV-2 infection in these patients.

Activity of NRTIs against HIV-2

NRTIs, in the triphosphate form, compete with natural substrates (dTTP, dCTP, dATP or dGTP) of RT [30]. All NRTIs lack 3´OH group that is present in substrates, consequently these inhibitors bind to RT active site and act as obligatory chain terminators, blocking the DNA synthesis.

NRTIs approved for clinical use include lamivudine (3TC), abacavir (ABC), zidovudine (AZT), staduvine (d4T), emtricitabine (FTC), tenofovir (TDF) and didanosine (ddI). NRTIs represent the backbone of commonly prescribed regimens against HIV-2, which are mainly constituted by two or more NRTIs associated to one PI [31-34].

Azvudine (FNC) is a novel NRTI with potent activity against HIV-1 and HIV-2 *in vitro* and it shows synergism in combination with six approved anti-HIV drugs in two different cell lines [35]. FNC potently inhibits some NRTI-resistant strains (L74V and T69N) and M184I appears to be the key resistance mutation selected by this drug [35]. BMS-986001 (also known as festinavir, censavudinea and OBP-601) is a novel NRTI, structurally related to stavudine (d4T) [36]. BMS-986001 seems to exhibit more potent activity against HIV-2 than against HIV-1 in culture [37]. BMS-986001 retains full or partial activity against HIV-2 variants that have mutations K65R, Q151M, and M184V [37]. In a clinical trial in HIV-1 infected patients BMS-986001 had similar efficacy to that of TDF with a smaller decrease in bone mineral density; however, greater resistance and gains in both peripheral and central fat accumulation were related [38]. Clinical studies are needed to determine the utility of this drug for HIV-2 infection.

As mentioned before, HIV-1 and HIV-2 RTs are highly conserved at the active site, which partially justifies the similar virological and therapeutic response to this class of drugs. However, HIV-2 seems to have a lower genetic barrier to resistance to NRTIs in comparison with HIV-1 [39].

Tenofovir alafenamide (TAF) is a novel prodrug of tenofovir, that is more stable in blood and plasma [40], has more favorable renal and bone safety profiles [41, 42] and has higher anti-HIV-1 activity at ten times lower oral doses in humans [43] in comparison with TDF.

For HIV-1, one *in vitro* study showed that TAF has higher resistance barrier than TDF [44], while two other studies showed that the development of resistance was rare in TAF regimens and similar to TDF regimens, after 48 and 96 weeks of treatment [45, 46].

One recent *in vitro* study performed with a limited number of isolates (n=3) has shown that TAF is also active against HIV-2 with a mean EC50 of 1.83 nM (compares with 3.63 nM for HIV-1) [47]. Bártolo et al. determined the activity of TAF against 11 HIV-2 primary isolates and one adapted strain and showed that TAF has strong activity against HIV-2 (IC_{50} TAF= 0.272 nM) [in preparation].

HIV-2 Resistance to NRTIs

HIV-1 resistance to NRTIs may evolve by two distinct pathways: i) exclusion mechanism, in which the mutated RT (main mutations: K65R, K70E, L74V, Q151M and M184I/V) discriminates against the incorporation of the drugs (3TC, FTC and TDF) (Figure 1) or ii) excision ATP-dependent mechanism by removal of incorporated inhibitors (d4T and AZT) from DNA primers, allowing DNA elongation (timidine analog-associated mutations-TAMs: M41L, D67N, K70R, L210W, T215Y/F and K219Q/E) [18]. Unlike HIV-1, HIV-2 rarely develops resistance to NRTIs through the excision pathway, relying exclusively on nucleotide discrimination [23]. Smith et al., showed that HIV-1 and HIV-2 do not share the same resistance pathways to NRTIs *in vitro* [48]. Whereas in HIV-1 high level of resistance to AZT/3TC involves the combination of multiple TAMs, in HIV-2 three key resistance mutations (Q151M, K65R, M184V) cause high level resistance to these inhibitors [49-51].

Mutation Q151M is a multi-drug resistance mutation, which is responsible for different levels of resistance to all NRTIs, with the exception of TDF [50]. d4T and ddI select this mutation and are not recommended for HIV-2 treatment. Combination of mutation Q151M and V111I confers high-level resistance to all NRTIs [50]. Mutation V111I was found to increase the fitness of viruses with NRTI resistance mutations K65R and Q151M, in HIV-2-infected patients [52].

Mutation Q151M was present in 45% of HIV-2-infected patients that were on NRTI-regimens, from southeastern France [53], mutation M184V was present in all HIV-2 patients on ART (n=17) studied in Cape Verde by Pina-Araujo et al. [54], and mutation K65R was selected in 40% of HIV-2 patients that were on treatment regimens containing 3TC and TDF [52]. An observational study performed in Belgium and Luxembourg showed that mutations K65R, Q151M and M184V were the most frequently observed mutations selected under therapy [55], while one study showed that the combination of these three mutations is sufficient for classwide NRTI resistance [48].

Phenotypic studies using recombinant HIV-2 have shown that Q151M has a small impact on the viral susceptibility to 3TC and TDF, confers moderate- to low-level resistance to ABC, and FTC, but is associated with high-level of resistance to AZT [48, 51, 52]. Mutation K65R arises in HIV-2-infected patients exposed to TDF and other NRTIs [12, 56] while K65R can be selected *in vitro* after HIV-2 exposure to increasing doses of TDF [57] and produces a 2- to 7-fold decrease

in viral susceptibility to the drug [52, 57]. In HIV-1, M184V confers high level resistance to 3TC and FTC [12]. This mutation is also selected in HIV-2 clinical isolates and leads to a 60-fold increase in IC_{50} for 3TC, and 7-fold increase for ABC, compared with wild type HIV-2ROD [51]. Overall these studies show that HIV-2 and HIV-1 develop distinct resistance pathways to this class of drugs. While in HIV-1 the TAMs D67N, K70R, L201W, T215Y/F and K219Q/E are typically selected by NRTIs, in HIV-2 they are rarely selected and M184V, K65R and Q151M resistance pathways are preferred.

Activity of Protease Inhibitors against HIV-2

HIV protease (PR) is an important target in the treatment of HIV infection due to its role in the proteolytic processing of the precursor Gag and Gag-Pol polyproteins during virus maturation, leading to the formation of mature, infection-competent virions. PR has two identical monomers with 99 amino acids and is constituted by three main domains: active site (which includes the conserved motif Asp-Thr-Gly), dimerization domain and flap region [58]. In the active dimeric state, it forms a central cavity where the natural substrates, the precursor Gag and Gag-Pol polyproteins, bind [59]. Each monomer contributes with one Asp25 residue in the catalytic site of the enzyme.

The PR of HIV-1 and HIV-2 have only 39%–48% homology at the amino acid sequence level [60]. However, in structural terms they are very similar, especially in the region of Asp catalytic residues (positions 23-30) as well in a small region that supports catalytic amino acids in an appropriate conformation for catalysis (residues 86-88) [61]. The substrate binding pockets are formed by residues in positions 8, 23, 25, 27-30, 32, 47-50, 53, 76, 80-82 and 84. Of these, only residues at positions 32, 47, 76, 82 are different between both viruses [62, 63]. The majority of the differences between the PRs are at surface level, while the regions essential to the enzyme function are conserved; however, certain polymorphisms decrease binding affinity of PR for certain inhibitors, and may lead to resistance to PIs [64].

Nine PR inhibitors (PI) are currently approved for clinical use: first generation – saquinavir (SQV), indinavir (IDV), nelfinavir (NFV) and ritonavir (RTV) which is used only to boost all PIs except nelfinavir; second generation – lopinavir (LPV), atazanavir (ATZ), tipranavir (TPV), fosamprenavir (FPV) and darunavir (DRV) [64]. PIs were design to bind to PR active site with higher affinity than the natural substrates and at the same time filling more space inside the active site cavity. Resistance mutations in PR lead to an expansion of the active site cavity, thereby reducing PI affinity to that region [65-67]. Second generation PIs were designed with the purpose of not only surpassing HIV-1 resistance that rapidly emerged with first generation PIs, but also improve bioavailability, dosing frequency, and minimize side effects.

The information on the activity of PIs against HIV-2 is still scarce and sometimes contradictory. Despite the structural similarities, HIV-1 and HIV-2 PRs show major disparities in susceptibility to HIV-1 PIs. LPV, DRV, SQV are the most potent HIV-2 inhibitors while ATV, NFV and APV demonstrate lower potency against HIV-2 [68-70]. In one study, susceptibility of HIV-2ROD to ATV was 8-fold lower compared to HIV-1BRU and to NFV and IDV was 3-fold lower [71]. Regarding TPV, the information is contradictory since two *in vitro* studies report high level resistance of HIV-2 to this inhibitor [64, 71], while Brower et al. suggests full activity of TPV against HIV-2

[62]. However, the study performed by Brower et al. was conducted with purified PRs instead of complete viruses.

HIV-2 natural resistance to APV has been demonstrated *in vitro*. APV failed to potently inhibit HIV-2 PR, *in vitro* [64] and HIV-2 clinical isolates exhibited IC_{50} values that were 8.8 to 31 times higher than those obtained for HIV-1 clinical isolates [71, 72].

Studies of phenotypic susceptibility show that the activity of DRV against HIV-2 is comparable to HIV-1 [71]. However, one study reported that 40% of patients with PI resistance mutations appeared to be resistant to DRV, which can be problematic as DRV is recommended as second-line therapy for HIV-2 patients [73].

HIV-2 resistance to PIs

HIV-1 resistance to PIs occurs by the emergence of mutations in Gag-PR cleavage sites [74]. Amino acid substitutions in the binding pocket or in a nearby site can have as consequence the reduction of the binding affinity of the PIs, ultimately resulting in failure in PR blocking [65-67]. In contrast with HIV-1, little is known about the role of resistance mutations in HIV-2 PR and their effects on viral replication [72].

Raugi et al. reported that replacing only four active-site amino acid residues in HIV-2 PR with the corresponding amino acids from HIV-1 (I32V, V47I, M76L, and I82V) results in a replication-competent virus which exhibits a pattern of class-wide PI sensitivity comparable to that of HIV-1 [70]. The authors suggest that four residues in the PR binding pocket are the primary determinants of intrinsic PI resistance in HIV-2. However, Deforche et al., showed, by bayesian network analysis, that HIV-2 resistance pathways to PIs are different from those observed in HIV-1, being the 54L/M, 82F, 90M and 99F mutations the four main resistance pathways to HIV-2 resistance to PIs [75] (Figure 1).

D30N mutation is selected in HIV-1 under selective pressure with NFV [76]. However, in HIV-2 the resistance mechanism to NFV appears to involve the combination of L90M, I82F or 54M/99F mutations [77]. The mutations I82F, I54M and V71I were found in an HIV-2 patient who received treatment with IDV for 12 months [78]. The mutation I82F confers high level of resistance to LPV (36.3-fold increase) and NFV (34-fold increase) in HIV-2 clinical isolates [76]. Two studies found a significant association between therapy with IDV or RTV and the emergence of I82F mutation in HIV-2 patients treated with these inhibitors [77, 78]. The I54M is the mutation most frequently observed in HIV-2 infected patients treated with PIs in several studies [72, 75, 77-80]. In addition, I54M was selected *in vitro* by APV, NFV and IDV in HIV-2 clinical isolates [76]. In phenotypic assays, this mutation confers high level resistance to APV, NFV, IDV, LPV with more than 10-fold increase in IC_{50} and moderate level of resistance to TPV with a 7-fold increase, compared to wild type virus [76]. In HIV-1, this mutation only occurs under selective pressure with APV [81]. The presence of the L99F+V62A combination was selected in HIV-2 clinical isolates under NFV and IDV pressure and it confers moderate resistance to these inhibitors (15 and 9-fold, respectively) and high level resistance to LPV (124-fold) comparing with the wild type clinical isolates [76].

The acquisition of the 54M and 82F mutations may lead to cross-resistance to multiple PIs [72]. It was reported that both mutations together cause a reduction in susceptibility to APV, IDV, NFV, ATV and LPV (33 to 2000-fold increase, compared with the wild type HIV-2) [72].

In the reference HIV-2ROD isolate the L90M mutation, which is a major resistance mutation to SQV and LPV in HIV-1 [82], conferred resistance to SQV but did not alter the susceptibility to LPV [83]. The mutation L90M has been found in HIV-2 infected patients treated with SQV, IDV, RTV or NFV [77, 80, 84, 85], frequently in association with other relevant mutations such as I54L/M, V71L, I82F or I84V [77, 84].

The mutation V47A is frequently found in HIV-2 patients failing LPV based treatment [72, 75, 79, 86]. Also, the mutations K45R and I64V were identified in HIV-2 individuals failing treatment with this inhibitor [72]. *In vitro*, the resistance mutation V47A confers more than 10-fold resistance to LPV in comparison with wild type virus and promotes cross-resistance to IDV, NFV and AMP [87]. However, this mutation causes hypersusceptibility to SQV [72, 86]. For that reason, it was recommended the favored use of LPV in first-line therapy, and the switch to SQV if resistance to LPV occurs, because SQV will still be active in a second-line therapy [34]. This mutation causes no resistance to RTV, NFV, TPV and DRV [86, 87].

Other mutations, K7R, V62A/T and L99F are often present in HIV-2 infected patients treated with PIs, but are rarely selected in HIV-1 infection [77, 79]. K7R was observed in HIV-2 patients under treatment with RTV, LPV or SQV, and in the majority of the cases it was present in association with 46I, 71I, 62A/T and L99F substitutions. L99F mutation was found in HIV-2 individuals treated with NFV, LPV/r, SQV or IDV [84].

HIV-1 and HIV-2 show different pathways of PI-resistance, so different approaches must be applied when choosing the appropriate ARV regimen. These findings highlight the importance of determining the resistance mutations present in HIV-2 infected patients receiving PIs, in order to make the best therapy choices for each case.

Activity of the INSTIs against HIV-2

IN is derived from the Gag-Pol polyprotein precursor and catalyzes integration of the provirus into the host cellular DNA [88]. Each IN monomer consists of three different domains: N-terminal domain (NTD, residues 1–49), catalytic core domain (CCD, residues 50–212), and C-terminal domain (CTD, residues 213–288) [88]. The CCD domain contains a conserved motif, catalytic triad (64D, 116D and 152E), that is crucial for the catalytic activity of IN. IN, contrary to the RT and the PR, do not have human homologous, which makes it an interesting pharmacological target for the development of new ARV drugs with less side effects [89].

The IN proteins of HIV-1 and HIV-2 share the same structure with 65% identity at the amino acid level [90]. Several assays have shown that all approved inhibitors on the market are effective against HIV-2 [91, 92] due to the conservation of certain catalytic motifs in the same positions as described for HIV-1. Positions involved in the zinc binding domain, the CCD and the DNA binding domain, all crucial to enzyme function, are 100% conserved in HIV-2 [90, 92, 93]. IN residues in positions that are essential to DNA binding (Q148), integration and replication (Q62, H67, N120, N144, Q148 e N155) in HIV-1 are also conserved in HIV-2 [92].

Integrase Strand Transfer Inhibitors (INSTIs) bind to divalent metals such as Mg^{2+}, required for IN catalytic reactions, thus competing with residues situated in CCD [94]. This process affects catalytic activity of IN and inhibits the joining of viral and cellular DNA (strand transfer reaction) [89, 94, 95]. The first INSTI, raltegravir (RAL) was approved in 2007 for clinical use in HIV-1 infection [94]. Roquebert et al. evaluated the phenotypic susceptibility of HIV-1BRU, HIV-2ROD and HIV-2 clinical isolates to RAL [92]. It was demonstrated that RAL had similar activity in HIV-1BRU (IC_{50}= 3nM), HIV-2ROD (IC_{50}= 4nM) and HIV-2 clinical isolates (IC_{50}=1.3-5nM) [92]. Damond et al., quantified the virological and immunological response to an ARV regimen containing RAL in two patients infected with HIV-2 [96]. Both cases reported were related to highly experienced patients, with multiple drug resistances, high viral loads (> 6000 copies/ml) and very low T CD4+ counts (< 25 cells/ µl). In 2007, when these patients started the regimen with RAL, consistent and significant increases were registered in T CD4+ cell counts that were maintained for 6 months after therapy start and the viral load was undetectable from the second month after initiation of therapy. The regimen with 3TC, ABC/DRV/r and RAL was well tolerated in both patients [96].

However, two other clinical cases with highly experienced HIV-2 patients showed slightly different results [97, 98]. After the start of RAL-based regimen the viral load decreased and T CD4+ cell count rose. After 4 and 8 months, the viral load increased and T CD4 + cell count decreased. Resistance testing in both cases showed the selection of the N155H mutation in the IN gene that has been previously identified has a resistance mutation to the INSTIs in HIV-1 [97, 98]. These reports showed that RAL was effective in suppressing viral load in HIV-2 experienced patients. However, they also showed that RAL-resistance mutations emerged rapidly.

Elvitegravir (EVG) was the second INSTI approved for HIV-1 treatment in 2014, available in a fixed-dose combination (cobicistat-boosted EVG+FTC+TDF), given once daily [99]. Zheng et al. were the first to report the successful treatment response of a treatment-naive HIV-2-infected patient, using EVG [100]. Roquebert et al. evaluated the activity of EVG against 14 HIV-2 clinical isolates obtained from INSTI-naive patients and concluded that all isolates were susceptible to EVG (IC_{50}=0.3-0.9nM) [92].

RAL and EVG are highly potent in HIV-1 treatment, however due to their similar structures, genetic pathways associated with resistance are mostly the same, leading to significant cross-resistance, which prevents sequential therapy with these two drugs, for both HIV-1 and HIV-2 infected patients [57, 101-103]

Dolutegravir (DTG) is the more recently approved INSTI for treatment of HIV-1 in ARV-naive and experienced patients [91, 104, 105]. DTG has a slower dissociation rate from IN and can adjust its conformation in response to structural changes in the active site of IN [106]. Besides having potent antiviral activity, DTG has limited cross-resistance *in vitro* to most RAL-resistant HIV mutants [107-109].

Although DTG has been extensively evaluated for HIV-1 treatment, few studies have examined its potential use in HIV-2– infected individuals. Two independent *in vitro* studies showed that DTG is active against HIV-2 isolates obtained from INSTI-naïve patients with an IC_{50} range similar to that of HIV-1 [110, 111]. Descamps et al. studied 13 HIV-2–infected adults failing a RAL-based therapy that received DTG 50mg twice daily [112]. The patients had virus harboring INSTI resistance mutations (at codons 66, 92, 97, 138, 140, 143, 147, 148, or 155) [112]. The results indicate that patients with virus harboring the integrase resistance pattern Y143C/G/H/R

at baseline achieved undetectable plasma viral load, but patients with mutations in codons 148 or 155 did not, suggesting that these profiles impact DTG activity in HIV-2 [112]. Trevino et al., in a retrospective observational study of HIV-2 patients receiving DTG, discovered that the majority of patients failing a RAL-based therapy selected for IN resistance mutations N155H, Y143G and Q148R [113]. DTG was prescribed for two patients with N155H mutation and both experienced an initial decrease in plasma viral load and an increase in CD4+ T cells number. However, one of the patients experienced viral rebound after 6 months [113].

In a recent work, DTG potently inhibited HIV-2 isolates obtained from RAL-failing patients (median IC_{50}, 0.718 nM), and was also highly active against all but two isolates obtained from INSTI-naive patients. Interestingly, primary resistance to DTG was found in two isolates obtained from one DTG-naïve patient, although mutations associated with INSTI-resistance were absent. Secondary resistance to RAL occurred via two mutational pathways, one with the signature mutation Q148K and the other with the minor mutations E92Q and T97A [submitted, under revision].

HIV-2 Resistance to INSTIs

The limited available evidence indicates that mutations conferring resistance to RAL in HIV-2 are similar to those found in HIV-1 involving important positions in the CCD [79, 90, 97, 114, 115], at codons 143, 148 and 155 [112, 114] (Figure 1). IN mutations Y143C, Q148K/R and N155H [90] associated with other secondary mutations in the IN protein (i.e., E92Q, T97A, G140S) [98, 114, 116] have been associated with failure to RAL-based regimens. Smith et al. showed *in vitro* that secondary mutations in the IN protein are very important in the development of RAL and EVG resistance in mutated HIV-2ROD [117]. Using site directed mutagenesis they identified three main mutational pathways: major mutation Y143C that can lead to high-level resistance to RAL in the combination E92Q/Y143C and high-level resistance to EVG in the combination T97A/Y143C; mutations G140S/Q148R, which are responsible for high-level resistance to RAL and EVG; and the combination of mutations E92Q/N155H that also leads to high-level resistance to RAL and EVG [117].

Salgado et al., quantified the impact, *in vitro*, of the N155H in a HIV-2 infected patient showing a 37-fold increase in resistance to RAL in comparison to HIV-2ROD wild type [97] and Roquebert et al., showed that mutation Q148R confers 55-fold resistance to RAL and 99-fold to EVG from baseline in an HIV-2 clinical isolate [118]. RAL-resistant virus can be detected by direct sequencing until 11 months after RAL withdrawal, which may compromise the efficacy of new INSTIs [119]. There is strong evidence of the extensive cross-resistance between RAL and EVG in HIV-2 in culture [57, 117].

In HIV-1, *in vitro* and clinical data indicate that mutations at codons 143 or 155 do not confer significant resistance to DTG, whereas mutations at codon 148 in combination with other secondary mutations can decrease DTG efficacy [95]. Regarding HIV-2, using site-directed mutagenesis in the HIV-2ROD9 isolate, Smith et al. showed *in vitro* that IN mutations E92Q, Y143C, E92Q/Y143C, and Q148R were associated with low levels of resistance to DTG in HIV-2ROD9 (2- to 6-fold), but Q148K, E92Q/N155H, T97A/N155H and G140S/Q148R mutations conferred moderate resistance (10- to 46-fold), and the combination T97A/Y143C in HIV-2ROD9 corresponded to high-level resistance (>5000-fold) [120].

Charpentier et al. studied 11 clinical isolates: nine were obtained from INSTI-naive HIV-2 patients and two were obtained from RAL-experienced patients [91]. In clinical isolates from RAL-experienced patients, they discovered that mutations T97A/Y143C lead to seven-fold increase in EC_{50} values for DTG, mutations G140S/Q148R lead to a 13-fold increase and mutations G140T/Q148R/N155H lead to 18-fold increase [91].

Descamps et al. studied HIV-2–infected patients that were not responding to RAL and were receiving a DTG-based regimen [112]. They concluded that all patients with mutations in codons 148 or 155 showed detectable viral load, after six months. This indicates that these mutations can impact DTG activity in HIV-2. Based on this work, cross-resistance of first-generation INSTIs with DTG seems to be higher in HIV-2 than in HIV-1 [112].

A recent work described the resistance mutations responsible for resistance to RAL and DTG in primary isolates obtained from HIV-2-infected patients. For RAL, combined mutations E92Q and T97A and mutation Q148K reduce the susceptibility of HIV-2, however these mutations did not affect the activity of DTG. This work is the first to report primary resistance to DTG in HIV-2, without the presence of INSTI-associated resistance mutations. In fact, two primary isolates obtained from the same patient showed natural resistance to DTG, and mutations K221Q and D222K, not yet described, might play a role in this resistance [submitted, under revision].

Given the small number of alternative therapies for HIV-2 treatment, this new class of ARVs represents a good option for patients infected with HIV-2 or co-infected with HIV-1 and HIV-2. Although emergence of resistance mutations is a concern, INSTIs are drugs that can potently inhibit HIV-2 replication and should be saved for second line therapy [33].

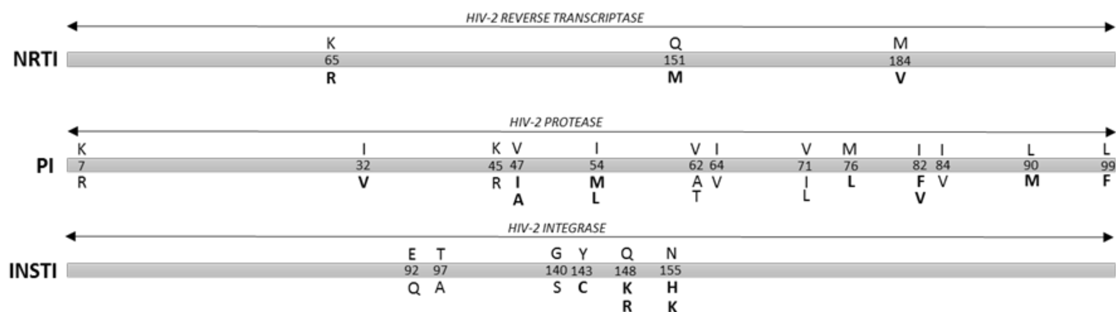

***Figure 1** – Main mutations associated with resistance to NRTIs, PIs and INSTIs. Bold residues represent high-level of resistance mutations.*

Entry inhibitors

Two types of entry inhibitors, fusion inhibitor (Enfuvirtide, T-20) and coreceptor binding inhibitor (maraviroc, MVC) were approved for HIV treatment [121, 122]. The fusion inhibitor T-20 is a peptide based on HIV-1 gp41 sequence (HR2 region) that blocks viral and membrane fusion by a mechanism of competitive binding to the HR1 region in gp41 (reviewed in [123, 124]). This inhibitor exhibits reduced efficacy against HIV-2, showing up to 100-fold increase in IC_{50} values *in vitro* comparing to HIV-1 [125]. Therefore, the use of T-20 in HIV-2 infection is not recommended [28].

Other peptides are being studied and some of them show promising results. Borrego et al. developed P3, an ancestral peptide derived from the HR2 domains of HIV-2 and SIV transmembrane glycoproteins [126]. P3 potently inhibits both HIV-1 and HIV-2 cell entry and replication (IC_{50} was 63.8nM for HIV-2 and 11nM for HIV-1, P<0.0001), and was also active against HIV-1 T-20 resistant viruses bearing the V38A and/or the N42D resistance mutations [126]. The authors also suggest that the genetic barrier for resistance to P3 might be significantly higher in HIV-2 than in HIV-1 [126]. 2P23 is also a promising short-peptide fusion inhibitor derived from HIV-1, HIV-2, and SIV [127]. This peptide was able to successfully inhibit HIV-1 isolates, T20- resistant HIV-1 mutants, and a panel of primary HIV-2 isolates, HIV-2 mutants, and SIV isolates [127]. Development of fusion inhibitor peptides allows the combination of different features in one small sized molecule, which is easier to formulate and has reduced production costs.

The coreceptor binding inhibitor MVC is an antagonist of co-receptor CCR5. This inhibitor binds to a CCR5 cavity within the 2, 3, 6 and 7 transmembranar helices (reviewed in [128]). MVC binds to CCR5 and induces conformational changes, particularly in the second extracellular loop of CCR5, preventing the interaction with the gp120 of the virus. Thus, MVC is only active against viruses with CCR5 tropism [129]. Consequently, it is mandatory a tropism test before starting therapy with this inhibitor. MVC has been successfully used in the treatment of HIV-1 infection [128, 130, 131]. However, the use of this drug in HIV-2 infection remains to be explored. There are also few data on the phenotypic susceptibility of HIV-2 to MVC *in vitro*.

In contrast to HIV-1, some HIV-2 isolates have the ability to use a wider range of coreceptors beyond CXCR4 and CCR5 including the CCR3, CCR1-4, GPR15 (BOB) and CXCR6 (BONZO) [5, 132-135]. However, most clinical isolates of HIV-2 exhibit CCR5 tropism and require the presence of this coreceptor in order to enter the host cell [125, 136]. Hence the effectiveness of MVC on the treatment of HIV-2 infected patients should be similar to that observed for HIV-1.

Three studies evaluated phenotypic susceptibility of HIV-2 clinical isolates to MVC [137-139]. It was shown that the potency of MVC is similar to HIV-1, with IC_{50} values between 0.175 to 2.1 nM [137-139]. However, Borrego et al. demonstrated that MVC inhibits R5 HIV-2 strains with higher IC_{90} values (IC_{90}= 42.7 nM) than those obtained for HIV-1 (IC_{90}= 9.7 nM) and also that inhibition of HIV-2 R5 viruses from AIDS patients required higher MVC concentrations than R5 viruses from early infections [137]. Consequently, the authors suggest that higher doses of this compound may be necessary to achieve full potency of MVC in HIV-2 treatment [137].

So far, only two clinical studies have reported the use of MVC in salvage therapy in HIV-2 patients with limited therapeutic options [140, 141]. It was demonstrated that both regimens, MVC and RAL or MVC and Foscarnet, increased T CD4+ cells count and decreased viral load to undetectable levels [140, 141]. However, these clinical cases do not provide valuable information about the relative efficacy of MVC as salvage therapy in HIV-2 infected patients since MVC was used in combination with other antiretroviral drugs and it was not performed a genotypic or phenotypic test before the treatment with this inhibitor. Recently, a MVC-containing regimen was prescribed to one R5 HIV-2 infected patient and resulted in undetectable viral load and an increase in the number of CD4+ T cells (140000 cells/µl), confirming the usefulness of MVC use in HIV-2 infection [submitted, under revision]. The recent development of a genotypic online tool to determine HIV-2 coreceptor usage can help clinicians decide if CCR5 antagonists such as MVC are a good treatment option [142]. However, the reduced clinical experience in the use of MVC in HIV-2, together with the fact that HIV-2 is able to use other cellular corecep-

tors for efficiently infect host cells, demands further research to better evaluate the potency of MVC *in vivo* and *in vitro* against HIV-2.

HIV-2 Resistance to CCR5 antagonists

Two main resistance pathways were proposed for CCR5 antagonists. The most common one is when R5-tropic viruses, that constituted the majority of viral species, are repressed and the small minority of X4-tropic viruses multiply, becoming the dominant viral species [143]. The selection of X4-tropic viruses can happen under pressure from drug treatment [125, 144]. The second mechanism through which resistance to CCR5 antagonists occurs involves the development of mutations in the *env* gene. Resistant viruses can increase affinity of gp120, allowing the connection gp120-CCR5 despite conformational changes from MVC binding [145, 146]. This can cause broad cross-resistance to CCR5 antagonists [146].

Another model for resistance to MVC is increasing the affinity for the binding site. HIV can develop mutations in the V3 loop that allow its binding to the extracellular loop (ECL) region domains in the presence of MVC [145]. However, mutations selected by MVC may not result in resistance for other CCR5 antagonists [147].

In HIV-1, amino acid changes in gp120, mainly in the V3 loop [148-150] but also in gp41 fusion peptide [151, 152] were identified and associated with *in vitro* resistance to CCR5 antagonists. Mutations I20F/Y21I conferred resistance to MVC [153] and substitutions A316T and I323V and a QAI deletion (positions 315-317 in V3 loop) *in vitro* are associated with resistance to MVC [148].

Mutations associated with HIV-2 resistance to entry inhibitors are not well documented, however, it has been suggested that higher concentrations of MVC may be necessary to inhibit HIV-2 infection in comparison with HIV-1 infection (IC_{90} 42.7 nM for HIV-2 and 9.7 nM for HIV-1) [137]. In addition, it was shown that MVC inhibits late-stage R5 HIV-2 infections with higher concentrations than early-stage R5 HIV-2 infection [137].

First and second line treatment of HIV-2 infection

The treatment regimens used for HIV-2 infection are usually very diverse especially in developed countries, reflecting the lack of guidelines to help clinicians select optimal treatment strategies in their practice [55, 154, 155]. Several small studies suggest poor responses in HIV-2 infected individuals treated with some ARV regimens including dual-NRTI regimens; regimens containing NNRTI plus 2NRTIs; and some unboosted PI-based regimens including NFV or IDV plus ZDV and 3TC; and ATV-based regimens [80, 85, 156-158].

U.S. guidelines of the Department of Health and Human Services (DHHS), as well as British HIV Association (BHIVA) guidelines recommend the use of two NRTIs and one boosted PI [28, 154] (Figure 2). BHIVA guidelines suggest as first line regimen TDF/ FTC/ LPV/r and as second line suggest TDF/FTC/SQV or DRV/RAL, especially if the mutation V47A arises [28].

For HIV-2, regimens containing boosted PIs have resulted in more favorable responses than two or three-NRTI-based regimens [55, 159, 160]. Two studies conducted in Europe compared the

therapeutic outcomes of HIV-2 patients on ART with three NRTIs with HIV-2 patients subject to treatment with boosted PIs and the results showed that regimens with boosted PIs are more effective than those of NRTIs [55, 161]. Recently, Balestre et al. also observed that boosted PI-regimens originated better immunologic responses than triple NRTI or unboosted PI-based regimens [162]. Peterson et al., also referred preference for regimens with two NRTIs (AZT/3TC) and one PI (LPV/r), despite the regimens with three NRTIs being more attractive in low income countries due to lower costs and the possibility of using PIs/r as second line regimens [56].

Low-income countries pose a critical challenge when deciding between the two first-line regimens. In countries with high tuberculosis prevalence, the use of a rifampin-based regimen requires double boosting of RTV in association with LPV or SQV, to treat patients infected with both tuberculosis and HIV. This strongly increases the risk of hepatotoxicity, which makes treatment with a combination of three NRTIs an interesting option, in these cases [163]. The risk of emergence of drug resistance has to be balanced against this fact as there are limited options for second-line regimens, in low-income countries.

For HIV-2, DRV, LPV and SQV are more active than other PIs [62, 71, 72], consequently one of these PIs, boosted with RTV, should be preferred when selecting a PI-based regimen. Regarding DRV, which is recommended as second-line therapy for HIV-2 patients, it is important to consider that one study reported that 40% of patients that developed resistance mutations to PIs appeared to be resistant to DRV [73].

Regarding HIV-2 therapeutic options, when resistance to NRTIs and PIs occurs, INSTIs and the CCR5 inhibitor MVC are the only remaining possibilities [162]. Recently, INSTIs (RAL, EVG and DTG) have been shown to exhibit potent efficacy against HIV-2 [91, 92, 112, 117, 120], and INSTI-based regimens have favorable treatment responses, being recommended as second line regimens for HIV-2 infection [100, 125, 164]. INSTIs are very effective in treatment naïve patients however, the use of INSTIs may be limited in patients previously treated with other ARV combinations and who harbor NRTI-resistant viruses due to the low genetic barrier to resistance of this drug class [162].

In case of therapeutic failure, the report of genotypic resistance must be taken into account for the choice of the next regimen. The CCR5 antagonist MVC is active *in vitro* against CCR5-tropic HIV-2 [138, 139], however, HIV-2 can use many other co-receptors besides CCR5 and CXCR4 [132]. The clinical efficacy of CCR5 antagonists in the treatment of HIV-2 infection is not completely understood, however, can be considered as part of a second or third line treatment for HIV-2 strains with R5 tropism [28, 125]. The potential use of INSTIs and MVC is also limited by the high costs of these medicines [162].

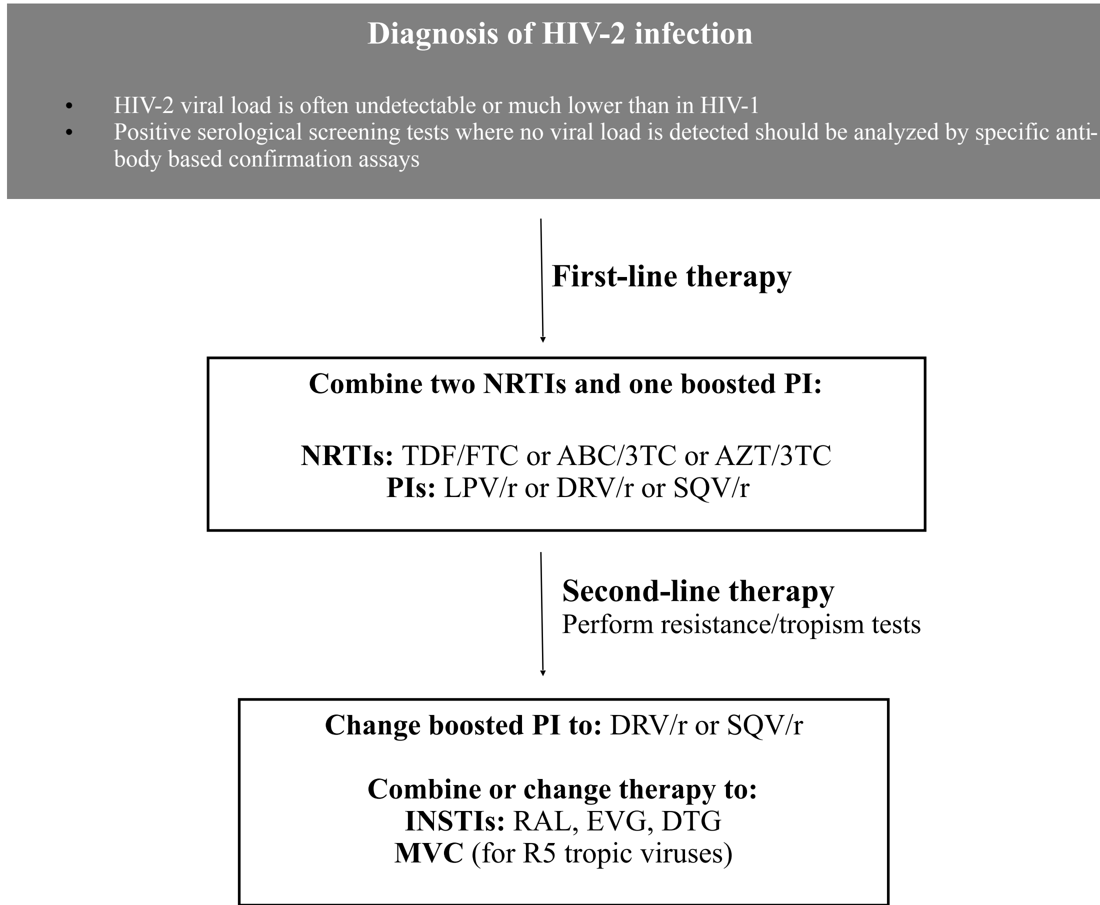

Figure 2 – *First and second-line therapy for HIV-2 infection [28, 154, 161, 162, 164].*

Conclusions

HIV-2 is naturally resistant to NNRTIs but is fully sensitive to NRTIs and INSTIs, with EC_{50}s comparable to those seen for HIV-1 [91, 92, 120, 165]. Due to the small number of HIV-2-treatment studies, deciding which therapeutic regimens to implement is a challenge. The first-line therapy most prescribed for HIV-2-infected patients are PI-based regimens (e.g. LPV/r), following the guidelines available [28, 56]. In lower-income countries three NRTI-based regimens are an alternative, particularly considering the prevalence of tuberculosis [166]. In West Africa, first-line HIV-2 treatments are chosen primarily based on cost and include regimens with combinations of two or three NRTIs with a PI/r usually LPV/r plus two NRTIs (e.g. AZT and 3TC) [37, 166].

Second-line therapy includes HIV-2 active PIs (DRV/r or SQV/r) or INSTIs-based regimens [166]. DTG is a good option for patients failing a RAL-based regimen, as DTG is active against most HIV-2 RAL-resistant mutants, while maintaining its potent antiviral activity [107-109]. CCR5 antagonist, MVC, appears to be an interesting option for HIV-2 treatment according to *in vitro* studies, however it is only active against HIV-2 strains with R5 tropism [138] and its use is also limited by the high cost. Further studies are needed to clarify the clinical efficacy of this compound.

When virological failure appears, there are limited drug options available for HIV-2 treatment and in some cases, cross-resistance strongly decreases therapeutic choices. In West Africa, the emergence of resistance to HIV-2 leaves patients with very few options for treatment. The majority of patients have no access to second-line therapy and have to remain on first-line regimens, being unable to successfully control the infection [37]. Considering the several challenges in HIV-2 infection, it is a priority to develop novel drugs specific for HIV-2, as well as to conduct randomized controlled trials among HIV-2 infected patients in order to define the best sequencing of first and second-line therapies for HIV-2, particularly in low-income countries.

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Section III
Pre-exposure prophylaxis for prevention of HIV infection

This section is an update of the following review article:

Diniz AR, Canhões R, Taveira T. (2015) Pre-exposure prophylaxis for prevention of HIV infection. Revista Portuguesa de Farmacoterapia, vol. 7, p. 15-33.

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Review article

Revista Portuguesa de Farmacoterapia (2015), vol. 7, p. 15-33

Abstract

Objectives: To review existing data on Pre-Exposure Prophylaxis (PrEP) for prevention of HIV infection, including the role of medical male circumcision, oral administration of antiretroviral drugs and topical microbicides. **Data Sources:** PubMed and http://www.clinicaltrials.gov. **Review methods:** Comprehensive review. **Results:** Medical male circumcision has been shown to prevent 48-60% of new HIV-1 infections. The efficacy rate of antiretroviral drugs given *per os* to prevent HIV infection varies in direct association with the adherence rate (62.2% in TDF2 study with 84% adherence; 44% in iPrEx study with <50% adherence; 48% in Bangkok study with 67% adherence; 67-75% in Partners PrEP study with 82% adherence; and 6% in FEM-PrEP study with 40% adherence). As for the use of topical microbicides, the CAPRISA 004 study showed 39% reduction in HIV infection using a 1% tenofovir gel. On the other hand, PRO2000 gel showed a modest reduction of 30% which was not statistically significant, and the VOICE study and FACTS 001 showed 14.5% and no reduction, respectively. DPV vaginal ring was associated with a 27% reduction of HIV infection in the ASPIRE study, but high levels of adherence showed 56% protection. The Ring study showed a protection of 31%. **Conclusions:** The studies suggest that medical male circumcision is highly cost-effective at preventing HIV infection but requires careful communication strategies to be successful. PrEP using antiretroviral drugs is also very effective but is highly dependent on the adherence rate. As for topical microbicides, 1% tenofovir gel is currently the only promising option, although further studies are recommended. Vaginal rings and long-acting formulations are very promising microbicides.

Keywords: PrEP; HIV infection; Male circumcision; Antiretrovirals; Microbicides; Vaginal rings.

Introduction

The HIV epidemic remains a significant global burden, especially in low and mid income countries [1]. While an HIV vaccine is still not available and treatment alone is not stopping HIV propagation, other preventive strategies are being investigated and implemented, in order to reduce morbidity and mortality.

Pre-exposure prophylaxis (PrEP) approaches may include topical or systemic interventions that are administered in a scheduled or exposure-driven manner to individuals at high risk of acquiring HIV infection. Target populations may include men who have sex with men (MSM), individuals with infected partners (serodiscordant couples), sex workers, intravenous drug users, or individuals who live in HIV endemic areas [2].

Several products, both topical and systemic, with and without active antiretrovirals, have been considered for use in PrEP. Oral formulations of tenofovir (TDF) and emtricitabine (FTC), once daily, are the only current PrEP agents approved and being prescribed for people who are at substantial risk of acquiring HIV [2].

Women are largely affected by the HIV epidemic and acquire the virus mostly by heterosexual exposure [3, 4]. Topical microbicides are formulated to be applied in the vagina or rectum and are being extensively studied for PrEP, due to their ability to protect women, acting on the site of exposure to HIV with reduced systemic side effects. Topical formulations being studied are TDF 1% vaginal gel [5-7] and the dapivirine (DPV) vaginal ring [8, 9]. However, results of clinical trials using topical microbicides often determine inconsistent rates of protection, probably due to the variable rate of adherence to treatment showed by the participants [5-7].

This paper intends to review current PrEP strategies, summarize important findings and critically analyze the main results.

Methods

Bibliographic research for this article was mainly performed at PubMed database website (http://www.ncbi.nlm.nih.gov/pubmed), using key words "PrEP", "Pre-Exposure Prophylaxis", "antiretrovirals", "microbicides" and "HIV infection". As complement, the website http://www.clinicaltrials.gov was used to analyze the results of the clinical trials for PrEP. Research articles and clinical trials reports were analyzed and the results compared, to obtain a critical analysis of the latest findings in HIV prevention of infection using PrEP.

Results

Male Circumcision

Worldwide, one third of men is circumcised for medical, religious, cultural or other reasons [10]. Male circumcision (MC) is associated with less frequency of genital ulcers that, together with microtears in the preputial mucosa, are an entrance way for HIV [11]. It has been estimated that 11% of the reduction in HIV acquisition in circumcised men is attributable to reductions in symptomatic genital ulcers and an additional 9% is mediated by reduced Herpes Simplex

Virus (HSV-2) incidence [12]. In HSV-2-infected men, there is an increased CD4+ T cell density in the foreskin [13], which explains why men infected with HSV-2 have superior risk of acquiring HIV.

HIV infection is higher among men with larger foreskin surface area [14]. Viruses and anaerobic bacteria may proliferate in the warm, moist, subpreputial cavity created by the male foreskin. In addition, Langerhans cells, CD4+ T cells, and CD8+ T cells, all target cells of HIV infection, are present in a large number in the foreskin mucosa [13, 15, 16], which HIV can penetrate [17]. Circumcision removes this rich vascular tissue containing a thin keratin layer, which is replaced with scar tissue [15].

Clinical trials have demonstrated that MC leads to a 48-60% risk reduction of HIV-1 infection in heterosexual men from sub-Saharian Africa [18-20]. In 2007, the World Health Organization (WHO), in conjunction with the Joint United Nations Program on HIV/AIDS (UNAIDS), formally declared the support of MC: "Male circumcision should now be recognized as an efficacious intervention for HIV prevention. Promoting male circumcision should be recognized as an additional, important strategy for the prevention of heterosexually acquired HIV infection in men" [21]. Five years later, in 2012, the American Academy of Pediatrics (AAP) took a similar step in support of the procedure among newborns, revising their policy statement to note that "preventive health benefits of elective circumcision of male newborns outweigh the risks of the procedure" [22].

Circumcision is an effective intervention that requires few resources and is one of the most cost-effective and affordable interventions that can be performed to prevent HIV acquisition [23]. However, this protective effect can be attenuated with the resume of sexual activity before total healing, which can even increase viral transmission risk due to the increased inflammation and bleeding [11]. In a stratified analysis, couples who recommenced sex before total healing of the circumcision wound had significantly increased risk of HIV transmission [24]. Sexual activity should only be restarted six weeks after surgery [25]. On the other hand, the protective effect conferred by MC can be compromised by increased risk behaviors, like reduction of condom use [26].

The role of MC in preventing HIV transmission to female partners is not clear. MC had no effect on male-to-female HIV transmission over two years of observation in a randomized controlled trial in Uganda [24]. This trial, however, was terminated prematurely due to futility. However, two observational studies found that MC decreased HIV transmission to female partners [27, 28]. Although the direct potential benefits of MC for male-to-female HIV transmission are not clear, modeling studies have suggested that by reducing HIV acquisition among male partners, MC will likely indirectly benefit females [29].

Although MC clearly reduces prevalence of sexually transmitted diseases (DSTs) in heterosexual men, its effect on homosexual men is not clear. Some observational studies indicate that MC in homosexual men is associated with a reduction of HIV infection [30, 31], but others did not find protection [32].

MC rate has been decreasing in recent years [11]. The WHO and UNAIDS, based on the promising results of clinical trials, recommend this practice in regions with low prevalence of MC, high prevalence of HIV and where the main way of viral transmission is heterosexual practice [11]. MC is an intervention that has shown clear preventive benefits in several studies, however to

better understand the advantages of this practice, more studies are needed to determine its efficacy in preventing HIV infection in male-female transmission and homosexual men. Besides, implementing MC requires a careful analysis of socio-economic conditions of the communities and the acceptance of this practice in men, especially in countries where MC is not common.

Oral antirretrovirals as PrEP

Oral antiretrovirals (ARVs) can be used to: (1) prevent HIV infection as antiretroviral treatment (ART) reducing the viral load of HIV in infected persons [33, 34]; (2) as post-exposure prophylaxis (PEP), where ARVs are used as chemoprophylaxis after a recognized high-risk exposure; (3) as pre-exposure prophylaxis (PrEP) where ARVs are used on a regular basis by individuals with repeated HIV exposures [35-37].

In PrEP, ARVs are administered to HIV-negative individuals, with ongoing and repeated exposure to HIV, as is the case of serodiscordant couples, to prevent HIV infection [38]. One of the advantages of PrEP over PEP is the fact that, at the time of risk exposure to HIV, ARV concentrations in plasma are already stable, and as a consequence the protection is much higher [39].

The drugs most intensively studied as PrEP are tenofovir (TDF), a nucleotide reverse transcriptase inhibitor and emtricitabine (FTC), a nucleoside reverse transcriptase inhibitor. TDF is a good PrEP candidate due to its potent antiretroviral activity against all HIV subtypes, it acts on the early steps of HIV infection, has few drug interactions and convenient dosing [39].

In the USA, the Center for Disease Control (CDC) and the Food and Drug Administration (FDA) recently approved the use of a combination of TDF/FTC as a strategy to reduce the risk of HIV-1 infection in seronegative individuals exposed to high-risk behaviors [40, 41].

It is essential that antiretroviral drugs used for PrEP reach vaginal and rectal tissues at the highest concentrations possible. TDF is available in concentrations 100 times higher in the rectal mucosa than in the vaginal or cervical mucosa [42]. FTC is 10 times more present in the cervical and vaginal mucosa than in the rectal mucosa, which justifies the combination of these two drugs [42-44]. TDF alone and in combination with FTC is widely used in antiretroviral therapy of HIV infection, and its safety profile is well documented [39].

Thigpen et al. (2012) performed a clinical trial named TDF2 in Francistown and Gaborone, Botswana with 1219 heterosexual participants from both genders, with ages between 18 and 39 years and sexually active, with the purpose of evaluating the safety and efficacy of TDF/FTC administration in HIV incidence in comparison with a placebo [37]. Half of the participants received prophylaxis with TDF/FTC [37]. According to what participants reported, the majority of the participants engaged in sexual relations with more than one partner in the previous month. From the individuals receiving antiretrovirals, 81,4% used condons in occasional sexual relations and 2,6% engaged in anal intercourse. A total of 36 participants suffered seroconversion, 10 in the FTC/TDF group and 26 in the placebo, showing a preventive efficacy of 61,7%. However, three participants were excluded (one in the TDF/FTC group and two in the placebo group), because it was proved that they suffered seroconversion at the start of the study, which means that the combined therapy had an efficacy of 62,2% in prevention of HIV infection [37]. The protective effect was superior when the analysis was restricted to the participants that declared to do a correct administration of the antiretroviral drugs in the previous 30 days,

which shows the influence of drug adherence for PrEP efficacy. The efficacy adjusted to adherence was 78% [37].

Clinical trial iPrEx, “Preexposure Prophylaxis Initiative”, was performed between July 2007 and December 2009 in several countries (Peru, Equator, Thailand, South Africa and the USA) and intended to evaluate the efficacy and safety of a daily dose of TDF/FCT for the prevention of HIV acquisition in men and transsexual women who have sex with men [36]. It was an important study because the risk of HIV infection through unprotected anal intercourse is 17-fold higher than vaginal intercourse [45], which can justify the increased prevalence of HIV infection in this group [36, 46]. Participants were 2499 man with ages between 18 and 67 years, and from those 29 were transsexual [36]. A total of 1251 participants received TDF/FTC once daily and 1248 participants received placebo [36]. There were diagnosed 110 cases of seroconversion, but in 10 cases a retrospective analysis revealed the presence of viral RNA in the initial visit (two cases in the TDF/FTC group and eight cases in the placebo group). From the remaining 100 seroconversions, 36 cases were from the TDF/FTC group and 64 from the placebo group, showing an additional protection of 44% in the incidence of HIV [36]. The efficacy was superior in individuals who performed receptive anal intercourse [36]. Cases with adherence ≥50% showed a reduction of 50% in the acquisition of HIV, reaching 70% in cases where adherence was ≥90%, during the previous 30 days [36].

In 2014, injectable drug users corresponded to 51% of HIV infections in eastern Europe and central Asia and 13% of HIV infections in Asia and the Pacific [1].

The Bangkok Tenofovir Study was a clinical trial performed between June 2005 and July 2010 that intended to evaluate the efficacy of TDF in the reduction of HIV transmission in injectable drug users. The study enrolled 2413 participants with an average of 31 years and 80% were male. The participants were followed during 4.6 years [47]. TDF was selected based on its potent action, long plasmatic half-life (12-18h), once daily administration, has few resistances and no significant interactions with methadone that was taken by 22 % of the participants [47, 48].

From 2413 participants, 1204 received TDF and 1209 received placebo. There were diagnosed 52 seroconversions: 17 individuals became HIV positives in the TDF group and 35 in the placebo group [47]. However, two participants were excluded from placebo group because they were already in the acute phase of the infection [47]. There was a decrease in the risk of infection of 48.9% with the daily administration of TDF. The efficacy was even higher in female participants (79%) and in participants over 40 years of age (89%) [47]. For the participants with high adherence to treatment and who showed detectable plasma levels of TDF, there was a reduction of 73.5% in the risk of HIV acquisition [47].

The clinical trial “Partners PrEP Study” was performed between July 2008 and November 2010 in nine places in Kenya e Uganda. It included serodiscordant couples who were in a stable relationship for at least three years and intended to evaluate the efficacy and safety of TDF and combination TDF/FTC on the reduction of HIV transmission [35]. The study enrolled 4747 participants. Adherence to treatment was very high: 98% by pill dispensing, 92% by pill count and 82% by plasmatic levels of the drugs [35]. During the study there were 82 seroconversions: 17 infections in the TDF group, 13 infections in the TDF/FTC group and 52 infections in the placebo group. They concluded that administration of TDF showed a reduction in 67% in the incidence of HIV-1 and the administration of TDF/FTC showed a reduction of 75% [35]. In a substudy that measured plasmatic levels of TDF in randomly selected participants in the TDF/FTC group, de-

tectable plasmatic levels were associated with a 90% reduction in the risk of acquiring HIV [35]. This study demonstrated that PrEP using TDF or TDF/FTC can be an effective preventive strategy in serodiscordant couples, reducing the incidence of HIV transmission for both genders [35].

Clinical trial FEM-PrEP was performed between June 2009 and April 2011, with women from Kenya, South Africa and Tanzania with ages between 18 and 35 years and intended to evaluate the efficacy and safety of a daily dose of TDF/FTC versus a placebo in the reduction of HIV infection risk [44]. This study was interrupted in 2011 when an interin analysis determined that it was very unlikely to prove a statistically significant difference between the two groups [44]. At the end of the study, 73 women showed seroconversion: 34 in the TDF/FTC group and 39 in the placebo group, without discrepant differences in T CD4+ cell count and viral RNA. They concluded that the efficacy of the antiretroviral prophylaxis was only 6%, thus it was not observed a significant reduction in the risk of acquiring HIV in comparison with placebo [44]. According to the authors, the low efficacy was attributed to poor adherence to treatment. In fact, although 95% of participants reported to always take the medication and the adherence rate by pill count was 88%, the analysis of TDF plasmatic levels revealed that only 20-37% of the participants had taken one dose of the drug in the previously 48h [44].

VOICE was a fase IIB clinical trial, performed between September 2009 and March 2013, that compared an oral dose of TDF or TDF/FTC with the topical vaginal application of TDF, versus oral and topic placebos. The study enrolled 5029 heterosexual women from South Africa, Uganda and Zimbabwe [49]. In 2011, after an interin analysis that determined futility, the groups that received oral TDF and topic TDF were closed. The group that received oral TDF/FTC continued the study until the end [49]. There were 52 new HIV infections in the oral TDF group, 61 in the TDF/FTC group and 60 in the oral placebo group. The efficacy was -49% for oral TDF, -4.4% for TDF/FTC and 14.5% for topic TDF gel [49]. This means that, like in FEM-PrEP study, no reduction in the risk of acquiring HIV was observed, in comparison with placebo. Reported adherence was 90% based on testimonies and monthly count of pills and unused gel devices [49]. However, analysis of blood samples detected TDF in only 30% of participants in oral TDF group, 29% in TDF/FTC group and 25% in TDF gel group [49]. These negative results were associated with very low adherence that occurred specially in young and single women [49], proving that it is necessary to improve adherence evaluation methods.

Maraviroc (MVC) is an entry inhibitor (CCR5-antagonist), concentrates in the rectum and genital tract and has recently been studied as a candidate for HIV-1 PrEP. Phase II NEXT-PrEP (HPTN 069) clinical trial enrolled 406 MSM in the USA and Puerto Rico. The participants were HIV-uninfected men and transgender women reporting condomless anal intercourse with ≥1 HIV-infected or unknown-serostatus man within 90 days and were randomly assigned to take either once-daily MVC alone, MVC plus TDF, MVC plus FTC, or TDF and FTC [50]. In a randomly selected subset, 77% demonstrated detectable drug concentrations at week 48 [50]. Five participants acquired HIV infection (4 in the MVC alone group, 1 in MVC/TDF group) [50]. From those five infections, two participants had undetectable drug concentrations at every visit, two had low concentrations at the seroconversion visit, and one had variable concentrations [50]. MVC-containing regimens were considered safe and well tolerated when compared with TDF/FTC-regimens. Participants who acquired HIV infection showed absent, low, or variable drug concentrations, likely indicating they were not consistently using PrEP [50]. These results indicate that MVC-containing regimens may warrant further study for PrEP, although it is important to con-

sider that MVC is only active against CCR5-tropic viruses [51], thus combinations of MVC with other ARVs should be preferred.

Overall, clinical trials using oral ARVs for the prevention of HIV transmission show that efficacy rates varied significantly with adherence (Table 1) and demonstrate that it is urgent to find preventive methods that provide higher adherence rates. Clinical trials TDF2 and Partners PrEP were the trials with best results and also higher adherence to treatment. Higher adherence in these two studies is probably related with the better perception of the risk of acquiring HIV in a stable relation with an infected partner. However, these results cannot be generalized to single women, women with multiple partners or women that although married do not know if the husband is infected with HIV.

On the other hand, FEM-PrEP and VOICE studies showed null or very low efficacy rates for women, due to poor adherence by the women involved in the studies. In clinical trial VOICE, young and single women were the group that showed lower rates of adherence and higher probability of becoming infected. Thus, it is urgent to find PrEP strategies with higher acceptance by women.

Understanding pharmacokinetics and pharmacodynamics of PrEP is extremely important to determine exactly when an individual is protected after starting therapy and also until when does PrEP confers protection after drug withdrawal. Seifert et al. showed in the clinical trial Cell-PrEP that optimal levels of PrEP are reached after 5-7 doses, meaning that PrEP should be started approximately one week before risk contact. The HIV Prevention Trials Network (HPTN) 066 study, also showed that optimal concentrations of TDF and FTC were achieved by 7 days [52].

Regarding when to safely stop PrEP after the last risk exposure, some considerations need to be made. Cell-PrEP study found that several days after ARVs were interrupted, high PrEP activity was still present. However, it is important to consider how long it takes for HIV to be completely cleared from the body, after the last exposure. Several factors may influence HIV clearance process: endocytosis by Langerhans cells, adherence to follicular dendritic cells and the occurrence of early cycles of replication [53, 54]. Thus it is recommended that PrEP is maintained until four weeks after the last risk exposure [55].

Table 1 – Reduction of HIV incidence of infection in PrEP clinical trials using oral ARVs.

Clinical trial (Country)	Population	ARV PrEP	HIV incidence reduction	Ref.
TDF2 (Botswana)	1219 heterosexual men and women	TDF/FTC and placebo	62.2% (adherence 84.1%)	Thigpen et al. (2012)
iPrEx (Brazil, Equator, Peru, South Africa, Thailand and USA)	2499 homosexual men and transgender women	TDF/FTC and placebo	44% (adherence <50%) 50% (adherence ≥50%) 70% (adherence ≥90%)	Grant RM et al. (2010)
Bangkok Tenofovir Study (Thailand)	2413 injectable drug users	TDF and placebo	48.9% (adherence 67%) 68% (adherence >90%)	Choopanya K et al. (2013)
Partners PrEP Study (Kenya and Uganda)	4747 heterosexual men and women (serodiscordant couples)	TDF, TDF/FTC and placebo	67% with TDF (adherence 82%) 75% with TDF/FTC (adherence 82%)	Baeten JM et al. (2012)
FEM-PrEP (Kenya, South Africa and, Tanzania)	2120 women	TDF/FTC and placebo	6% (adherence 40%)	Van Damme L et al. (2012)
VOICE (South Africa, Uganda and Zimbabwe)	5029 women	TDF, TDF/FTC e gel de TDF	-49% with TDF (adherence 30%) -4.4% with TDF/ FTC (adherence 29%) 14.5% with TDF gel (adherence 25%)	Marrazo et al. (2015)

Intermittent oral PrEP

Another PrEP strategy in study is intermittent PrEP. Intermittent PrEP includes any sporadic dosing schedule that differs from once daily dosing. The hypothesis for intermittent dosing is that comparable protection may be achieved with drug dosing at a time of high risk for HIV acquisition, while pill burden, side effects, and cost may be decreased by not using drug at times of low to no risk [6].

Three clinical trials using oral TDF/FTC intended to evaluate the potential of this strategy.

HPTN 067, also known as the ADAPT Study, compared the daily use and non-daily use of oral TDF/FTC to determine sex act coverage over 24 weeks. Self-reported adherence and plasma drug levels were evaluated [56]. The study enrolled more than 500 participants: women in Cape Town, South Africa, and MSM and transgender women (TGW) in Bangkok, Thailand, and Harlem, N.Y. [56]. The participants had a four-week period of once-a-week directly observed dosing, and after that were separated in three groups with different PrEP regimens for 24 weeks: a) daily, b) time-driven: twice weekly with a post-sex dose, or c) event-driven: before and after sex [56]. Highest levels of coverage and adherence were observed in Thai MSM in Bangkok, in the daily and time-driven dosing regimens, although the event-driven arm coverage was significantly lower (85%, 84% and 74% of all sex events were covered in the daily arm, time-driven arm and event-driven arm, respectively) [56]. In Harlem, N.Y., a cohort of young black MSM achieved 66% coverage of sex acts in the daily arm, 47% in the time-driven arm and 52% in the event-driven arm [56]. Daily arm group showed higher adherence than non-daily arms [56]. Of note is the fact that young, single, black women in South Africa had 75% coverage of sex acts in the daily arm, probably because they were aware that they were receiving the active product [56]. This study demonstrates that these women can take and adhere to a daily regimen of PrEP.

An open-label randomised trial named PROUD was directed at 13 sexual health clinics in England and included 544 HIV-negative homosexuals and other MSM and who had had anal intercourse without a condom in the previous 90 days [57]. Participants were separated in two groups: one group received daily TDF/FTC immediately and the other group received TDF/FTC after a deferral period of one year [57]. The study aimed to evaluate HIV infection incident during the deferral period, adherence, safety, and risk compensation [57]. The results showed that three HIV infections occurred in the immediate group and 20 in the deferred group, although 174 prescriptions of PEP in the deferred group, proving a relative reduction of 86% [57]. These results strongly support the introduction of PrEP to MSM at risk of HIV infection.

IPERGAY was a double-blind, randomized trial of PrEP, following 400 men MSM who had unprotected anal sex, for a median of 9.3 months [58]. The participants were divided in two groups: one group received a combination of TDF/FTC and the other received placebo, before and after sexual activity [58]. During follow-up, two infections occurred in the TDF/FTC group and 14 in the placebo group, corresponding to a relative reduction in the TDF/FTC group of 86% [58]. This study defended that administration of TDF/FTC before and after sexual risk exposure conferred protection against HIV-1 infection in MSM.

Topical microbicides as PrEP

Microbicides are compounds formulated as gels, films, capsules, tablets, foams, suppositories, creams or intra-vaginal rings (IVR), that when applied to the vagina or rectum might prevent the transmission of HIV, during sexual intercourse. Microbicides can be applied around the time of sexual exposure, used on a daily basis, or can be designed to release the drug over a prolonged period of time. The objective is to act on the earliest steps of the infection process, inhibiting or blocking viral entry at the vaginal or rectal mucosa.

Initial microbicide research was hampered by the incomplete understanding of sexual transmission of HIV. First developed microbicides, like surfactants and acidifying agents, were non-specific, acting either by disrupting viral and cellular membranes, or altering the vaginal environment in the genital tract to decrease viral transmission. Progress in understanding how HIV is transmitted and produces viable infection has allowed the design of specific compounds, that act on the early steps of HIV infection, leading to a more tailored strategy to microbicide development.

In the early 2000s, six topical surfactants, polyanions, and general antimicrobials (COL-1492, Cellulose sulfate, SAVVY, Carraguard, BufferGel, and PRO2000 Gel) were investigated for prevention of HIV. Although many showed promise in preclinical and Phase I studies, Phase II and III studies showed them to be either ineffective or even permissive for HIV infection [6].

Sub-Saharan Africa and Southeast Asia are the regions where the majority of microbicide studies are being performed [5]. Some studies defend that even a partially effective microbicide will be able to prevent millions of new HIV infections each year, globally [59], so an effective microbicide can have the power to alter the trajectory of the global HIV epidemic and will undoubtedly be used worldwide.

Topically delivered drugs that can be applied vaginally or rectally offer distinct advantages: higher local drug concentrations on tissues exposed to HIV, lower systemic drug levels and lower toxicity [60].

Microbicides should be able to act not only on the initial steps of HIV mucosal infection but should also have the ability to interrupt subsequent infection once HIV is intracellular. This way, an effective microbicide formulation will probably need the combination of several ARVs [61].

The ideal microbicide should be acceptable, affordable and effective. It should also be easy to use, not leak immediately after application and maintain high concentrations in the vagina for several hours to block HIV during and after sexual intercourse. Furthermore, the microbicide should have low toxicity, no effect on normal vaginal flora, and be compatible with condoms [62].

Several products are currently undergoing human testing. Different approaches to microbicide development are being explored (Table 2).

Gels

The HPTN 035 trial was a phase II/IIb, four-arm, multi-site, randomized controlled trial that intended to determine the safety and effectiveness of BufferGel and 0.5% PRO2000 Gel in preventing HIV male to female transmission [63]. The trial was conducted between 2005 and 2009 and enrolled sexually active HIV negative women, in Malawi, South Africa, Zimbabwe, Zambia

and USA [63]. BufferGel works by maintaining the normally acidic vaginal pH in the presence of ejaculate, preventing HIV acquisition and PRO2000 Gel prevents viral attachment and entry into susceptible cells [63].

Study participants were instructed to apply the study gel ≤1h before each sex act. Parameters analyzed during the study were: safety, sexual behavior, pregnancy, gel adherence, acceptability, and HIV serostatus [63]. Both products were safe. 0.5% PRO2000 Gel arm showed an incidence of HIV acquisition lower than both the Placebo Gel arm and the No Gel arm, while HIV incidence rates were similar in the BufferGel and both Placebo Gel and No Gel arms. The authors concluded that 0.5% PRO2000 Gel confers only 30% reduction in HIV acquisition in women, although the reduction observed was not statistically significant. The MDP 301 trial later confirmed that 0.5% PRO2000 has little or no protective effect [64]. BufferGel did not alter the risk of HIV infection [63].

TDF, a nucleotide reverse transcriptase inhibitor with potent activity against retroviruses, was the first ARV drug formulated as a microbicide to be tested. The CAPRISA 004 trial was the first study to demonstrated that an ARV formulated as a microbicide could prevent HIV infection in women [5]. It was a double-blind, randomized controlled trial that compared TDF gel (n=445) with placebo gel (n=444) in sexually active women from South Africa [5]. The study participants were instructed to apply no more than two gel doses in 24h; the first dose within 12h before sex and second dose as soon as possible but within 12h after sex. TDF gel was considered acceptable, safe and well tolerated [5]. Gel protection varied with adherence: high adherence to TDF gel (>80%) corresponded to 54% lower incidence of HIV; intermediate adherence (50-80%) and low adherence (<50%) corresponded to a reduction of 38% and 28% of HIV incidence, respectively. Overall, TDF gel reduced HIV infection by 39%, and by 54% in participants with high gel adherence [5].

In contrast to the CAPRISA 004 results, the VOICE study (MTN 003) stopped a daily 1% TDF gel arm early due to futility [6]. VOICE is a Phase II 5-arm study in 5000 women that intended to compare the efficacy of daily vaginal dosing of 1% TDF gel or a placebo gel, daily oral dosing of TDF 300mg, daily oral TDF 300mg combined with daily oral FTC 200mg, or an oral placebo tablet. The fact that this study was interrupted may be explained either by lack of adherence to the daily gel regimen, or by an unforeseen increase in genital tract mucosa permissiveness to HIV with frequent gel dosing [6].

FACTS 001 trial intended to study if a 1% TDF vaginal gel could prevent HIV and herpes (HSV) infections in women. The gel was used before and after sex. The results showed that the group of participants assigned to TDF gel did not have a reduction in HIV infections [65]. The majority of women in this trial showed low adherence to the gel, leading to disappointing results [65]. In 2059 participants, 61 HIV infections occurred in the TDF arm and 62 in the placebo group. In both groups, an average of four out of 100 women acquired HIV per year [65]. Based on returned applicators and self-reported number of sex acts, the women used the gel during an average of 50-60% of sex acts per month, with 13% of the participants using the gel during more than 80% of sex acts [65]. Detection of TDF in genital fluids was associated with a 52% reduction in HIV acquisition and women with no drug detected in genital samples were more likely to become infected [65]. Although women who used the gel consistently reached a protective effect, the overall adherence of the study population was too low to prove that TDF gel was effective [65].

Vaginal rings

Across trials of TDF-based prophylaxis, adherence is the most critical factor. Microbicides need consistent use in order to be effective and in the majority of trials, participants showed low adherence. The development of woman-initiated HIV prevention strategies that are safe, effective and of easy access in developing countries is a public health priority.

Vaginal rings are an attractive formulation to deliver ARVs for a long period of time, thus preventing heterosexual transmission of HIV-1, with the advantages of having better adherence and not requiring daily or pericoital use [66-70]. Vaginal rings are composed of hydrophobic polymers, like polyurethane, silicone elastomer or ethylene vinyl acetate [71, 72] and are divided in two categories: reservoir rings (contain the drug in an inner "core") and matrix rings (the drug is dispersed in a polymer matrix) [73]. The polymer must permit the permeation of the drug through the ring, to allow controlled drug release [73, 74].

Dapivirine (DPV), a non-nucleoside reverse transcriptase inhibitor (NNRTI), is being studied for vaginal ring microbicide formulation [8, 75, 76]. Several studies of DPV vaginal rings have been performed but to date, only two clinical trials assessed the safety and efficacy of a DPV vaginal ring against HIV infection: the ASPIRE study and the Ring study.

The ASPIRE study (MTN-020) was a phase III, randomized, double-blind, placebo-controlled trial that evaluated a DPV vaginal ring, changed once a month, performed between August 2012 and June 2015 and involved 2629 women from Malawi, South Africa, Uganda, and Zimbabwe [8]. This study intended to compare the efficacy and safety of the DPV vaginal ring with a placebo ring [8]. A total of 71 infections occurred in the DPV group and 97 in the placebo group, corresponding to a 27% reduction in the incidence of HIV-1 infection in the DPV group [8]. When two sites that had reduced rates of retention and adherence were excluded from the analysis, a reduction of 37% was observed [8]. Interestingly, women older than 21 years had higher rates of protection but that protection was not found in women between the ages of 18 and 21 years (-27% protection), probably due to the low levels of adherence of this age group [8]. Women older than 21 years showed high adherence and a protection of 56% [8]. This clinical trial showed that a DPV-vaginal ring can protect African women from HIV-1 infection, and greater protection is closely related to higher rates of adherence [8].

The Ring Study was a multicenter, randomized, double-blind, placebo-controlled, phase III trial involving 1959 women, who were enrolled at seven research centers in South Africa and Uganda [9]. The study intended to compare the safety and efficacy of a DPV-vaginal ring with a placebo ring, changed every 4 weeks, during 24 months [9]. In the DPV group, 77 women became infected with HIV-1 compared to 56 women in the placebo group, which means that DPV-vaginal ring produced a 31% reduction in the incidence of HIV-1 infection [9]. Contrary to the results of the ASPIRE trial, in the Ring Study the age effect was not significant, and no differences in protection rates were observed between age groups. Overall, DPV vaginal ring showed some level of protection against HIV-1 infection [9].

Formulation of vaginal rings containing multiple ARVs, with different mechanisms of action is an intelligent strategy, and some examples are vaginal rings releasing DPV and MVC [77], DPV and TDF [78] and TDF and acyclovir [79]. With these combinations, viral replication is inhibited at several stages of the viral cycle, increasing the effectiveness of the microbicide.

Other vaginal ring combinations being studied are the association of a microbicide with a contraceptive agent in order to prevent both HIV infection and unwanted pregnancies. These multipurpose prevention rings have been studied in the following combinations: DPV/Levonorgestrel, vicriviroc/progestin, TDF/Levonorgestrel [80-83]. Further studies are required to determine safety and efficacy of these combinations.

Films

Advances in vaginal drug pharmaceutics have generated new solid (e.g. quick dissolving films and tablets) and semi-solid dosage forms (e.g. suppositories) that can deliver active molecules with diverse physical properties, including hydrophobic or hydrophilic molecules and proteins that have proven challenging to deliver in aqueous-based formulations [84]. Most on demand microbicide products evaluated for effectiveness to date have been aqueous-based gels applied pericoitally. Quick dissolving films may be a suitable and inexpensive alternative vaginal dosage form for delivery of ARV microbicides.

Vaginal films have been studied for the prevention of HIV infection. In one study, TDF and efavirenz were incorporated in a vaginal film and its safety and ability to deliver the drugs were studied in mice [85]. The nanoparticle film administered vaginally once daily was considered safe to mice and effective in delivering the microbicides [85].

One study compared the use of vaginal gels with vaginal films containing DPV in women, for seven days [86]. Adverse events and plasma DPV concentrations were determined and biopsies from cervical and vaginal mucosa (extracted 2 hours after the last dose) were analyzed for drug concentration in the tissues and exposed to HIV *in* an *ex vivo* challenge assay [86]. All women had cervical and vaginal tissue concentrations of DPV higher than plasma concentrations [86]. Plasma DPV concentrations were similar in gel and film users, however, concentrations of DPV in cervical and vaginal tissues were three to five times superior in gel users compared with film users [86]. Nevertheless, in the *ex vivo* challenge assay, both film and gel released sufficient concentrations of DPV to inhibit HIV [86]. Concerning user's opinion, the film was considered more comfortable with less leakage, but more difficult to insert than the gel [86].

As is the case of vaginal rings, films may deliver microbicides efficiently to target cells and can also circumvent common user acceptability issues often associated with vaginal gels such as messiness or leakage [86, 87], which suggests that film formulations for microbicides merit further investigation.

Table 2 – Reduction of HIV incidence of infection in PrEP clinical trials using topical microbicides.

Clinical trial (Country)	Population	PrEP Microbicide	HIV incidence reduction	Ref.
HPTN035 **(South Africa, Zimbabwe, Zambia, USA and Malawi Republic)**	3050 sexually active women	0.5% PRO2000 Gel BufferGel Placebo gel No gel	30% (statistically not significant)	Abdool Karim et al. (2011)
CAPRISA 004 **(South Africa)**	889 sexually active women	TDF 1% gel and placebo	28% (adherence <50%) 39% (adherence 50-80%) 54% (adherence >80%)	Abdool Karim et al. (2010)
VOICE **(South Africa, Uganda e Zimbabwe)**	5029 women	TDF 1% gel and placebo	14.5% (adherence 25%) Stopped early due to futility	Marrazo et al. (2015)
FACTS 001 **(South Africa)**	2059 women	TDF 1% gel and placebo	No reduction	CROI (2015)
ASPIRE **(Malawi, South Africa, Uganda, and Zimbabwe)**	2629 women	Dapivirine vaginal ring and placebo ring	27%	Baeten et al. (2016)
The Ring Study **(South Africa and Uganda)**	1959 women	Dapivirine vaginal ring and placebo ring	31%	Nel et al. (2016)

Long acting injectable compounds as PrEP

A number of new drugs are being formulated in novel ways for PrEP. Two long acting (LA) injectable compounds are currently being developed for monthly to quarterly use as intramuscular injections for the prevention of HIV infection: rilpivirine (TMC278) [88] and cabotegravir (GSK1265744) [89]. LA ARVs may reduce the component of poor adherence linked to daily dosing. Preliminar studies performed with rilpivirine [90, 91] and cabotegravir [92-94], showed good results.

Preclinical studies have demonstrated the potential role of LA cabotegravir in the prevention of HIV transmission and advancement of this compound into clinical trials. Two phase II studies, ECLAIR and HPTN 077, have been designed to evaluate the safety, tolerability and acceptability of LA cabotegravir. The ECLAIR study took place in ten USA sites [95], whereas HPTN 077 in Brazil, sub-Saharan Africa, and the USA [96]. Both studies were double blind, similarly designed placebo controlled. HPTN 077 is ongoing. The ECLAIR study had a lead-in phase of 30 mg daily

oral cabotegravir for four weeks followed by a one-week washout phase and intramuscular administration of 800 mg of LA cabotegravir every 12 weeks for three doses [95]. The ECLAIR results demonstrated that both oral and LA cabotegravir were well tolerated, permitting continued development of cabotegravir for PrEP [95]. Participant satisfaction with intramuscular injections of cabotegravir was high, including a preference for injections compared with oral cabotegravir once-daily tablets [95].

Regarding the use of LA rilpivirine for HIV prevention, this compound is now being evaluated in HPTN 076, a phase IIa trial on safety and acceptability enrolling 132 HIV-uninfected low-risk women in the USA and sub-Saharan Africa in a 2:1 rilpivirine: placebo randomization [97]. The study consisted of a four-week oral lead-in followed by six injections of 1200 mg each, every eight weeks, followed by a 32-week observational period during drug washout [97]. LA rilpivirine was considered safe and acceptable [97].

Although LA agents eliminate the need for daily or pericoital pill-taking, adherence to injections is still required. In the clinical trials, injections were administered in a clinic-based setting, as a directly observed therapy strategy, but if a LA agent becomes approved for PrEP, issues regarding administration will require strict behavioral consideration. The injectable contraception literature suggests a high rate of nonadherence after initial injectable hormonal contraception use [98] and for this reason, even a LA injectable PrEP formulation would not be expected to solve adherence challenges for all patients.

Conclusions

HIV remains a significant global burden, while treatment alone may not be sufficient to stop the epidemics. Effective prevention strategies must be implemented to reduce morbidity and mortality. Although interventions such as counseling, condoms, and circumcision, allowed modest reductions in HIV spread, PrEP approaches are also very important. Clinical trials analyzed in this work strongly indicate that PrEP may have potential efficacy in reducing HIV-1 incidence in at risk groups – women, MSM, drug users and serodiscordant couples.

TDF and FTC were chosen initially to be studied for oral PrEP due to their efficacy in animal models [99-102], safety, and favorable pharmacokinetic profiles (long half-lives of active metabolites) [103, 104]. However, randomized clinical trials have resulted in mixed results with variable efficacy.

Clinical trials TDF2 [37] and Partners PrEP [35] were the trials with best rates of protection (62,2% and 67%, respectively) and higher adherence to treatment. However, FEM-PrEP [44] and VOICE [49] studies showed very low or null efficacy rates for women (6% and -49%, respectively), due to poor adherence.

One important factor implicated in adherence is the perceived risk of HIV acquisition in study participants. In FEM-PrEP, low levels of adherence may be explained by the fact that most women considered that they had low to no risk of acquiring HIV [44]. In contrast, the couples that were part of the Partners PrEP study considered that they had high risk of HIV infection because their stable partner was HIV infected [35]. Overall, efficacy rates were clearly related to adherence to treatment, which demonstrates that it is urgent to find preventive methods that provide higher adherence rates. One great concern is the fact that in this clinical trials,

PrEP was implemented in controlled environments with strategies to improve drug adherence and reduce risk behaviors; and even in this conditions, adherence was still very low, suggesting that PrEP implementation in the communities may be very challenging.

Topical microbicides (gels, films and rings) can provide discreet use and control of HIV prevention by women, and also provide high concentrations of ARVs in target tissues of HIV infection.

CAPRISA 004 was the first successful PrEP trial using an antiretroviral to protect against HIV infection. The 1% TDF topical gel used in this study lead to a reduction of 39% in the risk of HIV infection and a reduction of 54% in women who were >80% adherent [5]. However, the VOICE study stopped a daily 1% TDF gel arm early due to futility [49] and adherence in FACTS 001 was too low to prove TDF gel effectiveness [65]. The results of TDF gel trials are not consistent, suggesting that multiple factors may influence adherence to treatment and consequently the efficacy of TDF vaginal gel.

So far, only two clinical trials studied a DPV vaginal ring for the prevention of HIV infection. The ASPIRE study determine a 27% reduction in HIV incidence in the DPV group, but found lack of protection in the group of women between 18 and 21 years old [8]. Higher adherence to treatment was a key factor in increasing protection against HIV infection as women who were older than 21 years of age had a rate of adherence superior to 70% and the efficacy of HIV-1 protection was 56%. Physiologic differences in the genital tract, lower adherence and more frequent vaginal or anal sex are some of the reasons pointed out to justify low protection in women younger than 21 years [9]. In the Ring study, DPV vaginal ring conferred 31% of protection but there was no distinction between age groups [9]. Vaginal rings hold the promise of increasing adherence, by releasing the drug over prolonged periods of time, without requiring daily dosing. Sustained release of multiple ARVs in one vaginal ring may better prevent HIV infection and also reduce the emergence of resistant viruses. Moreover, the combination of multiple ARVs with different mechanisms of action may also protect against HIV-2, because some ARVs do not work against HIV-2, and a combination of multiple ARV in one vaginal ring could overcome this fact.

The future of PrEP will probably include long acting formulations like injectable compounds rilpivirine and cabotegravir that eliminate the need for daily or pericoital pill-taking, thus improving adherence. PrEP has the potential to be a valuable strategy to prevent the spread of HIV. Due to physiologic and behavioral differences between at-risk populations, optimal PrEP interventions will likely need to be tailored to the population and/or individual. The implementation of long-acting formulations or targeted, exposure-driven dosing strategies may improve overall adherence to PrEP.

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AIMS AND WORK PLAN

Aims and work plan

Worldwide, 1–2 million people are infected with HIV-2, mainly in West Africa but also in countries who have socio-economic ties with this region like Portugal, France, Brazil and India. HIV-2 is less pathogenic than HIV-1 doubling the average time from asymptomatic infection to AIDS from 10 to 20 years or more. However, if untreated, the disease evolves to AIDS and death in 20–25% of HIV-2 infected patients. A serious problem with treatment of HIV-2 infection is the fact that ART for HIV-2 relies entirely on compounds that were optimized for the treatment of HIV-1, thus several antiretroviral drugs used to fight HIV-1 are not effective against HIV-2, and in addiction, there is limited knowledge and experience in the treatment of HIV-2 infection.

INSTIs are a class of antiretroviral drugs active against HIV-1 and HIV-2. There are three INSTIs currently available: raltegravir (RAL), elvitegravir (EVG) and dolutegravir (DTG). DTG, the most recently approved INSTI, is active against HIV-2 clinical isolates in vitro with an IC50 range similar to that of HIV-1 and is used on ARV regimens for patients experiencing virological failure to RAL. However, there is a lack of studies characterizing the activity of DTG against HIV-2 clinical isolates obtained from patients failing RAL-based regimens.

In Chapter 2 we characterized the activity of DTG on a panel of 16 primary isolates of HIV-2 obtained from INSTI-naïve and RAL-failing patients from Portugal. We also characterized polymorphisms and mutations associated with resistance to INSTIs in these patients.

First we obtained blood samples from 14 HIV-2 infected Portuguese patients. Two patients were RAL-experienced, four patients were untreated and the others were on ART without INSTIs. Virus isolation and culture was performed, and we obtained 16 HIV-2 primary isolates. Next, we performed a single-round viral infectivity assay with TZM-bl reporter cells (CD4+, CCR5+ and CXCR4+) in the presence of several fold dilutions of DTG and RAL. 50% (IC50) and 90% (IC90) inhibitory concentrations and maximum percentage of inhibition (MPI) were determined for each drug. The nucleotide sequence of the integrase gene of each HIV-2 primary isolate was screened for resistance mutations and all sequences were compared to the sequence of HIV-2 ROD to identify the presence of relevant polymorphisms. The level of resistance for each polymorphism was determined based on GRADE Algorithm, drug susceptibility assays (fold-change) and what is described on the literature.

In contrast to the first two decades of the HIV pandemic, today girls and women make up more than half of people living with HIV, particularly in western and central Africa, where the virus is acquired largely by heterosexual exposure. Globally, young women are twice as likely to acquire HIV as their male counterparts and HIV is the leading cause of death among women aged 30–49. The HIV epidemic imposes a particular burden on women and girls. In addition to their greater physiological susceptibility to HIV acquisition, the pervasive social, legal and economic disadvantages faced by women reduce their ability to protect themselves from HIV infection.

Topical PrEP using microbicides is a strategy for preventing the transmission of HIV through sexual intercourse. One priority in this field is to find new microbicide candidates that interfere with the life cycle of HIV-1 and HIV-2 but are not based on antiretroviral drugs in current use, to prevent the selection and dissemination of resistant HIV strains.

Dendrimers are nanoscale well-defined hyper-branched polymers containing functionalized groups at their periphery that provide a strategy for the development of potent viral entry inhibitors, used as topical microbicides to prevent HIV acquisition. Polyanionic carbosilane dendrimers G2-S16, G2-NS16, and G3-Sh16 have shown great potential against HIV-1 infection in vitro and in vivo in previous studies.

In Chapter 3, we evaluated the activity of these dendrimers against R5- and X4-HIV-2 isolates, by performing a single-round viral infectivity assay with TZM-bl cells. The luciferase activity was measured and from the dose-response curve, the 50% cytotoxic concentration (CC50) and the half-maximal inhibitory concentration (IC50) of dendrimers were determined for each strain. We also studied the cytotoxicity, anti-sperm and antimicrobial activity of the dendrimers. Moreover, we researched the synergistic activity by triple combinations of these dendrimers with tenofovir and RAL against HIV-2 infection. In order to discover more about the mode of the antiviral action of these dendrimers, we used several in vitro experiments including: attachment and internalization of HIV-2 in PBMCs, HIV-2 inactivation, and cell-to-cell fusion inhibition. Cell-to-cell fusion inhibition was tested using a recombinant vaccinia virus (rVV) expressing HIV-2 ISY env gene. Hela-CD4- cells were transfected with pcDNA3.1+/Tat101-flag plasmid and infected with the recombinant vaccinia virus. After 3h, Hela-CD4- (effector cells) were collected and co-cultured with TZM-bl (indicator cells) at 1:1 cell density ratio in the absence or presence of increasing concentrations of dendrimers or controls. The percentage of membrane cell fusion was measured by luciferase activity and the level of syncytium formation was determined by direct microscopic observation. Finally, we tested if the vaginal application of 3% G2-S16 HEC gel formulation caused vaginal irritation or lesions after histological analysis in female BALB/c mice.

We have recently described a new fusion inhibitor peptide named P3 that potently inhibits both HIV-1 and HIV-2 replication. We consider that P3 is an attractive microbicide candidate for HIV prevention.

In Chapter 3, we evaluated the stability and antiviral function of P3 under different conditions of pH, temperature and oxidative stress, as well as its antiviral activity in a gel of hydroxyethyl cellulose (HEC), to be used as a vaginal microbicide. We also evaluated the safety profile of P3 in Balb/C mice. The long-term stability of P3 was evaluated in the presence of seminal plasma (SP) and vaginal fluid simulant (VFS). The antiviral activity of P3 in the presence of SP and/or VFS was determined with a single-round viral infectivity assay using TZM-bl reporter cells. Cells were infected with an HIV-1 isolate and antiviral activity was measured. Effect of temperature, pH and oxidation in the stability and antiviral activity of P3 were determined. P3 was formulated in 1.5% HEC-gel with 20% X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). The antiviral activity of this gel (designated P3/X-Gal HEC-gel) was evaluated using a single-round infectivity assay in TZM-bl reporter cells and HIV infection was quantified using the β-galactosidase reporter gene assay. Safety of P3 in the vagina was evaluated in BALB/c mice. A formulation of P3 diluted in PBS was applied intravaginally daily for 7 days in female BALB/c mice. Genital tract tissues were extracted and analyzed for signs of irritation, histological lesions and existence of injury in vaginal epithelium, inflammatory infiltrate, vascular congestion and/or edema in the submucosa.

CHAPTER 2
DIFFERENCES IN SUSCEPTIBILITY PATTERNS OF ISOLATES FROM HIV-2 INFECTED PATIENTS TO RALTEGRAVIR AND DOLUTEGRAVIR

Differences in susceptibility patterns of isolates from HIV-2 infected patients to raltegravir and dolutegravir

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Research article

Manuscript submitted to Scientific Reports, Novembre 2017

Abstract

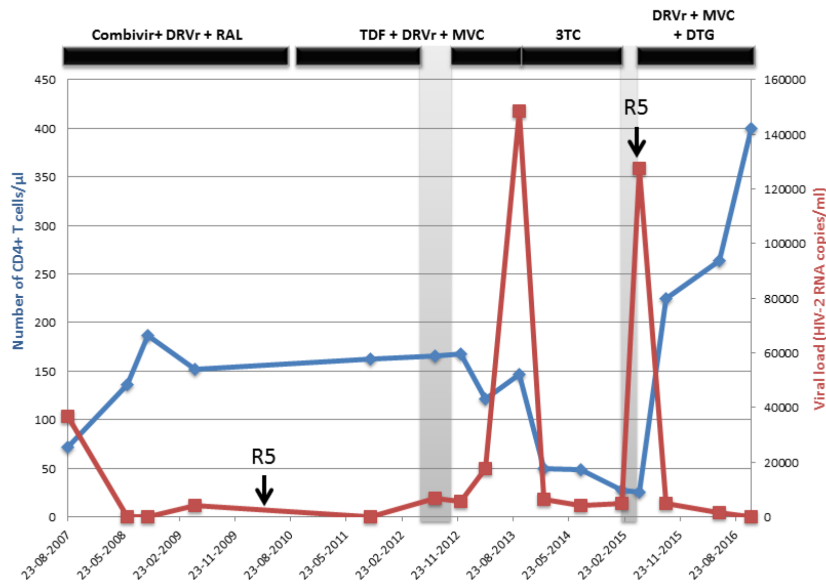
We assessed the activity of DTG and RAL on HIV-2 isolates obtained from twelve patients that were naïve to integrase inhibitors (INI) and from two RAL-failing patients, and identified resistance associated mutations and polymorphisms in matched integrase sequences. All but two isolates from an INI-naïve patient that exhibited resistance to DTG were sensitive to both drugs. To our knowledge this is the first description of HIV isolates exhibiting primary resistance to DTG. Genetic and evolutionary analyses suggested that mutations K221Q and D222K may play a role in primary resistance to DTG. The three isolates from RAL-failing patients were resistant to RAL. In two isolates this was caused either by mutation Q148K or E92Q + T97A. In the third isolate there were no known resistance mutations. Notably, all these isolates were sensitive to DTG. In summary, primary isolates from INI-naïve HIV-2 infected patients are generally sensitive to RAL and DTG. Nonetheless, some HIV-2 isolates may exhibit primary resistance to DTG which may be mediated via a potentially new mechanism. Mutations Q148K and E92Q + T97A associated with high-level resistance to RAL may not confer significant resistance to DTG implying different resistance mechanisms of HIV-2 to these drugs.

Introduction

Human immunodeficiency virus type 2 (HIV-2) was originated in West Africa around 1938 [1] and isolated from Guinea Bissau and Cape Verde patients with AIDS in 1986 [2]. These countries along with Senegal and Côte d'Ivoire were the main sources of extra-regional viral migration. Portugal and France, the two first targets of extra-regional viral dispersion from West Africa [3], report a relatively high prevalence of HIV-2 infection (4.5% [4] and 1.8% [5], respectively). HIV-2 has also spread to other European countries, the Americas, India and South Korea. Currently, it is estimated that approximately 1–2 million people worldwide are infected with HIV-2 [6, 7]. HIV-2 is composed of 9 groups termed A to I of which group A is by far the most disseminated [8]. HIV-2 is less pathogenic than HIV-1 doubling the average time from asymptomatic infection to AIDS (from 10 to 20 years or more) [9-11]. However, if untreated, the disease evolves to AIDS and death in 20–25% of HIV-2 infected patients. A major problem in this regard is the impaired or null susceptibility of HIV-2 to some of the currently available antiretroviral drugs [12]. HIV-2 is naturally resistant to all available non-nucleoside reverse transcriptase inhibitors and to the fusion inhibitor enfuvirtide [13-16]. HIV-2 shows some natural resistance to amprenavir *in vitro* and most viral isolates are partially resistant to nelfinavir, ritonavir, indinavir, atazanavir and tipranavir [13, 17-21]. Moreover, CD4+ T cell recovery following first-line combination ART is poorer in HIV-2 than in HIV-1- infected patients.

Raltegravir (RAL), the first integrase strand transfer inhibitor (INSTI, abbreviated to INI in this paper), is active against HIV-2 clinical isolates *in vitro* with an IC_{50} range similar to that of HIV-1 [22] and it can reduce viral load to undetectable levels in HIV-2 infected patients when combined with other suppressive antiretroviral drug [23-25]. Elvitegravir (EVG) also shows potent activity against HIV-2 *in vitro* [22] and it can reduce viral load in HIV-2 infected patients when combined with other suppressive antiretroviral drug [26]. Unfortunately, significant cross-resistance between RAL and EVG prevents sequential therapy with these drugs for both HIV-1 and HIV-2 infected patients [27-30]. DTG, the most recent INI, is active *in vitro* against HIV-2 isolates obtained from INI-naïve patients with an IC_{50} range similar to that of HIV-1 [31, 32]. Mutations conferring HIV-2 resistance to RAL and DTG are still incompletely defined but, in general, seem to be similar to HIV-1, mostly involving codons 92, 97, 140, 143, 148 and 155, either alone or in combinations [24, 32-35]. Few studies have examined the clinical effectiveness of DTG in HIV-2 infected individuals failing RAL-based regimens. In one study, DTG-containing regimens have shown substantial efficacy in HIV-2 infected patients harboring RAL- resistant viruses without mutations Q148H/R/K and N155H [33]. In other studies, virus replication was fully suppressed by DTG in patients failing RAL due to the N155H mutation [24, 25]. A modest increase (7 to 18-fold) in DTG EC_{50} values was observed in isolates obtained from two RAL-experienced patients with double (T97A + Y143C; G140S + Q148R) and triple (G140T + Q148R + N155H) resistance mutations [31]. Finally, major mutations Q148K, Q148R or G118R were detected in isolates from three HIV-2 patients that failed DTG treatment following RAL failure [25]. Overall, these studies showed a remarkable heterogeneity in RAL and DTG resistance mutations and pathways in HIV-2 infected patients challenging the prediction of HIV-2 susceptibility to these drugs based on integrase sequences and available algorithms. These tools are crucial for choosing the most potent antiretroviral combinations to maximize the long-term benefit of ART in HIV-2 infection. This is especially important in HIV-2 infection given the few therapeutic options available and the lower resistance barrier to these drugs relative to HIV-1 [12].

Figure 1A



In this study, we aimed to characterize the phenotypic and matched genotypic susceptibility of primary isolates obtained from INI-naïve and RAL-failing HIV-2 infected patients from Portugal to DTG and RAL. We found that DTG has a potent activity against primary HIV-2 isolates from INI-naïve and RAL-failing patients. Resistance mutations to RAL that do not confer significant resistance to DTG were identified implying different resistance mechanisms against these two drugs. Finally, primary resistance to DTG was observed for the first time in one patient and genetic and evolutionary analysis suggests it to be caused by a new mechanism.

MATERIALS AND METHODS

Ethics

Ethical approval for this study was obtained from the Ethics Committee of Hospital de S. José (DC-5125911). All patients provided written informed consent prior to the start of the study. This research complies with the Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/) and the Oviedo Bioethics Convention (http://conventions.coe.int/Treaty/en/Treaties/Html/164.htm) on medical research in humans.

Cells, plasmids and integrase inhibitors

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 and RAL [36-38]. DTG was provided by ViiV Healthcare. Wild-type pROD10 plasmid was a gift from Keith Peden [39]. HEK293T and TZM-bl cells were cultured in complete growth medium that consists of Dulbecco's minimal essential medium (DMEM) supplemented with 10% of fetal bovine serum, 100 U/ml of penicillin-streptomycin, 2

mM of L-Glutamine, 1mM sodium pyruvate and 1x of MEM non-essential amino acids (Gibco/ Invitrogen, USA). All cell cultures were maintained at 37ºC in 5% of CO_2.

Patient data

A total of sixteen primary isolates were obtained from fourteen HIV-2 infected Portuguese patients (Table 1). Thirteen isolates were obtained from twelve INI-naïve patients and three (10PTHSJIG, 15PTHSJIG and 15PTHCEC) from two patients failing a RAL-based therapeutic regimen. 10PTHSJIG and 15PTHSJIG were obtained from patient 10 in end of May 2010 and mid-June 2015, respectively. From 2007 to September 2010 this patient was given Combivir (AZT/ 3TC) + DRVr + RAL (Figure 1A). First evidence for virologic failure was obtained in May 2009. In October 2010 a regimen with TDF + DRVr + MVC150 was introduced with good results (undetectable viral load) until at least September 2011. From May to mid-August 2012 the patient interrupted the therapeutic due to travel to Guinea-Bissau; she resumed therapy on return but virologic failure emerged due to low adherence. From October 2013 to August 2015 the patient was on 3TC alone. From August 12 2015 onward she was on DRVr + MVC150 + DTG with continuous evidence of virologic suppression.

Isolate 15PTHCEC was obtained in March 23 2015 from patient 14 diagnosed with HIV-2 infection in 2010. Initial therapy in 2012 was DRVr + FTC/TDF (Figure 1B). Genotypic testing in end of August 2013 showed resistance to all NRTIs and PIs used for HIV-2 and therapy was changed to MVC+ RAL+ SQVr with limited success likely because the virus was already CXCR4-tropic at this time. Therapy was interrupted from April 15 to August 15 2014 because the patient traveled to Guinea Bissau and viral load on return was 17,575 copies/ml. Treatment with MVC+ RAL+ SQVr was resumed but genotypic tests performed in December 2014 showed resistance to all classes of ARVs and viral load of 5,630 copies/ml. In July 2015, the patient started DTG + AZT + 3TC, but anemia developed due to AZT and therapy was changed to DTG + FTC/TDF, with some success since viral load decreased to 1,677 copies/ml in November 30 2015.

Table 1 – Demographical and clinical characterization of the HIV-2 infected patients from which the viruses were isolated

Patient number	Isolate ID	Date of sample collection	Year of diagnosis	Gender	CD4+ T cell count/ µl[a]	RNA copies/ ml[a]	Date of starting therapy	Therapy	Co-receptor use
1	00PTHDECT	2000	1998	M	2919	na	Untreated	-	R5/X4
	03PTHDECT	2003	1998	M	209	20968	2003	d4T,3TC,LPVr	X4
2	01PTHDESC	2001	1992	F	44	1250	na	AZT, 3TC	X4
3	03PTHCC1	2003	2001	F	308	<200	2001	DDI, D4T, IDV	R5
4	03PTHCC6	2003	1992	F	615	<200	1996	AZT,3TC,IDV	R5
5	03PTHCC12	2003	1995	M	66	<200	Untreated	-	R5

6	03PTHCC19	2003	2003	F	175	<200	2005	D4T, 3TC, LPVr	R5
7	03PTHCC20	2003	1998	F	78	na	2005	TDF, ABC, LPVr	X4
8	03PTHSM2	2003	2002	M	275	<200	2002	AZT, 3TC, DDI	R5
9	04PTHSM10	2004	2001	F	265	4792	2002	AZT, 3TC, NVF	X4
10	10PTHSJIG	2010	2005	F	164	4257	na	AZT, 3TC, RAL	R5
	15PTHSJIG	2015	2005	F	40	1793	na	DRVr, MVC, DTG	R5
11	10PTHSMAK	2010	2009	F	40	1793	Untreat-ed	-	X4
12	10PTHSMAUC	2010	2010	M	177	<200	Untreat-ed	-	X4
13	10PTHSMNC	2010	2008	F	231	<200	na	SQV, ABC, 3TC	R5/X4
14	15PTHCEC	2015	2010	F	436	1677	2010	MVC, RAL SQVr	X4

Figure 1B

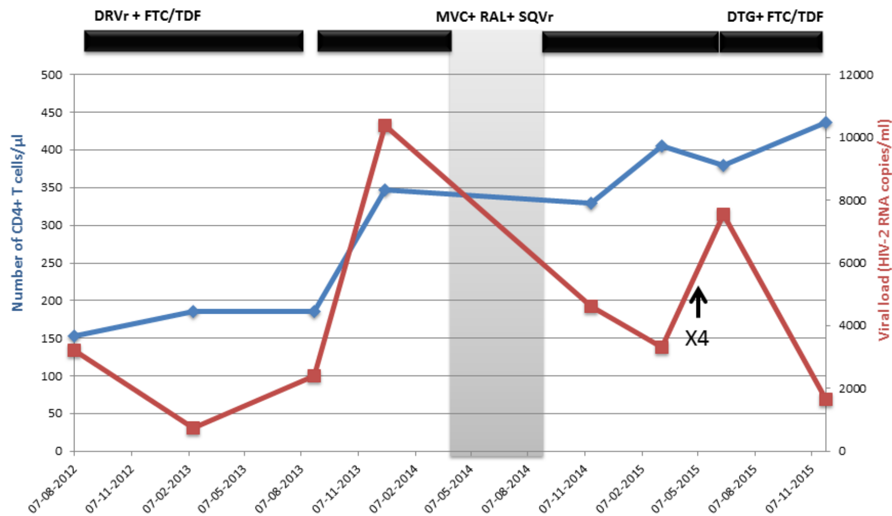


Figure 1 – *Evolution of viral load and CD4+ T cell counts in patients on a DTG- based treatment regimen after failing a RAL-based regimen.* ***A)*** *Patient 10;* ***B)*** *Patient 14. The arrow indicates the approximate time of virus isolation. R5 corresponds to CCR5 coreceptor use and X4 to CXCR4 coreceptor use as determined by genotypic analysis of the sequence of the V3 region of the isolates. The gray rectangles indicate periods of drug interruption. Combivir [lamivudine (3TC) and zidovudine (AZT)]; DRVr, ritonavir boosted darunavir; SQVr, ritonavir boosted saquinavir; FTC, emtricitabine; TDF, tenofovir disoproxil fumarate; MVC, maraviroc; RAL, raltegravir; DTG, dolutegravir.*

Virus stocks and titration

The 16 primary isolates described in this study were obtained by co-cultivation with peripheral blood mononuclear cells from seronegative subjects as described [40]. Different features of 14 primary isolates were previously described [41-44]; two new isolates (15PTHCEC and 15PTHJSIG) are presented here for the first time and their origin is described above. The HIV-2ROD lab-adapted strain was obtained by transient transfection of 293T cells with pROD10 plasmid using the jetPrime transfection reagent (Polyplus) according to manufacturer's instructions. Cell culture supernatant was collected 48h post-transfection, filtered and stored at -80ºC.

The 50% tissue culture infectious dose ($TCID_{50}$) of each isolate was determined in a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells. Firstly, 10,000 TZM-bl reporter cells were seeded in 96-well tissue culture plates and incubated overnight. 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. Cells were incubated with virus for 48h, before quantification of luciferase expression with the Pierce Firefly Luciferase Glow Assay Kit (Thermo Fisher, USA) according to manufacturer's instructions. Control wells containing only target cells and growth medium were used to measure background luminescence. The $TCID_{50}$ was calculated using the statistical method of Reed and Muench.

Drug susceptibility assays

The antiviral activity of DTG and RAL was evaluated using a single-round viral infectivity assay in TZM-bl cells. First, 10,000 TZM-bl reporter cells were seeded in 96-well tissue culture plates and incubated overnight. Next day, cells were incubated with several fold dilutions of DTG or RAL, for 1h at 37°C, in growth medium supplemented with 19.7 µg/ml of DEAE-dextran. The tested concentrations of DTG and RAL ranged from 5.99×10^{-7} nM to 31,086.79 nM. Cells were infected with 200 $TCID_{50}$ of each virus. After 48h of infection, luciferase expression was quantified with the Pierce Firefly Luciferase Glow Assay Kit (Thermo Fisher, USA) according to 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.

DNA extraction, PCR amplification and sequencing

Viral RNA was extracted from 1 ml of cell culture supernatant diluted 1/100, according to Biomérieux's easyMAG automatic extraction procedure, and used to amplify and sequence the last domain of the *pol* gene, comprising the integrase (293 amino acids). RNA was then retro-transcribed using Quiagen One-Step RT-PCR Kit. A nested PCR was then performed using the Thermo Scientific Taq DNA Polimerase (recombinant) reagent. Amplification products were checked on a 1% agarose gel and were subsequently purified using the ExoSAP-IT protocol. Dilution adjustments of the PCR products were made when necessary. The sequencing reaction was then performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Products were purified and run on an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were aligned against the HIV-2 ROD reference strain (Gen-

Bank accession # M15390), and edited with SeqScape and ChromasPro Software. Sequences were submitted to GenBank and were given the following accession numbers: KY962712-KY962727.

Phylogenetic analysis

The nucleotide sequences of the integrase gene were aligned using the Muscle program [45] implemented in SeaView version 4.5.4 [46] and manual adjustments were also made in SeaView. Maximum likelihood analysis was performed using the best-fit model of molecular evolution estimated by FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) under the Akaike Information Criterion. The model chosen was GTR+G. The phylogenetic tree was reconstructed using the PhyML program [47] implemented in SeaView version 4.5.4 [46] using the nearest-neighbor interchange (NNI) heuristic search strategy and 1000 bootstrap replications.

Analysis of genotypic drug resistance

The nucleotide sequence of the integrase gene of each HIV-2 primary isolate was analyzed using Genotypic Resistance-Algorithm Deutschland (GRADE) (http://www.hiv-grade.de/chain/deployed/grade.pl?program=hivalg), to identify any mutations associated to resistance to integrase inhibitors and the corresponding level of resistance determined by each mutation. The sequences of the integrase gene derived from HIV-2 primary isolates were also aligned as described and compared to the sequence of HIV-2 ROD to identify the presence of polymorphisms. The level of resistance for each polymorphism was determined based on GRADE Algorithm, drug susceptibility assays (fold-change) and what is described on the literature.

Statistical analysis

Statistical analyses were performed using Prism version 5.01 for Microsoft (GrahPad Software, San Diego, California USA, www.graphpad.com) with a level of significance of 5%. The 50% (IC_{50}) inhibitory concentrations were estimated by plotting the percent inhibition of infection (y axis) against the log10 concentration of each drug (x axis) and using the sigmoidal dose-response (variable slope) equation. The F-test was used to compare best-fit values between dose–response curves. The Man-Whitney U test was used to compare IC_{50} values between groups of isolates.

RESULTS

Phenotypic susceptibility of viral isolates to DTG and RAL

RAL was highly active against all viral isolates obtained from INI-naïve patients, median IC_{50} being 1000-fold lower relative to the historic isolate HIV-2 ROD which showed low level resistance to RAL (Figure 2; Table S1; Figure S1). In contrast, the three isolates obtained from RAL-failing patients (patients 10 and 14), were highly resistant to RAL their median IC_{50} being 8,888-fold higher relative to the isolates from naïve patients (53.860 nM vs 0.006 nM; P=0.0106) (Figure 2; Table S1; Figure S2). Median maximum percentage of inhibition (MPI) by RAL was similar for RAL-sensitive and RAL-resistant isolates (92.2% vs 97.7%).

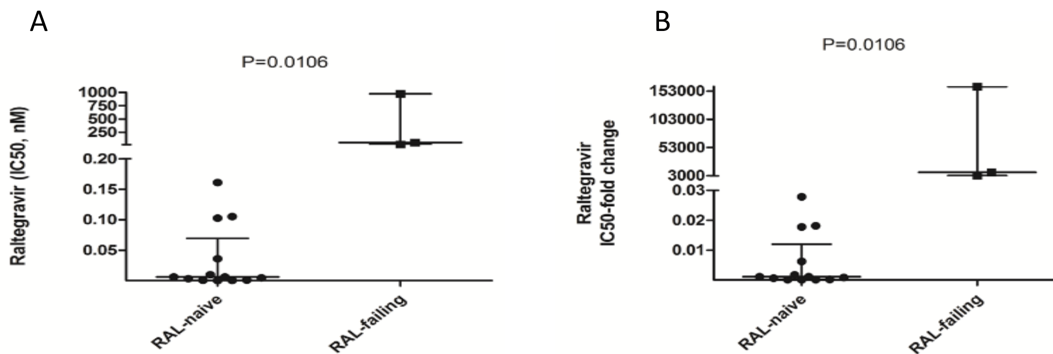


***Figure 2 –** Susceptibility of HIV-2 isolates obtained from RAL-naïve and RAL-failing patients to RAL as determined by the 50% inhibitory concentration (IC_{50}) (A) and IC_{50} fold-change (B). IC_{50} fold-change of isolates from RAL-naïve patients was relative to the historic HIV-ROD strain; IC_{50} fold-change of isolates from RAL-failing patients was relative to those from RAL-naïve patients. Lines indicate the median with interquartile range. Median values were compared using the Mann Whitney test.*

DTG was also highly active against primary viral isolates obtained from INI-naive patients, median IC_{50} being 2.1-fold lower relative to HIV-2ROD10 which was about 35-fold more sensitive to DTG than to RAL (Table S2; Figure S1). Of note, two viral isolates obtained 3 years apart from the same INI-naïve patient (patient 1, a child infected perinatally) required 95-fold (isolate 03PTHDECT) and 139-fold (isolate 00PTHDECT) higher concentration of DTG for 50% inhibition relative to HIV-2 ROD. Due to these two isolates the median IC_{50} of DTG was significantly higher than RAL for the isolates from INI-naïve patients (0.078 nM vs 0.006 nM; P=0.0210) (Figure 3). Remarkably, however, DTG was highly active against the RAL-resistant isolates with a median IC_{50} that was only 9.2-fold higher relative to the other primary isolates (0.718 nM vs 0.078 nM; P=0.1389) (Figure 4; Table S2; Figure S2). Similarly, to RAL, median MPI by DTG was similar for RAL-sensitive and RAL-resistant isolates (91% vs 92%).

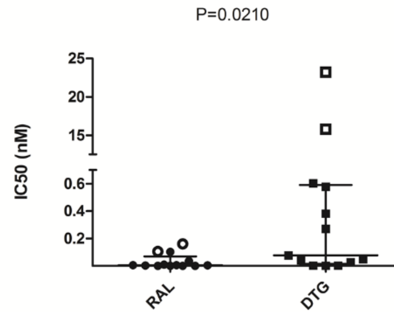


Figure 3 – *Susceptibility of HIV-2 isolates to RAL and DTG as determined by the 50% inhibitory concentration (IC_{50}). Open symbols indicate the two isolates from patient 1 showing primary resistance to DTG. Lines indicate the median with interquartile range. Median IC_{50} values were compared using the Mann Whitney test.*

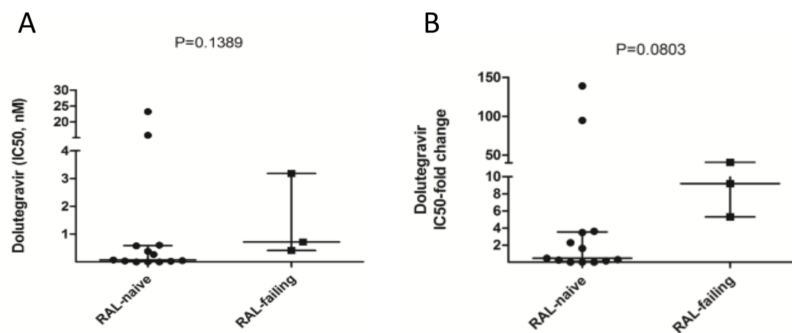


Figure 4 – *Susceptibility of HIV-2 isolates obtained from RAL-naïve and RAL-failing patients to DTG as determined by the 50% inhibitory concentration (IC_{50}) (A) and IC_{50} fold-change (B). IC_{50} fold-change of isolates from RAL-naïve patients was relative to the historic HIV-ROD strain; IC_{50} fold-change of isolates from RAL-failing patients was relative to those from RAL-naïve patients. Lines indicate the median with interquartile range. Median values were compared using the Mann Whitney test.*

Analysis of genotypic drug resistance

Integrase sequences were produced for all isolates and their evolutionary relationships as well as polymorphisms and resistance mutations were investigated. All new integrase sequences belonged to group A which is the most common HIV-2 group in Portugal and worldwide (Figure S3) [8]. As expected the sequences from the two isolates from patient 10 (10PTHJSIG and 15PTHJSIG) formed a monophyletic cluster supported by high bootstrap values. Likewise, the sequences from isolates of patient 1 (00PTHDECT and 03PTHDECT) formed a strongly supported cluster with the sequence from patient 7 isolate (03PTHCC20) confirming their common ancestry [48].

The catalytic triad DDE (positions 64, 116 and 152), the zinc coordination motif HHCC (12, 16, 40 and 43) and the RKK motif (231, 258 and 264), were completely conserved in the integrase of our isolates (Figure S4). We looked for drug resistance mutations in the isolates from RAL-

resistant patients 10 and 14. For patient 10, the isolate from 2010 (10PTHSJIG) had the secondary mutations E92Q and T97A (Figure 5; Table 2). These mutations have been associated with HIV-2 resistance to RAL when in combinations such as E92A/T97A/N155H, T97A/Y143C, E92Q/Y143C and E92Q/N155H [29, 31, 32, 49]. The isolate from 2015 (15PTHSJIG) had no mutations associated with resistance to RAL but harbored the I84V polymorphism which has been associated with resistance to RAL [50, 51]. Isolate from patient 14 (15PTHCEC) had the E92A and Q148K mutations (Figure 5; Table 2). The latter mutation has been associated with high level resistance to DTG, RAL and EVG in HIV-2 [25, 33].

Table 2 – Resistance mutations in the integrase protein of RAL-failing patients and susceptibility to RAL and DTG

Patient (isolate)	Resistance mutations	RAL IC_{50} Fold-change[1]	Predicted susceptibility to RAL[2]	DTG IC_{50} Fold-change[1]	Predicted susceptibility to DTG[2]
IG (2010)	E92Q, T97A	8,887.789	Low-level resistance	40.744	Sensitive
IG (2015)	None	3,297.030	Sensitive	5.308	Sensitive
EC (2015)	E92A, Q148K	160,726.073	High-level resistance	9.199	Sensitive

[1] Relative to median IC_{50} of primary isolates from INI-naïve patients; [2]Based on the GRADE algorithm and current literature.

As mentioned above, viral isolates 00PTHDECT and 03PTHDECT were isolated from patient 1 in 2000 and 2003, respectively, and are susceptible to RAL but naturally resistant to DTG. In 2000 this patient was not receiving ART (treatment-naïve) and in 2003 he was on d4T, 3TC and LPVr. To try to identify which polymorphisms might have been associated with resistance to DTG we compared IN sequences of these two isolates with those of HIV-2ROD, isolates from INI-experienced and a consensus sequence from INI-naïve patients (Figure 5). The following mutations were present only in 00PTHDECT: N170K, L200S, I201T, N202M, M203T, I204H and T206P in the catalytic core domain. Of these, only I201T and T206P have been associated to resistance to INIs in HIV-1 and HIV-2 [50]. However, the absence of these mutations in the isolate from 2003 (03PTHDECT) argues against their role in the resistance to DTG. Based on this alignment the most important mutations contributing to DTG resistance were probably K221Q and D222K as they involve significant changes in the charge of the protein and were present in both viral isolates. Unlike the isolates of patient 1 the isolate of his mother, patient 7, showed good sensitivity to DTG (Table S2; Figure S1). Alignment of the amino acid sequences from the mother and son isolates revealed only 18 changes (Figure 5B). Remarkably, only two changes, located at positions 221 and 222, were common to both isolates of patient 1 and different from patient 7 and from the consensus sequence of isolates from INI-naïve patients. Combined, these results suggest that mutations in codons 221 and 222 may have determined the resistance of 00PTHDECT and 03PTHDECT isolates to DTG.

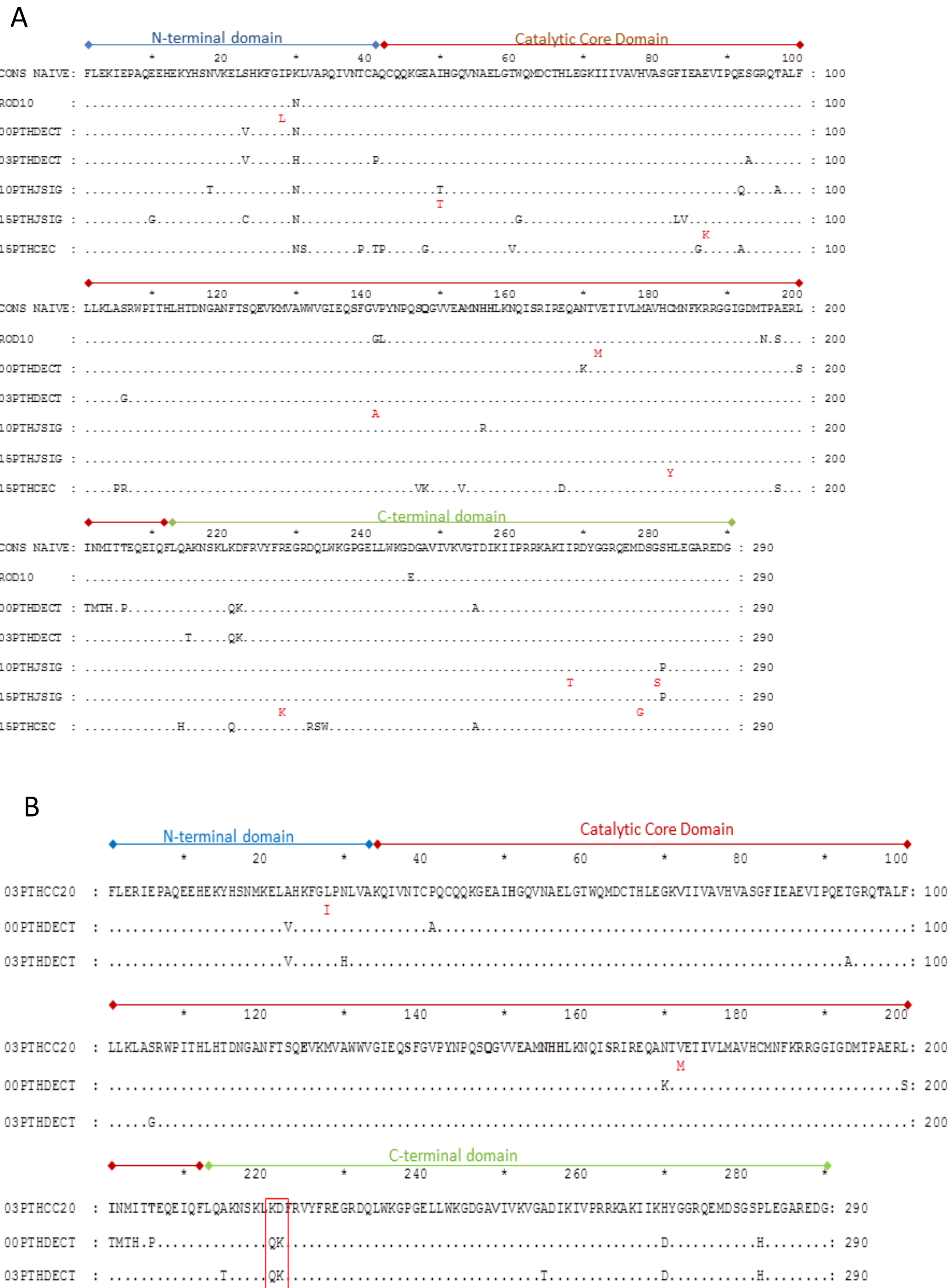


Figure 5 – *Alignment of the IN amino acid sequences of HIV-2 isolates showing resistance to RAL or DTG.* ***A)*** *Diversity in the amino acid sequences is shown relative to a consensus sequence (CONS NAIVE) obtained for the INI-naïve isolates, excluding isolates from patient 1 (00PTHDECT and 03PTHDECT) that show primary resistance to DTG. The consensus IN sequence was obtained using HIV Database Consensus Maker (https://www.hiv.lanl.gov/content/sequence/CONSENSUS/consensus.html). Isolates 10PTHJSIG and 15PTHJSIG are from patient 10 and isolate 15PTHCEC is from patient 14. ROD10 is the sequence from the reference and historic strain HIV-2ROD.* ***B)*** *Diversity in IN sequences of isolates from patient 1 is shown relative to the IN sequence from the isolate of his mother (03PTHCC20) that infected him perinatally in 1998 [48]. Conserved amino acids are represented with a dot. Polymorphic positions are represented with the amino acid letter. Positions corresponding to more than one amino acid in the viral sequence are marked with red on top of the corresponding sequence. Positions that are known to confer resistance to INIs in HIV-1 or HIV-2 are shown in bold in the consensus amino acid sequence.*

DISCUSSION

Using a validated phenotypic assay of HIV-2 susceptibility to antiretroviral drugs we showed that integrase inhibitors DTG and RAL were generally active against HIV-2 isolates from RAL-naïve patients from Portugal. Viruses from RAL-failing patients were highly resistant to RAL but were suppressed by DTG almost to the level of isolates from INI-naïve patients. These results are consistent with the observed clinical effectiveness of DTG on HIV-2 infected patients failing therapy with RAL [24, 25, 31-33, 35].

Two of the three RAL-resistant isolates had resistance-associated mutations in the IN already described in literature. Isolate 10PTHSJIG carried the E92Q and T97A mutations which are frequently selected as minor resistance mutations in HIV-1 infected patients failing therapy with RAL and EVG; E92Q alone can confer HIV-1 low level resistance to RAL and moderate resistance to EVG whereas T97A results in a 2-fold change in EVG susceptibility [52]. Thus far the role of these mutations in HIV-2 was still uncertain with one study reporting that E92Q alone does not confer resistance to RAL [49] and others reporting that it confers low-level resistance to RAL [29] and DTG [32]. T97A may improve the fitness of IN molecules carrying the resistance mutation N155H [49] and, when associated with the Y143C mutation, results in a 7-fold change in HIV-2 susceptibility to DTG [31]. Isolate 15PTHCEC carried the Q148K and E92A mutations. E92A is a mutation less frequently described at this residue and its role in INI susceptibility is still unclear [49, 50]. Q148K confers major resistance to RAL in HIV-1 and HIV-2, especially when in association with other accessory mutations (e.g., E92Q, T97A, G140S) [22, 33, 34, 50, 52]. However, its role in HIV-2 susceptibility to DTG is still unclear. Two recent clinical studies have shown that HIV-2 patients failing DTG after RAL failure caused by the major N155H mutation often select mutations at codon 148 (Q148K or Q148R) [25, 33]. This usually occurs in combination with accessory mutations at codons 151 and 153 and different polymorphisms. In contrast, one *in vitro* study with site-directed mutants showed that Q148K alone had only moderate effect on the susceptibility of reference isolate HIV-2ROD9 to DTG [32]. Likewise, in HIV-1 infected patients failing RAL, DTG lacked efficacy only in patients with changes at codon 148 plus two additional mutations at baseline [53]. Our results are in line with the former studies and indicate that in HIV-2, as in HIV-1, mutation at codon 148 alone is not enough to confer significant resistance to DTG. Finally, as in a previous study [25], one isolate was phenotypically resistant to RAL in the absence of any resistance mutation in the integrase. This again stresses the fact that our knowledge of HIV-2 resistance to RAL is still incomplete and that the currently available genotypic algorithms of drug resistance need to be perfected.

Virus isolates obtained from treatment-naïve patient 1 in 2000 and 2003 (00PTHDECT and 03PTHDECT) were sensitive to RAL but showed natural resistance to DTG. To our knowledge, this is the first study to report primary HIV resistance to DTG. Patient 1 was infected perinatally by his mother (patient 7) in 1998 [48]. Remarkably, the isolate obtained from his mother in 2003, also naïve to INIs, was fully sensitive to both RAL and DTG. Besides being similarly sensitive to RAL, the viral isolates from the mother and child had a similar sensitivity profile to other antiretroviral drugs [16, 42] indicating that the natural resistance of the infant's isolates to DTG is specific. No known INI resistance mutations were found in isolates from patient 1. A comparative inspection of the IN sequences from patient 1 with IN sequences from his mother and from the other INI-naïve patients revealed that mutations in codons 221 and 222 in the carboxy-terminal domain were the most likely determinants of the natural resistance to DTG. Codons located at the carboxi-terminal region of the HIV-1 integrase have been shown to de-

termine the ability of integrase to bind to reverse transcriptase (e.g. 231, 243, 247) and this seems to be important for reverse transcription and viral replication [54]. We hypothesize that in analogy to HIV-1, mutations at codons 221 and 222 in the HIV-2 integrase may mediate binding to the reverse transcriptase and increase reverse transcription and viral replication rates thereby contributing indirectly for natural resistance to DTG.

In summary, primary HIV-2 isolates from INI-naïve patients are generally sensitive to RAL and DTG. Combined mutations E92Q + T97A and mutation Q148K significantly reduce the sensitivity of HIV-2 to RAL but not to DTG implying different mechanisms of resistance against these two drugs. Primary HIV-2 resistance to DTG albeit rare can occur and mutations K221Q and D222K may play a role in this resistance. Our study confirms DTG and RAL as important therapeutic options for HIV-2 infected patients, highlights the added value of phenotypic assays to assess HIV-2 susceptibility to integrase inhibitors, and contributes for the production of better genotypic algorithms for prediction of HIV-2 susceptibility to integrase inhibitors.

ACKNOWLEDGMENTS

This work was supported by Fundação para a Ciência e Tecnologia (FCT), Portugal (PTDC/BIM-MEC/6631/2014 to Isabel Barahona and VIH/SAU/0029/2011, part of the EDCTP2 program supported by the European Union, to N.T.) and by the Luso-American Foundation for Development (FLAD) (Luso-American Collaborative Response Award on HIV/AIDS to N.T. and Isabel Barahona). Inês Bártolo is supported by a post-doc fellowship (SFRH/BPD/76225/2011) from Fundação para a Ciência e Tecnologia (FCT), Portugal. Ana Rita Diniz (SFRH/BD/89140/2012) and Francisco Martin (SFRH/BD/87488/2012) were supported by PhD grants from Fundação para a Ciência e Tecnologia (FCT), Portugal. ViiV Healthcare is acknowledged for providing DTG.

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SUPPLEMENTARY MATERIAL

Table S1 – Activity of RAL against primary HIV-2 isolates

Type of patient	Isolates	IC_{50} (nM)	Fold Change[1]	MPI (concentration, nM)
RAL-naive	ROD10	5.786	-	101.8 (3108.6)
	03PTHCC12	0.0002	0.00003	94.4 (11.7)
	03PTHCC19	0.0005	0.00009	95.7 (128.5)
	03PTHCC6	0.0007	0.0001	94.1 (1413.0)
	03PTHSM2	0.0009	0.0002	90.5 (1413.0)
	04PTHSM10	0.003	0.0006	95.6 (128.5)
	10PTHSMNC	0.005	0.0008	92.4 (1413.0)
	01PTHDESC	0.006	0.001	89.1 (1413.0)
	10PTHSMAUC	0.006	0.001	84.9 (128.5)
	10PTHSMAK	0.001	0.002	92.9 (1413.0)
	03PTHDECT	0.036	0.006	76.9 (15488.2)
	03PTHCC1	0.103	0.018	92.2 (15488.2)
	00PTHDECT	0.105	0.018	84.3 (15488.2)
	03PTHCC20	0.161	0.028	90.6 (15488.2)
	Median[2]	0.006	0.001	92.2 (1413.0)
RAL-Experienced	15PTHSJIG	19.980	3297.030	97.7 (31086.8)
	10PTHSJIG	53.860	8887.789	85.2 (31086.8)
	15PTHCEC	974.000	160726.073	101.1 (30902.9)
	Median	53.860	8887.789	97.7 (31086.8)

[1] IC_{50} fold change was calculated relative to reference HIV-2 strain ROD10 for isolates from RAL-naïve patients; for isolates from RAL-experienced patients, IC_{50} fold change was calculated relative to median IC_{50} values of primary isolates from INI-naïve patients. [2]HIV-2 ROD10 was excluded from median determination as it is not a primary isolate. MPI – Maximum percentage of inhibition.

Table S2 – Activity of DTG against primary HIV-2 isolates

Type of patient	Isolates	IC_{50} (nM)	Fold Change[1]	MPI (concentration, nM)
RAL-naïve	ROD10	0.167	-	98.7 (140.4)
	03PTHCC6	0.002	0.014	93.1 (140.4)
	03PTHCC19	0.003	0.017	95.6 (140.4)
	03PTHCC12	0.004	0.021	95.1 (140.4)
	03PTHSM2	0.028	0.168	89.7 (140.4)
	10PTHSMNC	0.042	0.251	92.4 (1544.8)
	04PTHSM10	0.050	0.299	94.8 (1544.8)
	10PTHSMAUC	0.078	0.466	85.4 (1544.8)
	03PTHCC1	0.271	1.623	88.7 (33884.4)
	03PTHCC20	0.382	2.287	92.4 (33884.4)
	10PTHSMAK	0.579	3.466	91.0 (140.4)
	01PTHDESC	0.603	3.611	90.3 (33985.8)
	03PTHDECT	15.810	94.670	74.1 (16982.4)
	00PTHDECT	23.220	139.041	82.2 (16982.4)
	Median [2]	0.078	0.467	91.0 (1544.8)
RAL-experienced	15PTHSJIG	0.414	5.308	94.9 (140.4)
	15PTHCEC	0.718	9.199	87.8 (33884.4)
	10PTHSJIG	3.178	40.744	92.0 (1544.8)
	Median	0.718	9.199	92.0 (1544.8)

[1]IC_{50} fold change was calculated relative to reference HIV-2 strain ROD10 for isolates from RAL-naïve patients; for isolates from RAL-experienced patients, IC_{50} fold change was calculated relative to median IC_{50} values of primary isolates from INI-naïve patients. [2]HIV-2 ROD10 was excluded from median determination as it is not a primary isolate. MPI – Maximum percentage of inhibition.

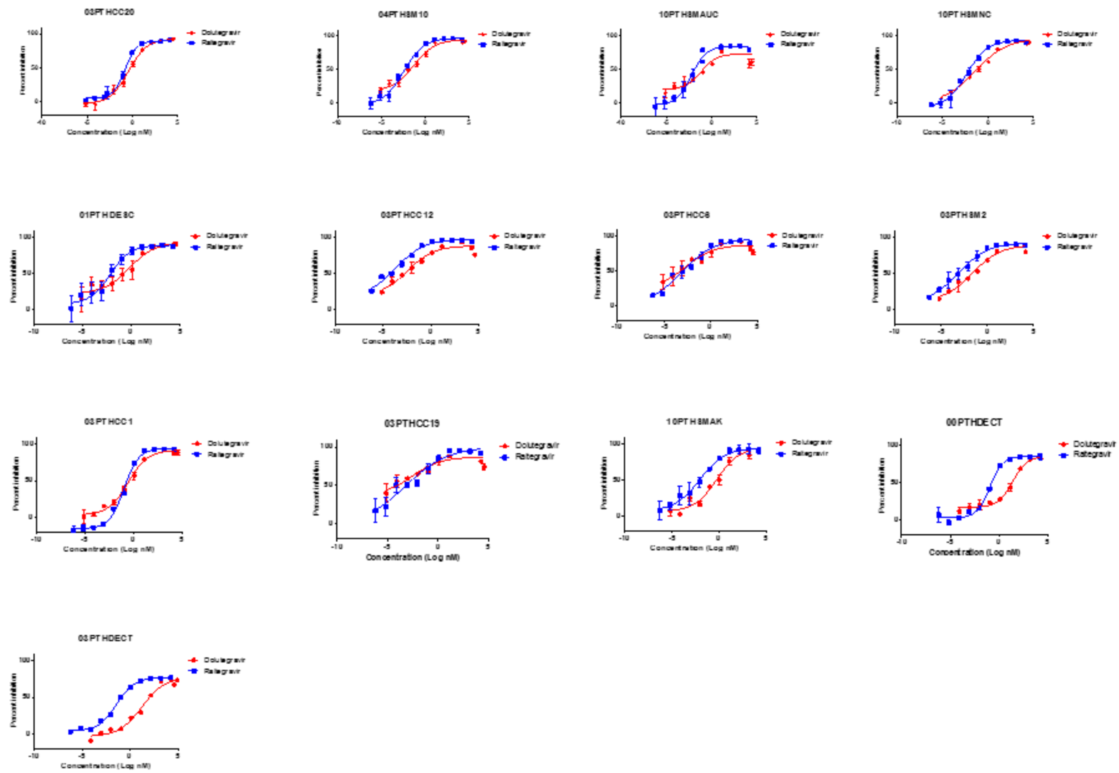

Figure S1 *– Dose-response curves showing the percent inhibition of replication of HIV-2 by DTG and RAL. Only isolates that were obtained from INI- naïve patients are shown.*

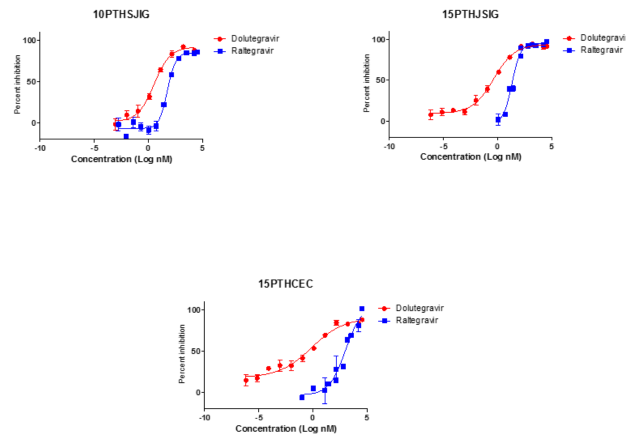

Figure S2 *– Dose-response curves showing the percent inhibition of replication of RAL resistant HIV-2 isolates by DTG. Only isolates that were obtained from patients failing a RAL-based therapeutic regime are shown.*

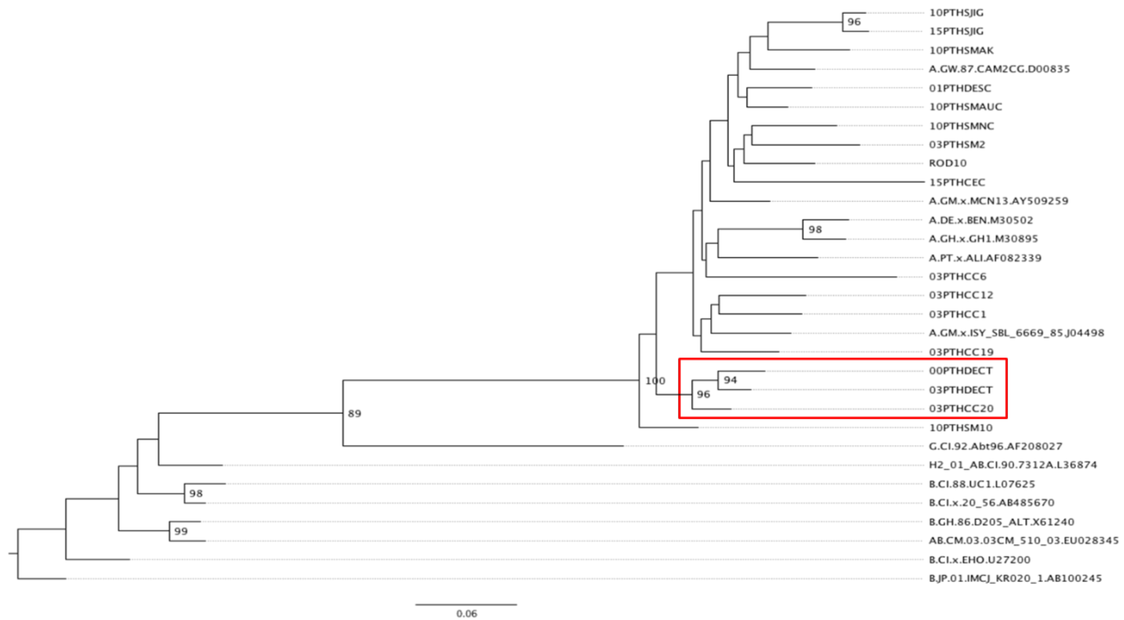

Figure S3 – *Phylogenetic tree of the integrase genes from HIV-2 primary isolates. The maximum likelihood phylogenetic tree was constructed with reference sequences from all HIV-2 subtypes. The bootstrap values supporting the internal branches are shown. Only bootstrap values above 70% are shown. The red box indicates the cluster containing the sequences of isolates from patients 1 (00PTHDECT and 03 PTHDECT) and 7 (03PTHCC20), son and mother, respectively, which form a pair of transmission.*

Figure S4 – *Logo sequence plot showing the variation in each amino acid residue of IN sequences. Results for the group of individuals with and without INI therapy are displayed side-by-side. The height of each box reflects the contribution of that amino acid to the position's total information, as determined by Shannon entropy. The color scale represents the water solubility and polarity of the amino acid residues. A transparent box represents gaps in sequences at a particular position. Positions are numbered according to HIV-2 ROD.*

CHAPTER 3
DEVELOPMENT OF WATER-SOLUBLE POLYANIONIC CARBOSILANE DENDRIMERS AS NOVEL AND HIGHLY POTENT TOPICAL ANTI-HIV-2 MICROBICIDES

Development of water-soluble polyanionic carbosilane dendrimers as novel and highly potent topical anti-HIV-2 microbicides

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Research article

Nanoscale, 2015; 7:14669-14683

ABSTRACT

The development of topical microbicide formulations for vaginal delivery to prevent HIV-2 sexual transmission is urgently needed. Second- and third-generation polyanionic carbosilane dendrimers with a silicon atom core and 16 sulfonate (G2-S16), napthylsulfonate (G2-NS16) and sulphate (G3-Sh16) end-groups have shown potent and broad-spectrum anti-HIV-1 activity. However, their antiviral activity against HIV-2 and mode of action have not been probed. Cytotoxicity, anti-HIV-2, anti-sperm and antimicrobial activities of dendrimers were determined. Analysis of combined effects of triple combinations with tenofovir and raltegravir was performed by using CalcuSyn software. We also assessed the mode of antiviral action on the inhibition of HIV-2 infection through a panel of different *in vitro* antiviral assays: attachment, internalization in PBMCs, inactivation and cell-based fusion. Vaginal irritation and histological analysis in female BALB/c mice were evaluated. Our results suggest that G2-S16, G2-NS16 and G3-Sh16 exert anti-HIV-2 activity at an early stage of viral replication inactivating the virus, inhibiting cell-to-cell HIV-2 transmission, and blocking the binding of gp120 to CD4, and the HIV-2 entry. Triple combinations with tenofovir and raltegravir increased the anti-HIV-2 activity, consistent with synergistic interactions (CI_{wt}: 0.33–0.66). No vaginal irritation was detected in BALB/c mice after two consecutive applications for 2 days with 3% G2-S16. Our results have clearly shown that G2-S16, G2-NS16 and G3-Sh16 have high potency against HIV-2 infection. The

modes of action confirm their multifactorial and non-specific ability, suggesting that these dendrimers deserve further studies as potential candidate microbicides to prevent vaginal/rectal HIV-1/HIV-2 transmission in humans.

INTRODUCTION

Human immunodeficiency virus (HIV) and other sexually transmitted infections are global threats to public health. Although HIV-1 strains are responsible for most of the global infections, HIV-2 strains are an important cause of disease in West African nations, Portugal, France, and in the United States [1,2]. Moreover, co-infection with both HIV-1 and HIV-2 occurs in some countries of West Africa where the viruses co-circulate [3,4].

Sexual transmission is responsible for the majority of HIV-2 infections due to infected semen or cervicovaginal secretions containing infected lymphocytes [5]. Ideally, a vaginal/rectal microbicide should have the following features: to be acceptable and affordable, offer maximal and immediate protection, remain in the vagina for a few hours to act against the sexual transmitted diseases (STDs) during and after sexual intercourse, not leak immediately after application, not accumulate to avoid toxicity effects, not affect the normal vaginal flora, and be compatible with male latex condom [6–8].

Dendrimers are a class of well-defined hyper-branched polymers with a nanoscale globular shape, well-defined functional groups at the periphery, and the presence of internal cavities to encapsulate guest molecules in the macromolecule interior. Their reasonable cost of manufacture, controlled synthesis, high biocompatibility, solubility, miscibility and reactivity, low polydispersity, and polyvalency distinguish them from conventional linear polymers [9]. Dendrimers offer unique opportunities in the synthesis of agents with broad-spectrum anti-HIV-1 activity and activity against Alzheimer's disease, herpes simplex virus (HSV), bacteria, and cancer [10–14]. A group of dendrimers with a carbosilane structure has demonstrated potential against HIV and other pathogens. These dendrimers are particularly suitable for this application due to the simplicity of their synthesis, which allows for large amounts of the polymer to be generated, the ability to obtain a polymer with a defined molecular weight and a number of terminal functions, their chemical and biochemical stability and biological inertness, and the low polarity of the C–Si bond, which imparts hydrophobicity to the carbosilane scaffold [15–17].

One of the most promising targets of the HIV cycle is the viral entry/fusion process, which is divided into three steps: (i) attachment of gp120 to CD4, (ii) binding to CCR5 and/or CXCR4, and (iii) fusion of the envelope with the cell membrane and release of the viral capsid into the cytoplasm of the host cell [18]. Dendrimers containing functionalized groups at their periphery can bind to their target in a multivalent manner, providing a strategy for the development of potent viral entry inhibitors. Although the antiviral activity and the mode of action of carbosilane dendrimers have been studied against HIV-1, their antiviral activity against HIV-2 is currently unknown. Dendrimers may be important to fight HIV-2 infection as few of the currently available antiretroviral drugs work well against this virus and drug resistance is rapidly selected against the drugs that do work [19].

In order to identify dendrimers with potent anti-HIV-2 activity, we considered several polyanionic carbosilane dendrimers successfully tested for HIV-1 applications with the objective of selecting those compounds with the best results obtained. In previous studies, we have shown

that polyanionic carbosilane dendrimers G2-S16, G2-NS16, and G3-Sh16 (generations described as the number of repeating layers of silicon atoms forming the dendrimer, Figure 1) had great anti-HIV-1 activity *in vitro* and *in vivo* [20–22]. G2-S16 and G2-NS16 consist of second generation carbosilane dendrimer scaffolds built from a silicon atom core, which is fully capped on the surface with 16 sulfonate and napthylsulfonate groups, respectively. G3-Sh16 is a sulphate-terminated generation 3 carbosilane dendrimer, silicon-cored and also with 16 anionic charges at the periphery. The anionic groups are in the form of sodium salts. Here, we have investigated the cytotoxicity, the anti-HIV-2 activity, and the anti-sperm and antimicrobial activities of G2-S16, G2-NS16, and G3-Sh16 dendrimers. Moreover, we researched the anti-HIV-2 activity of combinations of these dendrimers with tenofovir and raltegravir. In order to discover more about the antiviral mechanism of action of these dendrimers, we used several *in vitro* experiments including: attachment and internalization of HIV-2 in PBMCs, HIV-2 inactivation, and cell-based fusion assays. Finally, we show that vaginal application of 3% G2-S16 gel formulation does not cause vaginal irritation or lesions after histological analysis in female BALB/c mice.

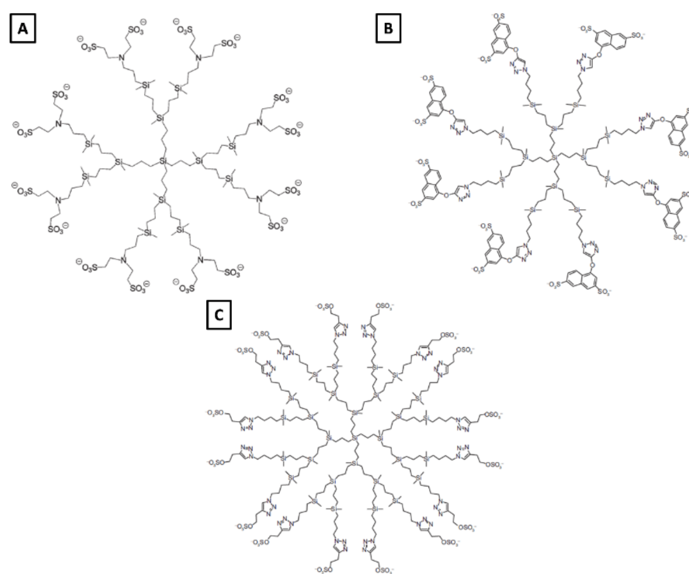


***Figure 1 –** Molecular representation of second- and third-generation dendrimers. (A) G2-S16 with 16 sulfonate end groups; (B) G2-NS16 with 16 naphthylsulfonate end groups; (C) G3-Sh16 with 16 sulphate end groups. The generation of dendrimers is determined by considering that each generation corresponds to the number of repeating layers of silicon atoms forming the dendrimer.*

Experimental section

a. Dendrimers and reagents

Polyanionic carbosilane dendrimers G2-S16 ($C_{112}H_{244}N_8Na_{16}$- $O_{48}S_{16}Si_{13}$; molecular weight, M_w: 3717.2 g mol−1), G2-NS16 ($C_{184}H_{244}N_{24}Na_{16}O_{56}S_{16}Si_{13}$; M_w: 4934.0 g mol−1) and G3-Sh16 ($C_{256}H_{508}N_{48}Na_{16}O_{64}S_{16}Si_{29}$; M_w: 6978.4 g mol−1) were synthesized as previously reported [15,17]. 1 mM stock solution of dendrimers and subsequent dilutions to obtain µM concentrations were prepared in distilled water. The reagents used as controls for the inhibition of viral replication were the peptide HIV fusion inhibitor T-1249 (Trimeris, Inc., Morrisville, NC, USA), tenofovir (TFV; Gilead Sciences, Foster City, CA, USA), and raltegravir (RAL; Merck Sharp & Dohme Corp., Whitehouse Station, NJ, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA), and serial dilutions using distilled water were completed to obtain a final concentration of DMSO in cells not more than 0.1% to avoid toxic effects by the solvent. The level of DMSO was previously shown to have no effect on cell growth/toxicity [23].

b. Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy blood donors by a standard Ficoll-Hypaque density gradient (Rafer, Zaragoza, Spain) and cultured following the procedures of Spanish HIV-HGM BioBank [24,25]. When indicated, PBMCs were stimulated with 2 µg ml^{-1} phytohemagglutinin (PHA; Remel, Santa Fe, NM, USA) and 20 U ml^{-1} IL-2 (Bachem, Bubendorf, Switzerland) for at least 2 days before the experiments began. The protocol for maintaining the human epithelial TZM-bl and HeLa cell lines (National Institute of Health AIDS Research and Reference Reagent Program, NIH-ARRRP) has been described previously [26,27].

c. Virus stocks and titration

CCR5- and CXCR4-tropic primary HIV-2 clade A strains were isolated by co-cultivation of PBMCs from infected subjects with PHA-activated PBMCs from healthy individuals [28]. The 50% tissue culture infectious dose ($TCID_{50}$) of viruses was determined in a single round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells as described previously [29]. The recombinant vaccinia virus vSC50 encodes the full-length *env* gene from HIV-2SBL/ISY, an X4-tropic infectious molecular clone, which was cloned into the vaccinia (WR) TK gene. *Env* gene expression is under control of the vaccinia virus P7.5 promoter [30]. Virus stocks were titered using the Reed & Muench method [31] in Rat2 cells.

d. Cytotoxicity assay

The safety and toxicity profiles of G2-S16, G2-NS16 and G3-Sh16 in TZM-bl, HeLa, and PBMCs cells were determined by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazo- liumbromide; Sigma) cytotoxicity assay according to the manufacturer’s instructions. Medium alone and 10% DMSO were used as untreated and cell death controls, respectively. 10 µM dextran was used as a harmless control to check and to verify the status of cells.

e. Antiviral assay

TZM-bl cells were pretreated with serial dilutions of dendrimers for 1 h at 37 °C, the optimum time pre-exposure at which the dendrimers retain their ability to prevent vaginal HIV-2 transmission. Cells were then infected with 200 $TCID_{50}$ of virus-containing primary R5- or X4-HIV-2 isolates for 2 h at 37 °C. After 48 h, the cells were washed and lysed, and the luciferase activity was measured by using a luciferase assay system kit (Promega, Madrid, Spain) according to the manufacturer's instructions. From the dose–response curve, the 50% cytotoxic concentration (CC_{50}) and the half-maximal inhibitory concentration (IC_{50}) of dendrimers were determined for each strain. The therapeutic index (TI) was determined by the following equation: $TI = CC_{50}/IC_{50}$ [32].

f. Inhibition of HIV-2 attachment and entry to PBMCs

PHA-activated PBMCs were pretreated with dendrimers or controls for 1 h. Then, the primary HIV-2 isolates were added to PBMCs (the equivalent to 40 ng capsid protein per 106 cells) and incubated for 2 h at 4 °C. Unbound virus was removed by washing with phosphate-buffer saline (PBS; Lonza, Walkersville, MD, USA) three times, the cells were then lysed, and cell-bound viruses were quantified by the capsid p24 content in cell lysates. To measure internalization, the same conditions were used, except that PBMCs were incubated with the virus for 2 h at 37 °C, and washed in acid wash (50 mM glycine, pH 3.2; Sigma) to strip the surface-bound viral particles, and the cell-internalized viruses were quantified by the capsid p24 content in cell lysates.

g. HIV-2 inactivation

The primary R5- or X4-HIV-2 isolates (the equivalent to 10 ng of the capsid protein) were incubated at 4 °C overnight into wells of a 96-well flat-bottom plate with poly-L-lysine to ensure the adherence of the viral particles to the bottom of the well. Then, the wells were washed three times with PBS to remove unbound HIV-2 and treated with dendrimers or controls for 1 h. PHA-activated PBMCs were then added. After 72 h, the cells were washed and lysed, and the HIV-2 p24 Gag level in the cell lysates was quantified by using a HIVp24gag ELISA kit (Innogenetics, Ghent, Belgium). Cell viability was measured by the MTT assay.

h. Cell-to-cell fusion inhibition

Cell-to-cell fusion (CTC) inhibition was tested using a newly developed method with recombinant vaccinia virus expressing HIV-2 ISY *env* gene (vSC50). HeLa cells were transfected using jetPRIME® reagent (Polyplus-transfection SA, Illkirch, France) with the Tat expressing plasmid pcDNA3.1+/Tat101-flag following the manufacturer's instructions and infected with the recombinant vaccinia virus. After 3 h, the HeLa cells were collected and co-cultured with TZM-bl (CD4+, CCR5+, CXCR4+, indicator cells) at 1 : 1 cell density ratio in the absence or presence of increasing concentrations of dendrimers or controls. The percentage of membrane cell fusion was measured by luciferase activity induced by the Tat protein. The level of syncytium formation was determined by direct microscopic observation.

i. Combination analysis between dendrimers and antiretrovirals

TZM-bl cells were pretreated with different concentrations of dendrimers/antiretrovirals (ARVs) at a constant ratio for 1 h at 37 °C. The cells were then infected with the equivalent to 10 ng of the capsid protein of the primary R5-HIV-2 or X4-HIV-2 strains. 48 h post-infection, the cells were washed and lysed and HIV-2 replication was quantified by luciferase activity. The IC_{50} and synergism were determined by using CalcuSyn software (Biosoft, Cambridge, UK). IC_{50} was determined by using the median-effect plot and the dose-reduction index [33]. Combination indices (CIs) were calculated based on the median-effect principle, [34] where CI < 0.9 indicates a synergistic effect, 0.9 < CI < 1.1 indicates an additive effect, and CI > 1.1 indicates an antagonism effect. Briefly, based on characterizing compounds that have similar modes of action (early stages of HIV-2 lifecycle), CIs were calculated as follows:

$$\mathrm{CI} = \frac{(f_\mathrm{a}/(1-f_\mathrm{a}))_1}{(f_\mathrm{a}/(1-f_\mathrm{a}))_\mathrm{C}} + \frac{(f_\mathrm{a}/(1-f_\mathrm{a}))_2}{(f_\mathrm{a}/(1-f_\mathrm{a}))_\mathrm{C}} + \frac{(f_\mathrm{a}/(1-f_\mathrm{a}))_3}{(f_\mathrm{a}/(1-f_\mathrm{a}))_\mathrm{C}}$$

In this scheme, f_a is the fractional inhibition caused by a compound relative to a no-compound control, and the subscripts refer to the compound (1, 2 or 3) or combination (C) used.

j. Sperm processing and spermicidal activity

Semen samples were collected from healthy volunteers by masturbation at the laboratory after 2–5 days of sexual abstinence and allowed to liquefy for 30–45 min at room temperature [35]. When completely liquefied, G2-S16 was selected for evaluating the sperm motility at 24 h according to the previously described method [36]. Only specimens with at least a final motile sperm concentration of 5 × 106 mL^{-1} were used. These parameters were evaluated by using the Sperm Class Analyzer software (Microptic S.L., Barcelona, Spain).

k. Antimicrobial assays

G2-S16 was evaluated for its antimicrobial activity against *Candida albicans* ATCC 10231, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 00603, *Lactobacillus plantarum* ATCC 14917, *Pseudomonas aeruginosa* ATCC 28753 and *Staphylococcus aureus* ATCC 29213 microorganisms. The antimicrobial activity of G2-S16 was measured using a broth microdilution test and serial 1 : 2 dilutions according to the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines.

l. BALB/c mice vaginal irritation test

Six- to eight-week-old female BALB/c mice (Charles River, Barcelona, Spain) were housed in a specific-pathogen-free animal facility at Centro de Biología Molecular 'Severo Ochoa' for at least one week before the experiments were conducted. All mice were maintained and treated according to protocols approved by the Institutional Animal Care and Research Committee. BALB/c mice were injected subcutaneously with 2 mg of medroxyprogesterone acetate (Depo-Provera, Pfizer, New York, NY, USA) five days before treatment. To determine mice number per group, we relied on a careful review of the-state-of-the-art of the use of compounds *in vivo* against HIV. Therefore, to assess and conduct an easy, low-cost, and a reliable statistical comparison of irritation studies with minimal damage, mice were randomized into 3 groups (n =3 in

each group). G2-S16 powder was dissolved in sterile-PBS at the indicated concentration, to which HEC was added to reach the final concentration of 2.0% followed by continuous stirring for 45 min until a translucent gel was formed. Forty microliters of 2% hydroxyethylcellulose (HEC; Bohm Laboratories, Madrid, Spain) gel containing 3% G2-S16 was carefully applied to the vaginal vault of the mice using a stainless steel feeding needle (a ball point needle that does not produce friction when it is inserted into the vagina) for one day or two consecutive days. On day 7, mice were euthanized and vaginal tissues were excised and fixed in 4% formaldehyde solution (Panreac, Barcelona, Spain) for histology. We observed daily the external appearance of the vagina, and analyzed important aspects that would involve visible signs of damage such as difficulty in inoculation, vaginal contraction, spasms, redness or burning. Animal housing and maintenance protocols followed the guidelines of Council of Europe Convention ETS123, recently revised as indicated in the Directive 86/609/EEC. Animal experiments were performed in compliance with the protocols approved by the Institutional Animal Care and Use Committee of the CBMSO (Comité de Ética de Experimentación Animal del CBM; CEEA-CBM, Madrid, Spain).

m. Histological studies in BALB/c

Formalin-fixed excised vaginal tissues were submitted to the Anatomic Pathology Laboratory (Anapath, Granada, Spain) for embedding, sectioning and evaluation of the hematoxylin-and-eosin-stained tissue by an experienced pathologist. Sections were mounted on slides and were subjected to a blind evaluation for epithelial erosion, leukocyte infiltration, thickening of the lamina propria (edema), and vascular congestion. The inflammation scores were assigned by a semiquantitative system [37,38].

n. Statistical analysis

The data are presented as mean values and standard deviations (SD). The statistical significance between a treatment group and untreated control was calculated by unpaired t-test using GraphPad Prism v5.0 software (GraphPad, San Diego, CA, USA).

Results

a. Cell biocompatibility

The biocompatibility of G2-S16, G2-NS16 and G3-Sh16 was evaluated in TZM-bl, HeLa, and PBMCs. Cells were treated for 48 h with increasing concentrations of dendrimers, which were considered toxic when the survival rate was <80%. G2-NS16 and G3-Sh16 were considered non-toxic at 10 µM in PBMCs; G2-S16 was toxic at 50 µM. G2-NS16 and G2-S16 were non-toxic at 100 µM in TZM-bl and HeLa cells, whereas G3-Sh16 was non-toxic up to 50 µM (Figure 2). Therefore, the *in vitro* working concentration selected as non-toxic for all types of cells to facilitate a better comparison between compounds was of 10 µM.

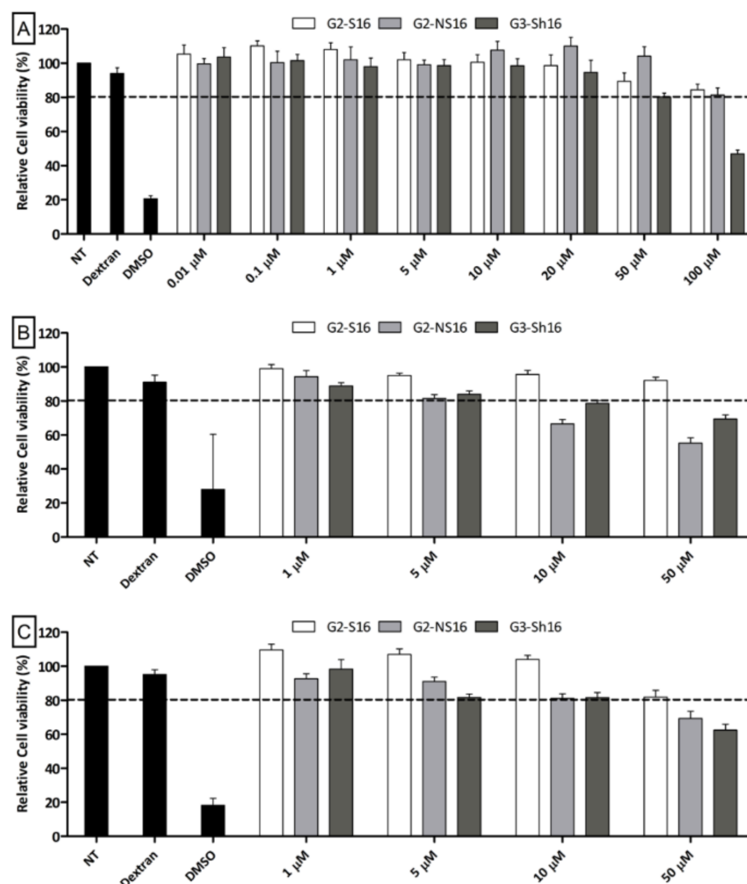


***Figure 2** – Cytotoxicity associated with polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16. **(A)** TZM-bl, **(B)** HeLa and **(C)** PBMC cells were loaded with increased amounts of dendrimers in a range between 0.01 and 100 µM; or treated with 10 µM dextran (innocuous control) or 10% of DMSO (control of cell death). Histograms represent the percentage of viable cells as the mean ± SD of at least three independent experiments performed in triplicate (vs. NT). Abbreviations: DMSO = dimethylsulfoxide; NT = non-treated (medium alone).*

b. Anti-HIV-2 activity

Although R5- and X4-HIV-1 viruses are present in body fluids (blood, semen, cervicovaginal and rectal secretions), R5 variants predominate in early stages during the process of sexual transmission, and persist throughout the course of HIV disease [39]. Moreover, late stage R5 HIV-1 variants show more rapid replication and higher cytopathicity relative to early stage R5 variants [40]. Previous studies have demonstrated a similar behavior of the viral coreceptor specificity during the course of HIV-2 infection [41,42]. Therefore, we analyzed the antiviral activity and the IC_{50} of G2-S16, G2-NS16 and G3-Sh16 against both early- and late-stage R5- and X4-HIV-2 isolates in TZM-bl cells.

The CC_{50} values were >100 µM for G2-S16 and G2-NS16 in TZM-bl cells. The CC50 for G3-Sh16 was 90 µM in TZM-bl cells (Table 1). The concentrations of G2-S16, at which R5- and X4-HIV-2 isolates were inhibited by 50% (IC_{50}), varied between 1.12 and 4.56 µM. The pre-treatment of TZM-bl cells with 10 µM G2-S16 resulted in 73–94% inhibition of infection by the primary R5- and X4-HIV-2 isolates (Figure 3A). Pretreatment of TZM-bl cells with G2-NS16 showed a dose

dependent inhibition of HIV-2 infection with IC_{50} values ranging from 0.73 to 1.07 µM (Table 1). G2-NS16 at 10 µM was the dendrimer with the best inhibition value against the primary R5- and X4-HIV-2 strains (>93%; Figure 3B). G3-Sh16 also showed dose dependent inhibition in HIV-2 infection with IC_{50} values ranging from 0.85 to 2.97 µM (Table 1). The maximum percentage of inhibition varied between 82–94% with 10 µM G3-Sh16 (Figure 3C). The TI of a compound is the ratio between the toxic and the therapeutic dose to measure its relative safety. Although TI values depend on many factors, it is generally considered that a drug has a good safety profile if its TI >10 [43]. The TI values of G2-S16 and G3-Sh16 were in a range of >21.9 to >89.3, and 43.5 to 105.9, respectively (Table 1). G2-NS16 showed the highest TI with values that varied between >93.5 and >137. In summary, the three dendrimers were highly active against HIV-2 infection in TZM-bl cells, G2-NS16 being the most potent and broad among them.

Table 1 – *In vitro* cytotoxicity and anti-HIV-2 activity of polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16 in TZM-bl cells

Dendrimer	**HIV-2**	**CC_{50} [a] (µM)**	**IC_{50} [b] (µM)**	**TI[c]**
G2-S16	**Early R5-HSM2.03**	>100	4.56	>21.9
	Late R5-HCC12.3		2.46	>40.7
	Early X4-CT03		1.12	>89.3
	Late X4-HCC10.3		3.72	>26.9
G2-NS16	**Early R5-HSM2.03**	>100	0.73	>137.0
	Late R5-HCC12.3		1.07	>93.5
	Early X4-CT03		0.81	>123.5
	Late X4-HCC10.3		0.88	>113.6
G3-Sh16	**Early R5-HSM2.03**	90	1.56	57.7
	Late R5-HCC12.3		2.07	43.5
	Early X4-CT03		0.85	105.9
	Late X4-HCC10.3		0.85	105.9

[a] CC_{50}: The cytotoxic concentration of the dendrimers that caused the reduction of viable cells by 50%. Data represent the mean ± SD of three independent experiments performed in triplicate. [b] IC_{50}: The concentration of the dendrimers that resulted in 50% inhibition in HIV-2 infection. Data represent the mean ± SD of three independent experiments performed in triplicate [c] TI: Therapeutic index is CC_{50}/ IC_{50}.

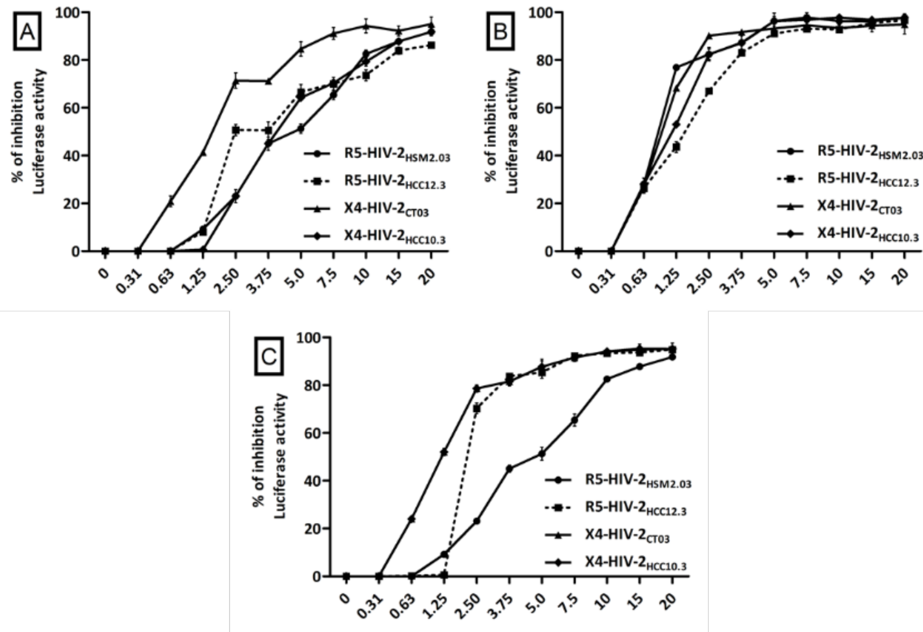


Figure 3 – *Anti-HIV-2 activity and dose–response curve obtained for polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16 in TZM-bl cells. TZM-bl cells were pretreated with increased concentrations of (A) G2-S16, (B) G2-NS16, and (C) G3-Sh16 for 1 h before HIV-2 infection (concentrations ranged from 0.31 to 20 µM). Equal amounts of virus-containing (2000 $TCID_{50}$) primary early/late R5- or X4-HIV-2 isolates were used. Luciferase activity was analyzed to 48 h post-infection vs. control non-treated cells. Data represent the mean ± SD of three independent experiments performed in triplicate.*

c. Virus-cell attachment and viral entry to the susceptible host cells

Viral entry is a process that involves the binding of a virus to the surface of a cell, fusion to the cell membrane, and internalization of the viral genome into the target cells. We evaluated whether G2-S16, G2-NS16 and G3-Sh16 are involved in HIV-2 binding or in entry steps in PHA-activated PBMCs. Virus attachment was measured at 4 °C, a temperature at which membrane fusion and endocytosis processes are ineffective. Virus internalization was evaluated by incubating the virus-cell mixture at 37 °C, a temperature that allows membrane fusion and viral entry. Treatment with 10 µM of G2-S16, G2-NS16 and G3-Sh16 significantly decreased the capacity of the primary X4-HIV-2 isolate to bind to PBMCs by 55%, 62%, and 52%, respectively ($p < 0.0001$; Figure 4A). This pattern was similar to that observed for the primary R5-HIV-2 particles (58–75% infection inhibition), but the reduction was not significant. As for the internalization of the viruses into PBMCs, albeit a reduction was observed for all dendrimers it was only significant for G2-S16 with X4-HIV-2 isolates (45% decrease at 10 µM; $p < 0.0001$; Figure 4B). In summary, the three dendrimers bound to target cells and perturbed the ability of the viral envelope to interact with its cell surface receptors. G2-NS16 was the dendrimer that best blocked the virus-cell binding processes.

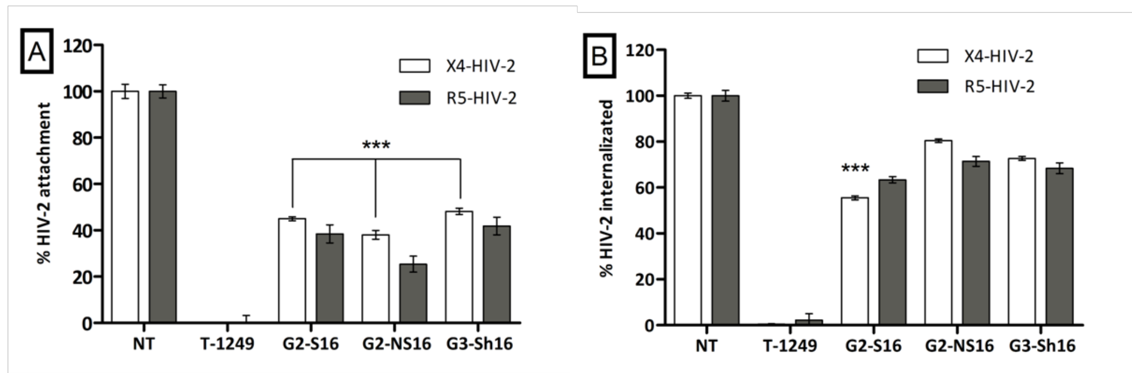


***Figure 4 –** Effect of polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16 on HIV-2 attachment and entry into PBMCs. PHA-activated PBMCs were pretreated with dendrimers (10 µM) or control (5 µM T-1249) for 1 h before infection with the primary R5- or X4- HIV-2 isolates. After infection for 2 h (A) at 4 °C (binding) or (B) at 37 °C (internalization), the cells were washed extensively (with glycine-acid washing for internalization assay) and lysed with 0.1% Triton X-100 buffer. Attachment and internalization levels were quantified by the measure of p24 in cell lysates by p24 ELISA. Results represent the mean ± SD of three independent experiments performed in triplicate. ***p < 0.0001 vs. untreated control. Abbreviations: PBMCs = peripheral blood mononuclear cells; PHA = phytohaemagglutinin.*

d. HIV-2 inactivation

The mechanism of inhibition of polyanionic carbosilane dendrimers against HIV-2 could also be associated with a direct viral inactivation, without forgetting the ability of dendrimers to block the gp120/CD4 interaction. Therefore, we evaluated the ability of G2-S16, G2-NS16 and G3-Sh16 to directly inactivate the primary HIV-2 isolates before contact with PHA-activated PBMCs. The pre-treatment of primary X4-HIV-2 with 10 µM of G2-S16, G2-NS16 or G3-Sh16 significantly decreased the infectivity of X4-HIV-2 after 1 h of exposure (82–91% reduction; p < 0.0001). This pattern was similar to that observed for the primary R5-HIV-2 (68–81% reduction), but the decrease was not significant (Figure 5). The cell viability was always over 80%. To summarize, the pre-treatment with the dendrimers for 1 h decreased the infectivity of the HIV-2 particles in a tropism-dependent manner, suggesting that the dendrimers act strongly on the virion. The best results of HIV-2 inactivation were obtained with the G2-NS16 dendrimer.

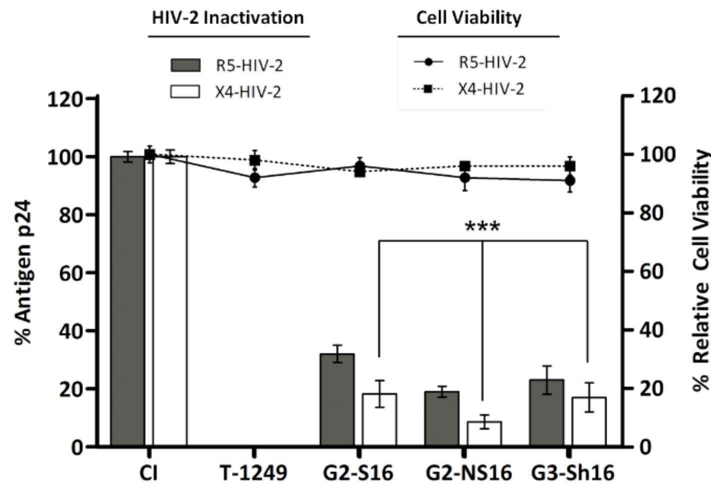
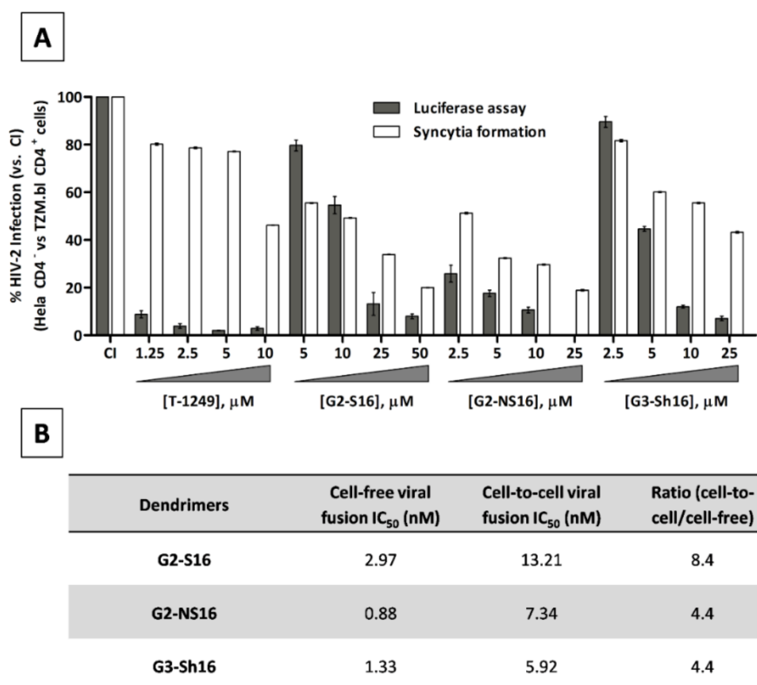


Figure 5 – *HIV-2 virus inactivation by polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16 prior to infection the PBMCs. The primary R5- and X4- HIV-2 isolates (grey and white bars, respectively) were stuck on poly-L-lysine coated plates and were treated with dendrimers (10 µM) or control (5 µM T-1249) 1 h before exposure to PHA-activated PBMCs. After 72 h, PBMCs supernatants were collected and p24 levels were measured by p24 capture ELISA. Cell viability was measured by the MTT assay (black solid and dashed lines). Data are represented as the mean ± SD of three independent experiments performed in triplicate. ***p < 0.0001 vs. untreated control. Abbreviations: CI = control of infection; PBMCs = peripheral blood mononuclear cells; PHA = phytohaemagglutinin.*

e. Inhibition of cell-to-cell fusion and syncytium formation

A Tat and luciferase-based fusion assay and a syncytium counting assay were developed and used to assess whether the dendrimers block cell-associated virus entry. In the presence of a HeLa/TZM-bl cell mixture, G2-S16, G2-NS16 and G3-Sh16 efficiently blocked fusion between both cell lines in a dose-dependent manner (Figure 6A). We also observed a significant decrease in the number of syncytia when the cells were pre-treated with the dendrimers, in particular for G2-NS16, which acts like the fusion inhibitor control T-1249 (Figure 6A). Inhibition of HIV-2 CTC fusion requires higher concentrations of each dendrimer than inhibition of HIV-2 cell-free infection. The IC_{50} of G2-S16 for CTC inhibition was 8.4-fold higher than the IC_{50} for cell-free virus infection. The IC_{50} of G2-NS16 and G3-Sh16 for CTC inhibition was 4.4-fold higher than the IC_{50} for cell-free virus infection (Figure 6B). In summary, our results indicate that the three dendrimers inhibit cell-associated HIV-2 infection in a dose-dependent manner, in particular G2-NS16, albeit at a higher concentration relative to the inhibition of cell-free infection.



Dendrimers	Cell-free viral fusion IC_{50} (nM)	Cell-to-cell viral fusion IC_{50} (nM)	Ratio (cell-to-cell/cell-free)
G2-S16	2.97	13.21	8.4
G2-NS16	0.88	7.34	4.4
G3-Sh16	1.33	5.92	4.4

Figure 6 – *Inhibition of Env/CD4-mediated membrane fusion. The plasmid Tat+ (pcDNA 3.1/Tat 101-flag) was transfected using the jetPRIME® transfection reagent into HeLa-CD4− cells. The cells were then infected with the rVVenv virus (rVV/ISY). After 3 h, TZM-bl (indicator cells) and HeLa (effector cells) were co-cultured and treated with dendrimers at a range of concentrations (2.5–50 µM) or T-1249 (1.25–10 µM) for 48 h. (A) Percentage of fusion was measured by luciferase activity vs. cell-to-cell fusion without treatment (NT) and under direct microscopic observation by quantification of the syncytium formation. Data represent the mean ± SD of three independent experiments performed in triplicate. (B) The half-maximal inhibitory concentration (IC_{50}) for cell-free and cell-to-cell viral fusion.*

f. Combination of dendrimers and antiretrovirals against HIV-2 infection

The anti-HIV-2 activity of triple drug combinations was assessed in a single cycle assay in TZM-bl cells. For these studies the dendrimers were combined with TFV, a nucleoside RT inhibitor, and RAL, an integrase inhibitor. The 1 : 1 : 1 fixed ratio was selected as the optimum ratio after a preliminary study of the combinations using different ratios. In G2-S16/TFV/RAL, the IC_{50} for G2-S16 decreased against the primary R5- and X4-HIV-2 isolates (IC_{50}: 3.0–60.0 nM) compared with dendrimer treatment alone. A similar result was also observed for the ARVs against the two primary HIV-2 infections (IC_{50}:60–310 nM for TFV; IC_{50}: 60 nM for RAL) compared with single-drug treatment (Figure 7A). CI was then calculated to determine whether synergistic, additive or antagonistic effects against all primary HIV-2 isolates occurred after this combination. CI calculations showed synergism at 75, 90, and 95% inhibition of R5-HIV-2 (CI: 0.38–0.53) and X4-HIV-2 infection (CI: 0.47–0.61) (Table 2). For G2-NS16/TFV/RAL, the IC_{50} for G2-NS16 dropped against the primary R5- and X4-HIV-2 isolates (IC_{50}: 50.0–60.0 nM) compared with the dendrimers used alone. Reductions were also observed in the IC_{50} for TFV (IC_{50}: 250–610 nM) and RAL (IC_{50}: 100–240 nM) against the primary R5-HIV-2 and X4-HIV-2 infection compared to the

drug treatment alone (Figure 7B). The average CI displayed stronger synergy at the calculated EC_{90}, and EC_{95} inhibitory concentrations against R5-HIV-2 infection (CI: 0.24–0.30). CI values indicated a good synergistic inhibitory profile at the three EC_{75}, EC_{90}, and EC_{95} concentrations against X4-HIV-2 infection (CI: 0.46–0.65) (Table 2). With G3-Sh16/TFV/RAL, a reduction in G3-Sh16 concentration was observed (IC_{50}:10–110 nM) against the primary R5- and X4-HIV-2 infection. The IC_{50} for TFV dropped against R5-and X4-HIV-2 infection (IC_{50}: 260–620 nM) compared with TFV alone; and for RAL decreased against R5- and X4-HIV-2 infection (IC_{50}: 100–360 nM) compared with the drug used alone (Figure 7C). CI determination showed synergistic interactions at the calculated EC_{75}, EC_{90}, and EC_{95} inhibitory concentrations against R5-HIV-2 infection (CI: 0.31–0.42). This combination exhibited synergism at 90%, and 95% inhibition of X4-HIV-2 (CI: 0.60–0.64) (Table 2). In summary, the strongest synergistic interactions and the highest inhibition of infection in TZM-bl cells were observed with G2-NS16/TFV/RAL, which is consistent with the most potent activity of G2-NS16 alone relative to the other dendrimers.

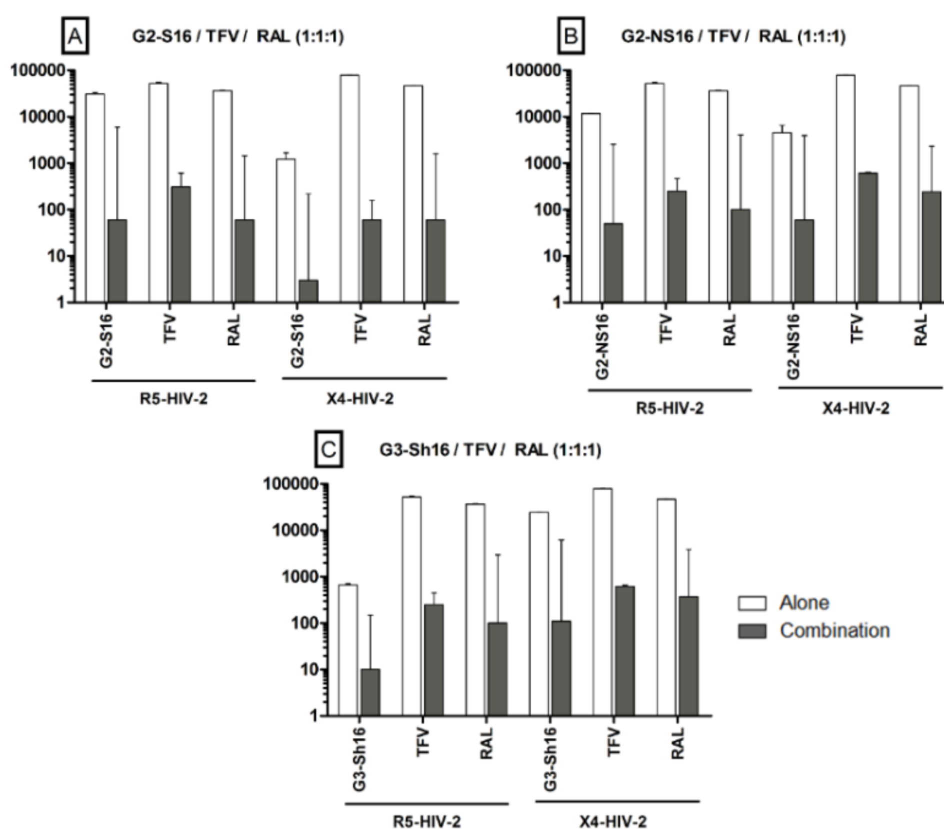


Figure 7 – *The half-maximal inhibitory concentration (IC_{50}) of polyanionic carbosilane dendrimers G2-S16, G2-NS16 and G3-Sh16 and antiretrovirals (tenofovir or raltegravir) alone and in combination in TZM-bl cells. Mean 50% effective concentrations (IC_{50}, nM) for (A) G2-S16/ TFV/RAL, (B) G2- NS16/TFV/RAL, and (C) G3-Sh16/TFV/RAL at an equimolar ratio (1 : 1 : 1) after infection with the primary R5- and X4-HIV-2 isolates in TZM-bl cells. The white bars indicate the mean IC_{50} calculated for the compounds when added alone, and the gray bars indicate the mean IC_{50} calculated for the compounds when used in combination. Data are shown as the mean ± SD from three independent experiments performed in duplicate. Abbreviations: TFV = tenofovir; RAL = raltegravir.*

Table 2 – Combination indices of three-drug combinations against HIV-2 infection in TZM-bl cells. Computer-simulated combination indices at 50, 75, 90 and 95% inhibition of primary R5- and X4-HIV-2 infection *in vitro* in TZM-bl cells. CIs are presented as the mean ± SD from the average of three experiments performed in triplicate.

Triple combination (combination ratios)	HIV-2	CI values at inhibition of: [a] 50%	75%	90%	95%	CI_{wt}-values[c]
G2-S16 + TFV + RAL (1 : 1 : 1)	R5	0.66 ± 0.04[b] +++	0.53 ± 0.01 +++	0.43 ± 0.01 +++	0.38 ± 0.02 +++	0.45 ± 0.02 +++
	X4	0.73 ± 0.05 ++	0.61 ± 0.01 +++	0.52 ± 0.03 +++	0.47 ± 0.05 +++	0.54 ± 0.03 +++
G2-NS16 + TFV + RAL (1 : 1 : 1)	R5	0.63 ± 0.09 +++	0.42 ± 0.02 +++	0.30 ± 0.01 ++++	0.24 ± 0.03 ++++	0.33 ± 0.04 +++
	X4	0.84 ± 0.02 ++	0.65 ± 0.05 +++	0.52 ± 0.08 +++	0.46 ± 0.09 +++	0.55 ± 0.06 +++
G3-Sh16 + TFV + RAL (1 : 1 : 1)	R5	0.51 ± 0.05 +++	0.42 ± 0.05 +++	0.35 ± 0.02 +++	0.31 ± 0.01 +++	0.36 ± 0.02 +++
	X4	0.84 ± 0.03 ++	0.73 ± 0.02 +++	0.64 ± 0.05 +++	0.60 ± 0.06 +++	0.66 ± 0.04 +++

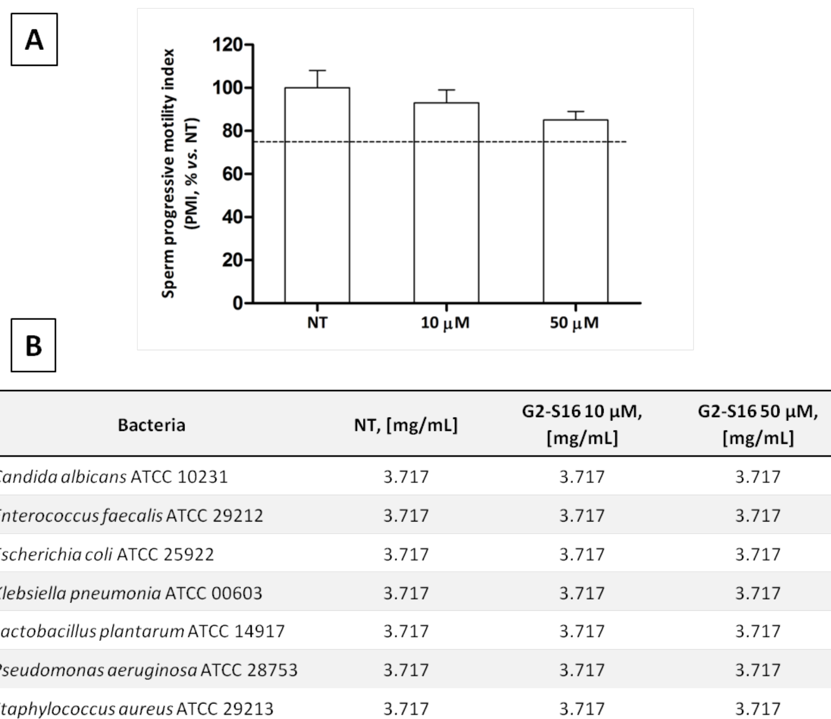
[a] CI > 1.1 indicates antagonism (−); 1.1 > CI > 0.9 indicates the additive effect (ad) and CI < 0.9 indicates a synergistic effect. [b] Synergy levels: 0.9 > CI > 0.85: + (sight synergism); 0.85 > CI > 0.7: ++ (moderate synergism); 0.7 > CI > 0.3: +++ (synergism); 0.3 > CI > 0.1: ++++ (strong synergism); CI < 0.1; +++++ (very strong synergism). [c] Because high degrees of effects are more important to the treatment than the low degrees of effects, the weighted average CI value was assigned as $CI_{wt} = [CI_{50} + 2CI_{75} + 3CI_{90} + 4CI_{95}]/10$, where CI_{50}, CI_{75}, CI_{90}, and CI_{95} are the CI values at 50, 75, 90, and 95% inhibition, respectively [33,34] Abbreviations: CI = combination index; RAL = raltegravir; TFV = tenofovir.

g. In vitro spermicidal activity

To identify whether the topical microbicide candidate G2-S16 is spermostatic or spermicidal, the sperm was cultured in the presence or absence of dendrimers and the progressive motility of the sperm was analyzed. No significant changes in sperm progressive motility of G2-S16 (at 10 and 50 µM) at 24 h post-treatment were obtained compared with untreated control (Figure 8A). This result indicates that G2-S16 can be considered safe to be used as topical vaginal microbicide because it did not alter the sperm motility and did not affect other sperm functions. Moreover, this finding is consistent with the results obtained with G2-NS16 and G3-Sh16 in previous studies [22].

h. Antimicrobial activity

The normal vaginal microbiota contains a wide variety of bacterial species that maintain an acidic pH by hydrogen peroxide and lactic acid production [44]. Alterations in this ecosystem can cause several vaginal infections, such as bacterial vaginosis and *Candida* vaginitis, which represent the majority of these infections among women [45]. A successful microbicide has to be stable and biocompatible with normal vaginal flora, preventing HIV-2 transmission in this highly complex microenvironment. Therefore, toxicity of G2-S16 against a composite population of bacteria observed in normal vaginal microbiota was analyzed. No antimicrobial activity of G2-S16 (at 10 and 50 µM) at 24 h post-treatment against the list of microorganisms cited in the Experimental section was observed (Figure 8B). In summary, our findings are consistent with the results obtained with G2-NS16 and G3-Sh16 in previous studies [22]. It suggests that G2-S16 is a good potential candidate for the first biological barrier encountered by the viruses because G2-S16 did not have negative effects on the normal vaginal flora.

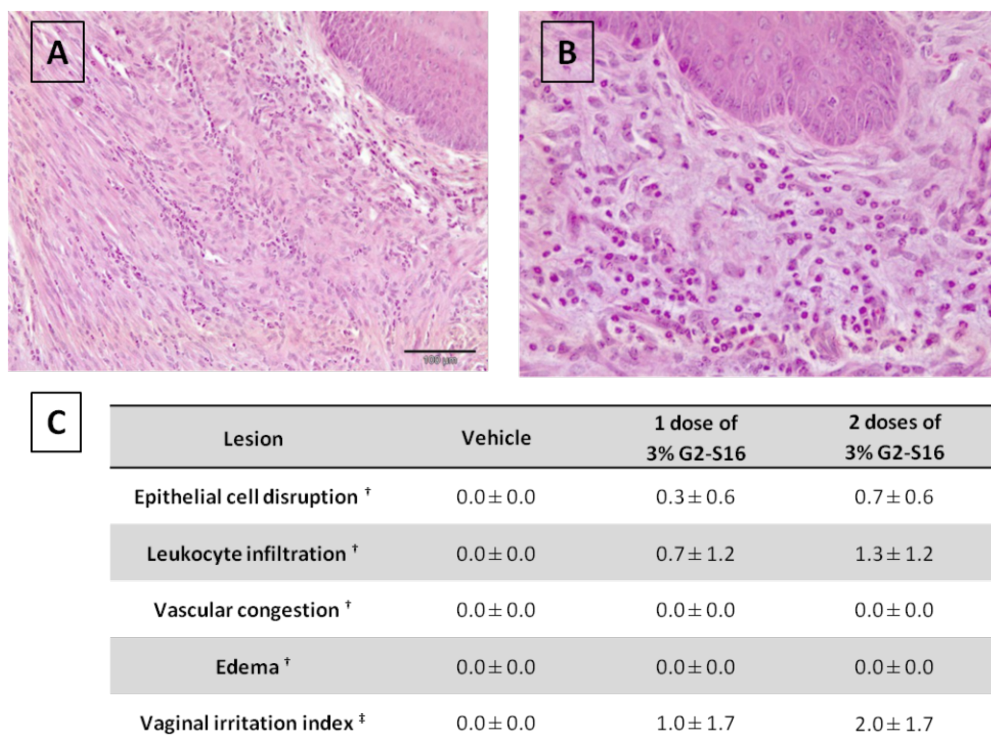


Bacteria	NT, [mg/mL]	G2-S16 10 μM, [mg/mL]	G2-S16 50 μM, [mg/mL]
Candida albicans ATCC 10231	3.717	3.717	3.717
Enterococcus faecalis ATCC 29212	3.717	3.717	3.717
Escherichia coli ATCC 25922	3.717	3.717	3.717
Klebsiella pneumonia ATCC 00603	3.717	3.717	3.717
Lactobacillus plantarum ATCC 14917	3.717	3.717	3.717
Pseudomonas aeruginosa ATCC 28753	3.717	3.717	3.717
Staphylococcus aureus ATCC 29213	3.717	3.717	3.717

Figure 8 – *Sperm survival index and antimicrobial activity after 24 h of treatment with G2-S16. (A) Sperm were cultured in seminal plasma, in the presence or absence of different concentrations of G2-S16 (10 and 50 μM). The sperm progressive motility was measured by using the Sperm Class Analyzer software. A calculated sperm motility index value <75% was used to indicate sperm toxicity. Data are represented as the mean ± SD of three different donors. (B) The determination of the minimum inhibitory concentration (antimicrobial susceptibility) of G2-S16 at 10 and 50 μM, defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation at 35 °C, is calculated by a modified Kirby–Bauer disk diffusion technique.*

i. In vivo *assay of G2-S16 in the BALB/c mice model*

To evaluate whether microbicide exposure of G2-S16 resulted in toxicity and inflammation of the vaginal mucosa, G2-S16 at 3% was applied intravaginally to BALB/c mice, and pathological examination of the vaginal tissues was performed at 7 days post-application. Histopathological examination indicated that the application of one dose or two doses of 3% of G2-S16 gel-treated BALB/c mice did not induce vaginal irritation or damage in the vaginal mucosa (Figure 9).



Lesion	Vehicle	1 dose of 3% G2-S16	2 doses of 3% G2-S16
Epithelial cell disruption †	0.0 ± 0.0	0.3 ± 0.6	0.7 ± 0.6
Leukocyte infiltration †	0.0 ± 0.0	0.7 ± 1.2	1.3 ± 1.2
Vascular congestion †	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Edema †	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Vaginal irritation index ‡	0.0 ± 0.0	1.0 ± 1.7	2.0 ± 1.7

Figure 9 – *Effects of 3% G2-S16 on the vaginal mucosa in the BALB/c mice model. Mice were inoculated (A) once or (B) twice for two consecutive days (with an interval of 24 h between sessions) intravaginally with 40 µl of 2% HEC containing G2-S16 at 3% (8 mM). The vaginal tract was harvested from the mice at 7 days post-application. Formalin-fixed, paraffin-embedded tissues sections were stained with hematoxylin-eosin for evidence of morphological damage or evidence of inflammation. HEC-treated mice were included as reference control (data not shown). Photographs are representative of all treated mice. Original magnification 100×. (C) Histopathological examination of vaginal tissues. Data were calculated as the mean ± SD of the scores estimated at the vaginas of three mice in each group. †Individual score: 0 = absence, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe irritation. ‡The cumulative score for epithelial disruption, submucosal leukocyte infiltration, edema and vascular congestion were correlated to human vaginal irritation potential as follows: vaginal irritation index ≤8: acceptable; 9–10: borderline; ≥11: unacceptable. The scoring system was established according to Eckstein et al. [37] and Zhong et al. [38] Abbreviations: HEC = hydroxyethylcellulose gel.*

Discussion

Sexual transmission is the main route of HIV spread throughout the world [46]. In the absence of a prophylactic anti-HIV vaccine, microbicides could offer to women a new strategy to prevent sexually transmitted HIV [47–49]. To identify alternative medicines that provide significant advantages compared to existing therapies, different mechanisms to inhibit the viral lifecycle before the integration should be considered, such as the process of receptor-mediated viral entry.

Dendrimers containing several types of functionalized groups at their periphery have shown effective anti-HIV activity as non-specific microbicides [18, 50, 51]. SPL7013 (the active product of VivaGel®), a fourth generation polylysine-based dendrimer with 32 napthylsulfonate groups at the periphery, is the only topical nanomicrobicide that has advanced to human clinical trials

for HIV-1/HSV-2 prophylaxis. However, VivaGel® provided low activity against R5-HIV-1 isolates and epithelial injury after 7–14 days of twice-daily administration and has been associated with an increased risk for HIV infection [52]. New water-soluble dendrimers with a carbosilane structure, which are characterized by the easy availability of reagents, short reaction times, high reproducibility and quantitative yields of reaction, have been synthesized [15–17]. These polyanionic carbosilane dendrimers are stable compounds and their microbicide capacity against HIV-1 has been previously reported [22,53]. However, the potential microbicide capacity against HIV-2 infection is still unknown. Here, the anti-HIV-2 activity of G2-S16, G2-NS16 and G3-Sh16 and the mechanism of action were determined.

It has been reported that in heterosexual HIV transmission, R5-tropic HIV variants dominate in the early stages of HIV disease [54], although X4-tropic HIV variants are also present in body fluids [55]. We compared the half cytotoxic concentration of G2-S16, G2-NS16 and G3-Sh16 when exposed to TZM-bl cells (CC_{50} >90 µM) with the concentration of these dendrimers at which R5- and X4-HIV-2 infectivity was inhibited by 50% (IC_{50}: 0.73–4.56 µM). Then, the therapeutic index was determined (TI: 21.9–>137) and used as an indicator of overall efficacy and safety. Despite the fact that TI was high (>10), G2-S16, G2-NS16 and G3-Sh16 are compounds that should be studied more thoroughly and carefully before passing to clinical trials. In the virucidal and HIV-2 inactivation assays, we observed the capacity to diminish the residual infectivity of HIV-2 particles after 1 h of incubation with the dendrimers. These results suggest that diverse mechanisms of HIV-2 inactivation may be involved. The dendrimers can (i) disrupt the integrity of the HIV-2 membrane by decreasing the stability of viral RNA [56], (ii) bind to the V3 loop [20,57], or (iii) interact and modify by denaturing the two disulfide bonds located on the HIV-2 gp120 protein (an area implicated in binding to the CD4 receptor) [58]. However, additional research is needed to define the mechanism of inactivation occurring. The observations previously obtained by molecular dynamics simulations support this idea because the mechanism of action is associated with electrostatic interactions between HIV gp120 and different functional groups of dendrimers [20]. However, the HIV-2 inactivation has not still been studied and further experiments by molecular modeling should be performed. The ability to prevent HIV-2 binding to PBMCs but not entry can be explained by the existence of other effective mechanisms of attachment besides the direct HIV-2 fusion with the CD4 cell surface (i.e., through galactosyl-ceramide, ICAM-1, LFA-1 or heparan sulfate) [59], which are beyond the mode of action of the dendrimers. Another possibility is that virus-cell fusion occurs with the endosomal membrane following the endocytic uptake of virus particles [60]. However, all alternatives of HIV-2 entry can probably coexist, which is the reason why G2-S16, G2-NS16 and G3-Sh16 inhibit binding but do not inhibit completely the internalization. Finally, the HIV-2 capsid protein can be altered by the dendrimers during the passage to the cell and this leads to problems in its disaggregation, most likely the p24 antigen enters the cell but RT, integrase and viral RNA do not. Consequently, HIV-2 particles are not infectious.

The ability of HIV to spread between cells determines its virulence, with direct HIV CTC up to thousand fold more potent and efficient than infection by cell-free virus particles [61,62]. It is known that CTC protects viruses from humoral immune responses and antiviral treatments, allowing the persistence of residual replication, and the establishment and maintenance of viral reservoirs. Previous studies have demonstrated that HIV-1 CTC is susceptible to ARVs and neutralizing antibodies [63–66]. Here, we have developed a simple and highly effective assay to assess the activity of dendrimers and other drugs in CTC promoted by the HIV-2 envelope. We

show that dendrimers can efficiently prevent cell HIV-2 CTC albeit at a higher concentration relative to cell-free infection. These results confirm that cell-to-cell fusion is more difficult to inhibit than cell-free HIV infection [61] and, more importantly, indicate that dendrimers can prevent cell-associated HIV-2 infection.

ARVs display great potential in the prevention of sexual HIV transmission. However, ARV-based microbicides could increase the risk of emergence of multidrug-resistant mutants, drug–drug interactions, systematic absorption and adverse side effects associated with high drug concentrations and life-long therapy [29,67,68]. Therefore, to prevent these problems the ideal HIV microbicide should combine different classes of antiretroviral drugs acting in different targets with compounds that act in a non-specific way [18]. Here, we have shown that dendrimer/TFV/RAL combinations at a fixed 1 : 1 : 1 ratio have significant synergistic interactions against the primary R5- and X4-HIV-2 isolates. Similar results have been previously obtained for HIV-1 using a combination of these dendrimers with TFV and/or maraviroc [69–71]. G2-NS16/TFV/RAL was the most potent combination regimen against HIV-2 isolates, which is consistent with the higher anti-HIV-2 potency of the G2-NS16 dendrimer when given alone relative to the other dendrimers. The advantage of these combinations, over time, is that dendrimers can act against X4/R5 viruses and strengthen their inhibitory activity acting at different stages of HIV-2 life-cycle. Interestingly, the different ARVs did not block the non-specific functionality of dendrimers, and dendrimers did not interfere in the antiviral activity of ARVs, as shown in previous studies [69–71].

The safety profile of a microbicide candidate should preserve its activity against HIV infection and other STDs (i.e., HSV-2) and be carefully evaluated before moving the candidate into clinical trials. It is also important to note that an algorithm focused on the prevention of HIV infection should address whether the lead topical microbicide candidates are spermicidal. There is some evidence of harm through genital lesions with the spermicide nonoxynol-9 when it is vaginally administered for preventing HIV and other STDs [72]. Previously we have shown that G2-NS16 and G3-Sh16 do not induce changes in sperm motility [22]. In this work, we studied the anti-sperm and antimicrobial activities of G2-S16. The sperm motility and the different bacteria present in normal vaginal flora showed very similar response patterns compared to untreated control, indicating that G2-S16 is not an antimicrobial and spermicidal compound. The results confirm that this class of dendrimers is not spermicidal.

Several polyanionic polymers as entry inhibitors (i.e., Ushercell, Carraguard or PRO2000) showed an increased risk of HIV-1 infection because of the disruption of the integrity of the mucosal epithelial surface [73]. In the present work, we studied the safety of 3% G2-S16 gel on topical mucosal site using the BALB/c mice model. This concentration was selected on the basis of published data on VivaGel®, a water-based vaginal product, and of preliminary drugs used as microbicides with concentrations on the same order of magnitude to permit a direct comparison between the different system designs [74–76]. We showed that G2-S16 displayed a good safety profile and did not cause alterations to the vaginal epithelium. For the topical application, HEC gel is spread all over the vaginal surface, and remains at the surface for a required length of time. The choice of an appropriate vehicle is very important for the release of drugs intravaginally because the mode of delivery affects the adherence. Liquid formulations are inappropriate for controlled drug release due to their short residence time in the vaginal cavity. HEC gel (semisolid preparation), which is characterized for its acceptability, feasibility and low cost, enables the active drug to act in the right site of activity, to distribute throughout the

vaginal surface, and to remain at the surfaces for an adequate period of time. The dendrimer establishes a film or a physical barrier to prevent the dissemination of infected cells from the local mucosa to the regional lymph nodes and acts against the infection once the virus has crossed the epithelial barrier [77–79].

Conclusions

To summarize, our studies reveal that G2-S16, G2-NS16 and G3-Sh16 are non-specific compounds that inhibit HIV-2 infection acting at different and early steps in the HIV-2 lifecycle. These dendrimers act directly on the virus, block HIV-2 replication at steps prior to the integration of proviral DNA into the infected host cell genome (binding and fusion/entry into target cells), block HIV-2 CTC and are effective at non-cytotoxic concentrations easily reachable in the mice model. The use of a combined therapy blocking HIV-2 infection at early steps in the HIV-2 lifecycle is highly effective to stop the infection over existing therapeutic approaches, as they may avoid virus entry into new target cells and accelerate the decay of the latent reservoirs for HIV. The delivery vehicle for the formulation of the dendrimers is important for a good distribution throughout the vagina or rectum. Therefore, novel routes of administration to those already known (topical gels, intravaginal rings or locally applied solid films and tablets) should be explored to improve the activity, acceptability and adherence of these dendrimers for the prevention of HIV-2 infection and other STDs that enhances HIV. It would be also interesting to test the anti-HIV-2 activity of the microbicide in the presence of semen and to study the hypersensitivity, photosensitivity and condom integrity in the presence of the microbicide. Finally, further studies including humanized mice to complement *in vitro* and *in vivo* findings should be considered avoiding high economic losses and time before reaching human clinical trials.

Our data suggest that these dendrimers are promising candidates for future microbicide clinical trials in the field for prevention of HIV-2 infection. The role of these parameters in G2-S16/TFV/RAL, G2-NS16/TFV/RAL and G3-Sh16/TFV/RAL, and other combination activities should be assessed in future studies.

Financial support

This work was (partially) funded by RD12/0017/0037, project as part of Acción Estratégica en Salud, Plan Nacional Investigación Científica, Desarrollo e Innovación Tecnológica 2008–2011 and cofinanced by Instituto de Salud Carlos III (ISCIII) (Subdirección General Evaluación) and Fondo Europeo Desarrollo Regional (FEDER), RETIC PT13/0010/0028, Fondo de Investigacion Sanitaria (FIS) (PI13/02016), CTQ2011-23245, CTQ-2014-54004-P (MINECO), “Fundación para la Investigación y la Prevención del Sida en España” (FIPSE), Comunidad de Madrid (grant numbers S-2010/BMD-2351 and S-2010/ BMD-2332], PENTA, CYTED 214RT0482. CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008–2011, Iniciativa Ingenio 2010, the Consolider Program, and CIBER Actions and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. Veronica Briz is supported by the Sara Borrel program (CD09/00433). Fundação para a Ciência e Tecnologia (FCT) (http://www.fct.pt), Portugal, (grant VIH/SAU/0029/2011). Ana Rita Diniz is supported by a PhD grant SFRH/BD/89140/2012 from Fundação para a Ciência e Tecnologia (FCT), Portugal.

Acknowledgements

The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: vSC50 from Dr Sekhar Chrakabarti and Dr Bernard Moss. Trimeris Inc. (USA) provided T-1249.

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CHAPTER 4
EVALUATION OF THE FUSION INHIBITOR P3 PEPTIDE AS A POTENTIAL MICROBICIDE TO PREVENT HIV TRANSMISSION IN WOMEN

Evaluation of the fusion inhibitor P3 peptide as a potential microbicide to prevent HIV transmission in women

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Research article

Manuscript submitted to PlosOne, February 2018

Abstract

Microbicides are an important strategy for preventing the sexual transmission of HIV but, so far, the most advanced tenofovir-based microbicides have had modest efficacy. This has been related to adherence problems and high prevalence of tenofovir-resistant HIV-1 strains. P3 is a new peptide with potent activity against HIV that may be a good microbicide candidate. In this work P3 was formulated in a gel of hydroxyethyl cellulose and its activity, stability and safety profile in BALB/c mice were evaluated.

HIV infection was fully blocked by a 1.5% gel containing P3 at the IC_{90} (366.4 nM) concentration. The antiviral activity did not change at 4°C during 4 months and at 25, 37 and 65°C for 1 week. P3 was stable and fully functional at acidic pH up to 24h, under different concentrations of hydrogen peroxide and in the presence of genital fluids up to 48h. P3 had no antibacterial activity and did not affect sperm motility and vitality. Finally, P3 did not cause significant alterations in the vaginal epithelium of BALB/c mice at 0.06 and 0.2 mg/day doses. These findings indicate that P3 is an excellent candidate for further development as a microbicide gel for the prevention of HIV transmission in women.

Introduction

At the end of 2015 almost 37 million people were living with HIV [1,2]. Sub-Saharan Africa encompasses the majority of the infected population (25.5 million people). Women, who acquire the virus mainly by heterosexual exposure, now account for approximately half of the infected population worldwide (17.8 million) and greater than 58% in sub-Saharan Africa.

New HIV infections have fallen by 38% since 2001 [1]. Nonetheless, an estimated 2.1 million people became newly infected with HIV in 2015. The control of HIV pandemic requires the development and use of safe and effective prevention methods. Condoms can be an effective barrier against HIV transmission but its use is unreliable and often not within the woman's control [3]. Oral pre-exposure prophylaxis (PrEP) with tenofovir (TDF) or tenofovir disoproxil/emtricitabine (TDF/FTC) can play an important role in HIV prevention in women [4,5]. Its effectiveness is highly dependent on adherence levels and, because of the TDF component, it can cause a decrease in spine and total hip bone mineral density [6] and a decline in renal function [7,8]. Moreover, the time from initiation of daily TDF/FTC to maximal protection against HIV infection is still unknown. Topical PrEP using microbicides is an additional strategy for preventing the transmission of HIV through sexual intercourse. Microbicides are products (rings, gels, films or inserts) used topically, either vaginally or rectally, to prevent entry and/or replication of HIV in the cells of those mucosae [9,10]. The ideal microbicide candidate for HIV prevention should observe the following requirements: be active on HIV-1 and HIV-2, easy to use and discrete, painless to the user, cost-effective, and safe and well tolerated for chronic administration [9,10]. It should allow self-administration with minimal interference in body function and daily life. Finally, it should provide long-standing protection and maintain its activity in the presence of vaginal fluid and semen. Most of the first generation microbicides, which include surfactants, polyanionic and acidifying agents, were abandoned due to safety issues and lack of efficacy [11-15]. The new generation of microbicides contain antiretroviral drugs (ARV) with potent activity against HIV, which are also, in most cases, used to treat HIV infection [12]. The CAPRISA 004 trial carried out on women from South Africa using a daily dose of 1% TDF vaginal gel reported a reduction of 39% in HIV acquisition; impressively, in high adherents (>80%) HIV acquisition was reduced by 54% [1]. However, in the VOICE study, a confirmatory study carried out with women from South Africa, Uganda and Zimbabwe the rate of HIV-1 acquisition was only reduced by 15%; this was associated with very low adherence [16]. Likewise, in the FACTS 001 trial which took place in South Africa the effectiveness of the 1% TDF gel was null when the entire population of trial participants was analyzed [17]. More recently in the ASPIRE study the risk of acquiring HIV was reduced up to 37% using a dapivirine-infused vaginal ring [18]. This phase 3 clinical trial was conducted in women from Malawi, South Africa, Uganda and Zimbabwe. Interestingly, while no protection was observed in women younger than 21 years which appeared to use the ring inconsistently, in women older than 21 years the dapivirine ring reduced the risk of HIV infection by 56%. Taken together, these results indicate that current microbicides confer partial protection from HIV in women from sub-Saharan Africa.

As recommended by the 2013 WHO guidelines, TDF is increasingly being used in first-line regimens with efavirenz and lamivudine or emtricitabine for the treatment of adult HIV-1 infection [19]. TDF treatment selects for TDF-resistant HIV-1 strains which already reach high proportions in low- and middle-income countries [20]. A TDF-based microbicide will likely be ineffec-

tive against such TDF-resistant strains. Moreover, its regular use may promote the selection and dissemination of TDF- resistant strains that will not respond to TDF-based therapeutic regimens [11]. Hence, one priority in this field is to find new microbicide candidates that interfere with the life cycle of HIV-1 and HIV-2 but are not based on antiretroviral drugs in current use. Recent studies indicate that entry inhibitors may be good microbicides as they effectively prevent vaginal HIV-1 transmission in humanized mice models [21].

We have recently described a new fusion inhibitor peptide named P3 based on ancestral sequences of the transmembrane glycoproteins of HIV-2 and SIV [22]. P3 forms a typical α-helix structure in solution, binds strongly to its target in the transmembrane glycoprotein and potently inhibits both HIV-1 and HIV-2 replication [22]. The high α-helical and reduced random coil contents of P3 are thought to determine its high target binding affinity, and predict that P3 should be more resistant to degradation by proteolytic cleavage in biologic fluids than other fusion inhibitor peptides [23]. Combined, these features make P3 an attractive microbicide candidate for HIV prevention. In the current study we evaluated the stability and antiviral function of P3 under different conditions of pH, temperature and oxidative stress, as well as its antiviral activity in a gel of hydroxyethyl cellulose (HEC). We also evaluated the safety profile of P3 in BALB/C mice.

Methods

Therapeutically active compound

P3 is a peptide with 34 amino acid residues and a molecular weight of 4,379.87 g/mol whose sequence overlaps the N-terminal pocket-binding region and core of the heptad repeat (HR) 2 region in the HIV/SIV transmembrane glycoprotein (P3 amino acid sequence: WQEWEQQVRYLEANISQRLEQAQIQQEKNMYELQ). P3 was derived from ancestral sequences of the transmembrane glycoproteins of HIV-2 and SIV and potently inhibits HIV-1 and HIV-2 replication (mean IC_{50} for HIV-1, 11.0 nM; mean IC_{50} for HIV-2, 63.8 nM) [22]. The P3 peptide was produced commercially by Genemed Synthesis (San Antonio, Texas, USA). It was modified with the N-terminus acetylated and the C-terminus as a carboxamide, the salt form being acetate. Reverse-phase high-pressure liquid chromatography (HPLC) was used for purification (>95%) and mass spectrometry for confirmation analysis.

Cell lines and virus

The cell lines, virus and culture protocols were described in supplementary information (Method S1).

Seminal plasma and vaginal fluid simulant

Semen samples were collected in a sterile container from seven HIV-1-seronegative healthy individuals by masturbation. The volunteers had no recent history of sexual transmitted infec-

tions, no urogenital abnormalities, and abstinence from sexual intercourse for 48h prior to collection. Vaginal fluid simulant (VFS) was prepared as described elsewhere [48]. The preparation of seminal plasma (SP) and VFS is described in supplementary information (Method S2).

SP and VFS cytotoxicity was assessed in TZM-bl cells. Cells were cultured in serial-fold dilutions of the biologic fluids and cellular viability was investigated using the alamarBlue assay [49].

Stability and antiviral activity of P3 in the presence of biologic fluids

The antiviral activity of P3 in the presence of SP and VFS was determined with a single-round viral infectivity assay using TZM-bl reporter cells, as previously described [50]. Briefly, TZM-bl cells were plated ($1x10^4$ cells/well) in a 96-plate and incubated overnight at 37ºC with 5% CO_2. In the following day, the IC_{90} concentration of the peptide (366.4 nM) was added to each well in a total volume of 100 µl of complete growth medium in the presence of either 0.5% or 1% (v/v) SP. The same procedure was done with VFS and with SP+VFS. Cells were then infected with 200 $TCID_{50}$ (in 100 µl) of HIV-1 isolate 93AOHDC250 and incubated for 48h before measuring the luciferase activity with the Pierce Firefly Luc One-Step Glow Assay Kit (ThermoFisher Scientific, Rockford, USA) according to the manufacturer's instructions. At least two independent experiments, each in triplicate, were performed for each antiviral activity analysis. For these experiments, the positive control was made of cells plus virus, the negative control was made of cells plus complete growth medium, compound controls were made of cells plus P3 alone or P3 in SP, VFS or SP+VPS, and the biological fluids control were made of cells plus SP, VFS or SP+VFS.

The long-term stability of P3 was evaluated in the presence of SP [0.5% or 1% (v/v)] or VFS at 37°C for a period of 24h, 48h and 1 week before performing the assay, as described above. In all cases, P3 was considered stable in the presence of biologic fluids if its antiviral activity was unaffected by SP, VFS and SP+VFS. In addition, peptide concentration was determined after exposure to the above conditions by mass spectrometry as described below.

Liquid chromatography and mass spectrometry (LC-MS/MS) analysis

Individual standard solutions (0.25-250 ppm) of P3 were prepared in acetonitrile (99.9% LC–MS) (J.T.Baker, Deventer, the Netherlands) in the presence of SP (0.5 or 1.0%), VFS or complete growth medium (GM) and analyzed after 24h, 48h and 1 week. In order to precipitate the proteins in the biological fluids, 1 ml of acetonitrile (1:1) was added to 1 ml of sample and samples were centrifuged at 13,000 rpm for 10 min and filtered (0.20 mm PVDF membrane; Chromafil®Xtra from Macherey-Nagel®) just before analysis. All samples were analyzed as triplicates. Experiments were performed to confirm that this procedure did not interfere with the concentration of the analyte in the standard solutions.

The characteristics of the Liquid chromatography and the Tandem mass spectrometry systems is reported in supplementary information (Method S3).

Effect of temperature, pH and oxidation in the stability and antiviral activity of P3

The stability and antiviral activity of P3 were evaluated at different conditions of temperature, pH and oxidation. Briefly, P3 solutions at concentrations corresponding to the IC_{50} and IC_{90} were prepared in GM and were incubated at 25, 37, and 65°C for a period of 1 week, at 37ºC for a period of a month and at 4°C for a period of 4 months. The antiviral activity of each peptide solution was then evaluated with a single-round viral infectivity assay using TZM-bl reporter cells and HIV-1 isolate 93AOHDC250, as described above.

The effect of pH on P3 was evaluated at pH ranging from 4 to 8. Briefly, IC_{50} peptide solutions were prepared in GM and the pH was adjusted using HCl (1M) or NaOH (1M). Each solution was then incubated at 37ºC for 2, 8 and 24 hours and their antiviral activity was determined in a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells as described above.

To test for oxidation by H_2O_2, P3 solutions at concentrations corresponding to the IC_{50} and IC_{90} were treated with H_2O_2 at biologic relevant concentrations (1.2µM and 5µM) [51]. The antiviral activity of the different solutions was determined in a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells, as described above.

Controls for these experiments were the following: positive controls (cells plus virus), negative controls (cells plus complete growth medium); pH compound controls (cells plus P3 in different pH growth medium); H_2O_2 compound controls (cells plus P3 in H_2O_2, 1.2µM or 5µM); cell controls were also performed under the same incubation conditions. H_2O_2 and pH cytotoxicity were assessed in TZM-bl cells using alamarBlue assay [49]. In all cases, P3 was considered stable when its antiviral activity was unaffected under the different conditions tested.

Hydroxyethyl cellulose P3 gel

Hydroxyethyl cellulose (HEC) powder (Ashland, Switzerland) was sterilized by dry heat at 150ºC for 24h. The sterility was confirmed by direct inoculation of HEC powder (0.1mg) in Sabouraud agar, blood agar and trypticase soy agar. Blood and trypticase soy agar plates were incubated 24h at 37ºC and Sabouraud agar plates 48h at 37ºC. Aqueous systems containing decreasing concentrations (%) of HEC (10, 7.5, 5, 2.5, 1.5, 1, 0.5, 0.25 and 0.05) were prepared to assess the HEC-gel cytotoxicity in TZM-bl cells using the alamarBlue assay [49].

P3 (366.4 nM corresponding to the IC_{90}) was formulated in 1.5% HEC-gel with 20% X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). This concentration of HEC was chosen based in the results of the cytotoxicity assay and the feasibility of the *in vitro* antiviral assay.

The antiviral activity of this gel (designated P3/X-Gal HEC-gel) was evaluated using a single-round infectivity assay in TZM-bl reporter cells (Method S4). TZM-bl cell line has an integrated copy of the β-galactosidase gene under control of the HIV-1 promoter enabling simple and qualitative analysis of HIV infection using β-galactosidase as a reporter gene. When HIV infects TZM-bl cells, β-galactosidase gene is expressed and the X-gal present in the medium is hydrolyzed by the β-galactosidase enzyme producing an intensely blue product (5,5'-dibromo-4,4'-dichloro-indigo) [52]. The cells were observed directly in an inverted microscope

coupled with a Leica DFC490 camera at 100X magnification. At least two independent experiments were performed, each in triplicate wells. Controls for these experiments were the following: positive control (cells + X-Gal HEC-gel + virus); negative control (cells + X-Gal HEC-gel); compound control (cells + P3/X-Gal HEC-gel); all of the above controls without X-Gal.

Spermicidal activity

Sperm was obtained from normal donors (n=3) after signing a written informed consent. The sperm was incubated at 37°C with a 114 µM P3 solution in water (40%) and PBS (60%) (~300-fold higher concentration than the IC_{90}) and sperm motility and viability were analyzed at various time intervals (0, 30, 60, 120 and 240 min). Spermatozoa viability was evaluated by dye exclusion method using a solution of Eosin Y (5mg/mL). Two hundred spermatozoa were counted with a phase-contrast microscope (Olympus, Modell BH-2), differentiating the live (unstained) spermatozoa from the dead (stained) cells. Sperm motility was evaluated in a Makler chamber using a computer-aided sperm analysis system (CASA, Hamilton Thorne Research, MA, USA). At least five microscopic fields were assessed in a systematic way to classify 200 spermatozoa motility. Each spermatozoon is graded according to cell velocity: progressive motility (i.e.,>5 µm/s), non-progressive motility (<5 µm/s) and immotility [53].

Anti-bacterial activity

Anti-bacterial activity of P3 was determined according to CLSI guidelines [54]. The Minimum Inhibitory Concentration (MIC) was determined by the agar diffusion method, in plates of Mueller-Hinton agar or Rogosa agar (for Lactobacilli) [55]. The bacteria used in this experiment and MIC determination protocol are described in supplementary information (Methods S4). The maximum concentration of P3 tested was 228.4 µM (~600-fold higher concentration than the IC_{90}).

BALB/c mice vaginal irritation test

Female BALB/c mice (6-8 weeks-old) (Charles River Laboratories, Wilmington, MA) were pretreated subcutaneously with 2mg of medroxyprogesterone acetate (Depo-Provera; Pfizer, NY, USA) in PBS (Lonza, Verviers, Belgium). After five days, 30µl of test formulation with different doses of P3 diluted in PBS was applied intravaginally daily for 7 days by vaginal gavage to animals previously anesthetized with isoflurane (Forane, Abbott, Madrid). Controls included mice that received PBS (placebo group) and 3% nonoxynol-9 (N9) in PBS (irritation control group). On the eighth day, mice were sacrificed and genital tract tissue were extracted and fixed in 4% paraformaldehyde for histological analysis. The animals were distributed in groups of three mice each: group placebo only treated with PBS (1-3), group A treated with dose of 0.06mg/day of P3 in PBS (4-6), group B treated with dose of 0.2mg/day of P3 in PBS (7-9), group C treated with dose of 0.4mg/day of P3 in PBS (10-12) and group irritation control treated with 3% N9 in PBS (13-15).

Histological studies in BALB/c

The presence of histological lesions in mice vaginas was evaluated with hematoxylin-eosin staining. The staining protocol is described in supplementary information (Method S5).

The existence of injury in vaginal epithelium, inflammatory infiltrate, vascular congestion and/or edema in the submucosa was evaluated in each biological sample. The values (score) assigned for each of these lesions are described in supplementary information (Method S5).

Statistical analysis

Results of stability studies were analyzed by one-way ANOVA (Friedman test) with a post-test of multiple comparisons (Dunn´s test). Sperm motility and viability statistical data analysis was made using the one-way ANOVA with a post-test of multiple comparisons (Sidak´s correction) between the drug vehicle group (control) and the P3 group, for each incubation time (significance at $p<0.05$).

Ethic statement

The study was approved by the ethics committees of the participating institutions. The methods were performed in accordance with the relevant guidelines and regulations. All experiments involving human participants were performed according to the guidelines and protocols approved by the Ethics Committee of Faculdade de Farmácia da Universidade de Lisboa. All animal experiments were performed according to the guidelines and protocols approved by the Ethics Committee for Animal Experimentation of Comunidad Autónoma de Madrid, CAM, Spain. Oral informed consent was obtained from all subjects enrolled in this study.

RESULTS

Antiviral activity of P3 is unaffected in the presence of seminal plasma and vaginal fluid simulant

The anti-HIV activity of a microbicide candidate should be tested in the presence of the biological fluids encountered during the sexual transmission event [24]. Thus, the antiviral activity of P3 was evaluated in the presence of seminal plasma (SP), vaginal fluid simulant (VFS) and the combination of both. We first confirmed that SP and VFS were not cytotoxic for TZM-bl cells at the concentrations used in these assays (0.5 and 1.0%) (Supplementary Fig. S1). The antiviral activity of P3 was unaffected by the human fluids up to 1 week of contact (Figure 1). The only exception was SP 1% that caused a significant decrease of antiviral activity at 1 week. Consistent with the virological results, P3 was readily detectable in the presence of VFS by mass spectrometry. However, there was a decrease to undetectable levels of P3 [below the limit of quantification of the method which is 2.5μg/mL (2.5 ppm)] after one week in the presence of 0.5% of SP and after 48h in the presence of 1% SP (Figure 1C). This indicates that P3 is degraded to some extent in the presence of SP and explains the significantly lower antiviral activity of P3

after one week with SP. Overall these results indicate that P3 maintain full antiviral activity in the presence of human biological fluids during at least 48h.

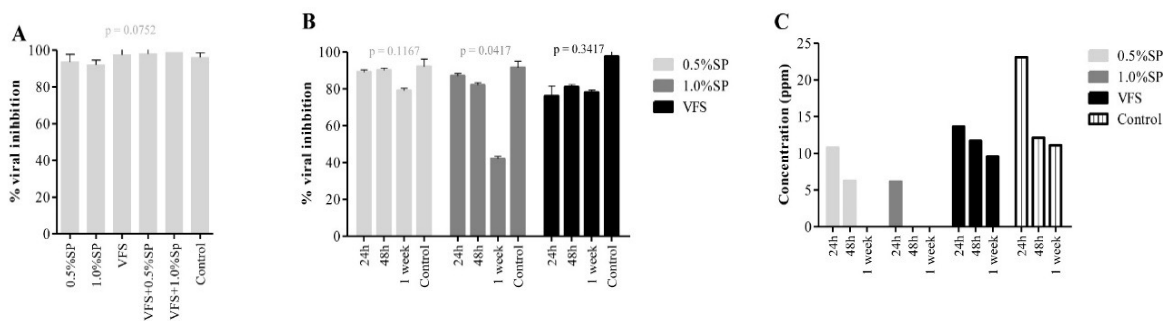

__Figure 1 –__ Evaluation of P3 anti-HIV1 activity in the presence of genital fluids: (A) SP, VFS and the combination of SP and VFS, (B) SP and VFS in different time points. (C) Effect of SP and VFS on P3 as determined by mass spectrometry. Solutions of P3 (366.4nM) were prepared in complete growth medium in the presence of 0.5 or 1% (v/v) SP, VFS, VFS with 0.5 or 1% (v/v) SP. The antiviral activity of each solution was evaluated with a single-round viral infectivity assay using TZM-bl reporter cells and HIV-1 primary isolate 93AOHDC250 in the presence of growth medium alone, SP and VFS to define the effects of biologic fluids on antiviral activity.

Antiviral activity of P3 is preserved at stringent temperature, pH and oxidative stress conditions

The P3 peptide must remain stable at different temperatures during the manufacturing process of the microbicide, during the shelf-life of the compound and in the human body [25]. Since the microbicide will be applied vaginally P3 must be stable in the acidic environment of the healthy vagina (pH 3.5–4.5) [26] and in the near neutral pH environment after ejaculation [27]. Finally, it should not be oxidized by the H_2O_2 present in the vaginal lumen [28,29]. To test for thermal stability, P3 concentrations corresponding to the IC_{50} (11nM) and IC_{90} (366.4nM) were incubated at 25°C, 37°C and 65°C for a period of one week, at 37ºC for a month and at 4°C during 4 months and then its antiviral activity was determined and compared to the control in which antiviral activity was determined after 48h at 37°C. Similar mean viral inhibition levels were obtained after a week of incubation at 25°C (IC_{50}, 57.5% vs control 43.5% p=0.3338 and IC_{90}, 99% vs control 99% p=0.5, Friedman Test) and at 37°C (59.5% vs 43.5% p=0.3366 and 99% vs 99% p=0.5) (Figure 2A). Although not statistically significant, antiviral activity of P3 increased at 65°C (99.5% vs 43.5% p=0.3301 and 100% vs 99% p=0.5). P3 stored at 37ºC for a month and stored at 4°C during four months maintained full anti-HIV-1 activity (Figure 2B and 2C). Together, these results highlight the very high thermal stability of P3.

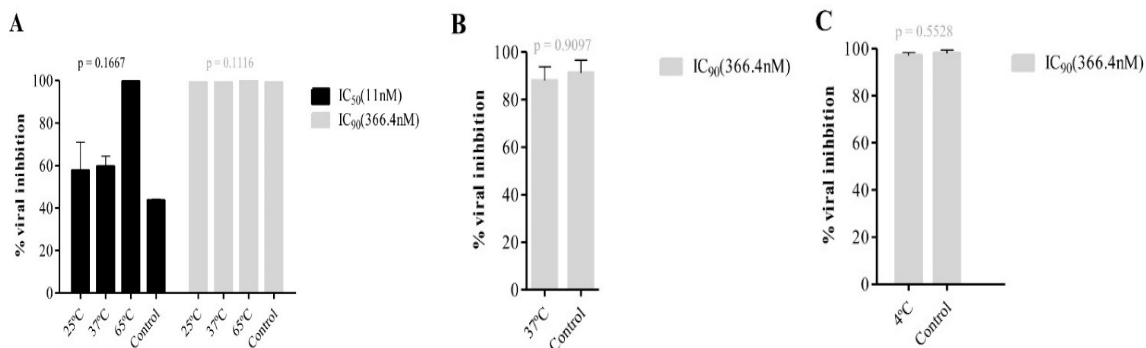

Figure 2 – *Biological stability of P3 at different temperatures. The stability of P3 solutions (11 and/or 366.4nM) in growth medium were evaluated in a biological assay as a function of temperature. Thermal degradation studies were conducted at 25, 37, and 65°C for a period of 1 week (A), 37ºC for a period of a month (B) and a 4ºC for a period of 4 months (C). The antiviral activity of each solution was evaluated with a single-round viral infectivity assay using TZM-bl reporter cells and HIV-1 primary isolate 93AOHDC250.*

Next we accessed the stability of P3 at different pH (4, 6, 8) and for different periods of time (2h, 8h and 24h). For these experiments a concentration equivalent to the IC_{50} was used. TZM-bl cells cytotoxicity was first analyzed. Basic P3 solution (pH=8) did not affect the cells; acidic solutions (pH=4 and 6) affected only minimally cell viability (Supplementary Fig. S2). For each pH condition tested, P3 always inhibited around 50% viral replication indicating that the pH did not significantly affect its antiviral activity (after 2h incubation at pH=4, mean=56.67±9.504%; pH=6, 51.33±11.68%; pH=8, 43.67±19.66%; pH=7.5 GM (control), 42.33±15.31%; p=0.3004; after 8h incubation- pH4, 62.67%±0.5774; pH6, 46.33%±6.658; pH8, 36.33%±12.420; pH7.5 GM (control), 40.67%±17.01; p=0.1476; 24h- pH4, 70.67%±4.509; pH6, 46.00%±5.000; pH8, 49.67% ±13.650; pH7.7 GM (control), 58.33%±1.155; p=0.0510) (Figure 3). P3 activity seemed to be potentiated at pH=4 after 24h (Figure 3B) but no significant differences were found when compared with the other pH conditions and with the control (P3 in GM, pH=7.5). Finally, P3 was stable and maintained its antiviral activity in the presence of biological concentrations of H_2O_2 (1.2µM and 5µM) (Figure 4). H_2O_2 at these concentrations did not affect the viability of TZM-bl cells (Supplementary Fig. S3). Taken together, the high stability of P3 at different temperature, pH and oxidative conditions indicate that it is an ideal candidate for a vaginal microbicide.

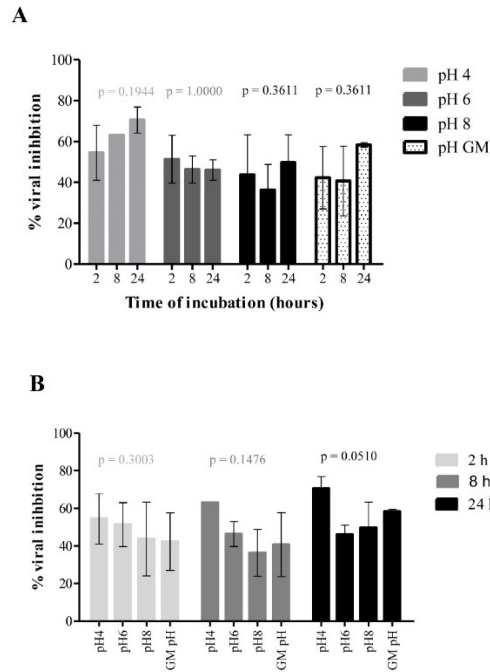

Figure 3 – *Biological stability of P3 at different pH conditions. 11nM solutions of P3 (corresponding to the IC_{50}) in growth medium (GM) at pH 4, 6 and 8 were incubated at 37ºC for 2, 8, 24 hours. The antiviral activity of each solution was evaluated with a single-round viral infectivity assay using TZM-bl reporter cells and HIV-1 primary isolate 93AOHDC250.*

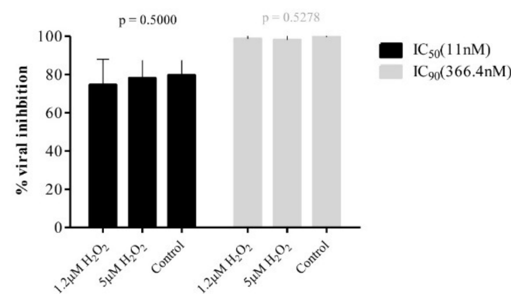

Figure 4 – *Antiviral activity of P3 in the presence of H_2O_2. Solutions of P3 (11 and 366.4nM) were prepared in complete growth medium in the presence of 1.2μM of H_2O_2 and 5μM of H_2O_2. The antiviral activity of each solution was evaluated with a single-round viral infectivity assay using TZM-bl reporter cells and HIV-1 primary isolate 93AOHDC250.*

P3 exerts full antiviral activity in the context of the HEC-gel

As the aim of the research was to develop a P3-based HEC gel, it was important to assess whether the HEC-gel interferes with the stability and antiviral activity of P3. We first determined that HEC-gel at concentration ≤1.5% was not cytotoxic to TZM-bl cells (Supplementary Fig. S4). We therefore formulated P3 peptide at 366.4 nM concentration (equivalent to the IC_{90})

in 1.5% HEC-gel. This gel showed full activity against HIV-1 thereby confirming that the P3 peptide is effectively delivered in the HEC-gel formulation (Figure 5).

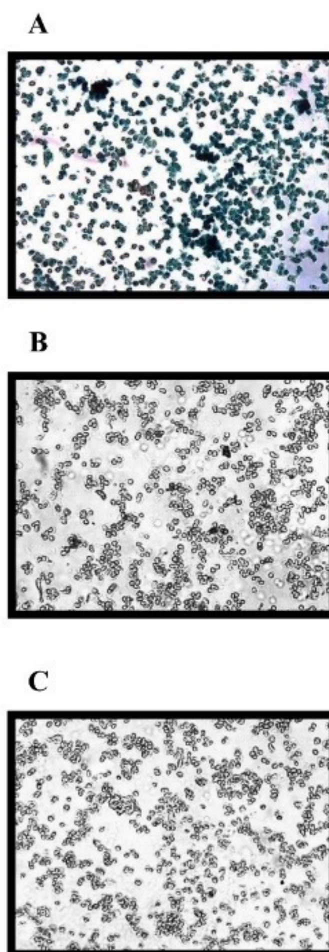

Figure 5 – *Antiviral activity of P3 formulated in 1.5% HEC-gel in TZM-bl cells. (A) 1.5% HEC-gel + X-Gal + HIV-1 (positive control); (B) 1.5% of HEC-gel + X-Gal (negative control); (C) 1.5% HEC-gel + P3 (366.4 nM) + HIV-1 + X-Gal. HIV infected cells produce β-galactosidase which converts X-Gal into 5,5'-dibromo-4,4'-dichloro-indigo, an intensely blue product which is insoluble. The cells were observed in an inverted microscope coupled with Leica DFC490 camera at 100X magnification.*

P3 lacks spermicidal activity

As a vaginal microbicide P3 will be in contact with semen and it is crucial that it does not alter the sperm characteristics. To assess the effect of P3 peptide in sperm cells, the semen was cultured in the presence of 114 µM of P3 (~300-fold higher than the IC_{90}) and the progressive motility and vitality of the sperm cells was analyzed at 0, 30, 60, 120 and 240 min post-treatment. No significant changes were found in spermatozoa motility and vitality (Figure 6) when compared with control without P3. These findings indicate that P3 lacks spermicidal activity and could be safely used as a vaginal microbicide.

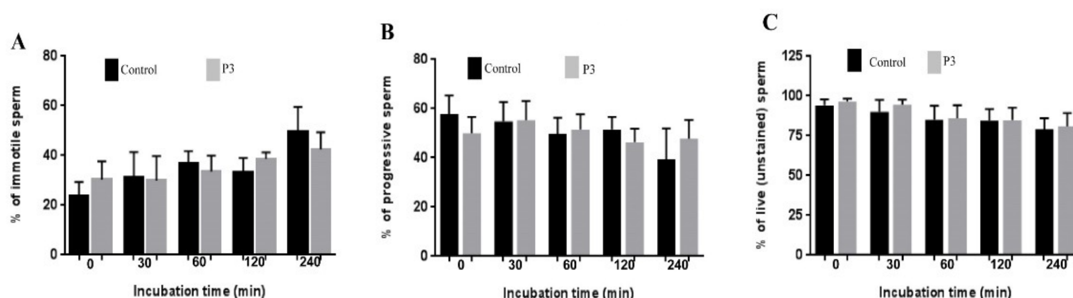

Figure 6 – *Effect of P3 in human spermatozoa motility and viability. Motility and vitality were assessed at different time intervals (0, 30, 60, 120 and 240 min). (A) Percentage of immotile sperm in the presence or absence (control) of P3; (B) Percentage of progressive sperm in the presence or absence (control) of P3; (C) Percentage of live sperm in the presence or absence (control) of P3. No differences (p>0.05) were observed between control group (drug vehicle) and P3 treated group, for each incubation time.*

P3 lacks anti-bacterial activity

Lactobacillus species and other bacterial species that are present in a healthy vagina crucially contribute to maintain the 3.5 to 4.5 acidic pH and produce several antiviral and antimicrobial substances that inhibit pathogenic organisms [30]. Alterations of normal vaginal microbiota may lead to several vaginal infections [31] and affect the risk for vaginal HIV transmission [24,32]. Hence, P3 must not interfere with the normal vaginal microbiota. The effect of P3 on vaginal bacteria was investigated using different species of bacteria including two common clinical species of lactobacillus, *Lactobacillus rhamnosus* and *Lactobacillus plantarum* (Table 1). P3 did not affect bacteria growth up to 228.4 µM concentration (~600-fold higher than the IC_{90}) (Table 1). These findings suggest that P3 will not affect the normal vaginal microbiota.

Table 1 – Activity of the P3 peptide against Gram positive and Gram negative bacteria

Bacteria	P3 (MIC)
Bacillus subtilis ATCC 6633	> 228.4µM
E. coli ATCC 10536	> 228.4µM
E. coli (clinical strain)	> 228.4µM
Enterococcus faecalis ATCC 29212	> 228.4µM
Lactobacillus plantarum	> 228.4µM
Lactobacillus rhamnosus	> 228.4µM
Pseudomonas aeruginosa (clinical strain)	> 228.4µM
Staphylococcus aureus ATCC 6538	> 228.4µM

MIC- Minimum Inhibitory Concentration at 228.4µM which is ~600-fold higher than the IC_{90}

P3 does not significantly irritate or damage the vaginal mucosa

To see if exposure to P3 induced alterations in the vaginal mucosa, histological evaluation of lesions found in the vaginal wall of 15 BALB-c mice treated with PBS, with P3 at doses of 0.06 mg, 0.4 mg and 0.6 mg (in 30µl) in PBS and with 3% N9 in PBS daily for 7 consecutive days, was done. In the placebo group treated with PBS, no significant histological lesions were observed in any of the three samples tested (Supplementary Fig. S5 A-D). The epithelium was composed of several layers of cells, being the last one, a layer of mucous cells that was present in the proestrus phase of the estrous cycle. No inflammatory infiltrate or vascular congestion was found in any layer of the wall.

In mice treated with 3% N9 considered as mucosal irritation control group (Supplementary Fig. S5 E-H), no epithelial mucosal cell layer was found, because it had been replaced by a stratified epithelium such as the one present in estrus. Hyperplasia of the epithelium was observed, with a higher number of cell layers than normal condition, with evidence of papillary formations into the submucosa (Supplementary Fig. S5 E-H). There was also an inflammatory infiltrate constituted mainly by polymorphonuclear neutrophils between epithelial cells which sometimes formed microabscesses (Supplementary Fig.S5 H). Focal ulceration also appeared in some areas of the tissue. This inflammatory infiltrate had spread to the submucosa with a moderate degree of severity. In this submucosa, light congestion and vasodilation were also observed (Supplementary Fig. S5 E-H). The total score obtained in these three samples indicate the presence of an average irritation of the vaginal wall (Table 2).

In group A treated with 0.06 mg/day of the compound, injuries were minimal or even absent (mouse 6), histological alterations were not showed (Supplementary Fig. S6). As in the case of the samples treated only with PBS, mucosal epithelial cells were maintained and only minimal epithelial hyperplasia without presence of inflammatory cells appeared. In sample 4, scarce congestion and vasodilatation were observed and the sample 5 showed a minimal infiltration of neutrophils in the submucosa.

In group B (Supplementary Fig. S7) treated with 0.2 mg/day, lesions were less obvious than in mice treated with doses of 0.4 mg/day. Only in sample 7 appeared minimal inflammatory infiltrate in the epithelium, like in the beginning of the metaestrus at the estrous cycle. This inflammation was extended into the submucosa but vascular congestion and vasodilation were minimal in this layer (Supplementary Fig. S7 A-B). In sample 9, the inflammatory infiltrate and congestion were light (Supplementary Fig. S7 E-F). In this group the irritation of the vaginal wall was minimal.

Finally, in group C treated with 0.4 mg/day of P3 peptide, two of the samples (mice 10 and 12) showed the most obvious lesions of the groups studied. The epithelium kept mucosal cells, but many neutrophils were observed inducing microabscesses. In addition, hyperplasia remained and numerous apoptotic cells appeared. The injuries were severe. In the submucosa, an inflammatory infiltrate consistent of neutrophils was found and a moderate level of vasodilation and congestion occurred (Supplementary Fig. S8 A-B, E-F). Score of vaginal irritation was moderate in both cases. However, sample 11 had only a slight epithelial hyperplasia and minimal vasodilatation in the submucosa (Supplementary Fig. S8 C-D) (Table 2). Overall, the most severe injuries were found in two mice belonging to the group treated with 0.4 mg/day, with moderate vaginal irritation. However, in another mouse of that same group, the irritation of the vagi-

nal epithelium was minimal. The samples belonging to mice treated with 3% N9 showed an average degree of vaginal irritation. In the rest of mice, treated groups and placebo group, mild histological lesions were observed in their samples. Taken together these results indicate that P3 could be safely administered as a microbicide at a dose up to 0.2 mg/day.

Table 2 – Histological score after intravaginal administration of compounds in different groups of female BALB/c mice during 7 consecutive days.

	PBS			0.06 mg/day P3			0.2 mg/day P3			0.4 mg/day P3			3% N9 P3		
Mouse ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Epithelial lesion	0	0	0	1	1	0	1	0	0	4	2	4	3	2	3
Inflammatory infiltrate	0	0	0	0	1	0	1	0	2	3	0	2	3	3	3
Vascular congestion	0	0	0	1	0	0	1	1	2	3	1	2	2	2	2
Edema/fibrosis	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
Total Score*	0	0	0	2	3	0	3	1	4	10	3	9	8	7	8

**The values (score) assigned for each of these lesions were: 0 (no change) when no injury or the observed changes were within normal range; 1 (minimum) when changes were sparse but exceeded those considered normal; 2 (light) when injuries were identifiable but with no severity; 3 (moderate) for significant injury that could increase in severity; 4 (very serious) for very serious injuries that occupy most of the analyzed tissue. These values were added up and determined the level of vaginal irritation as minimum 1-4, average 5-8, moderate 9-11 and severe [12-16].*

Discussion

There is an urgent need for female-controlled HIV prevention strategies. A safe and effective vaccine against HIV would be the best solution but despite more than two decades of HIV-1 vaccine research ,there is still no efficacious vaccine. The use of vaginal microbicides is one strategy to provide women with the ability to prevent HIV transmission from their infected partners. However, the most promising microbicides tested up to now, which contain TDF or dapivirine as active drugs, have conferred only modest protection against HIV-1 acquisition in women [1,16,18]. Concerns are also raised regarding the efficacy of TDF-based microbicides against infection by TDF-resistant strains, which are now common in low- and middle-income regions, and possible selection of TDF-resistant strains which will not respond to a TDF-based treatment regimen [11,20].

We have recently described a fusion inhibitor peptide, named P3, with very potent activity against HIV-1, HIV-2 and SIV [22,33]. Potent and specific entry inhibitors like P3, that are not in clinical use may be interesting microbicide candidates for HIV prevention. In this work, we evaluated the activity of P3 peptide in a HEC gel using a newly developed *in vitro* assay. HEC has been used as the universal placebo gel in several vaginal gel formulations and is considered safe [34,35]. We found that P3 at the IC_{90} concentration was effectively delivered to the target cells in a 1.5% HEC-gel and was fully active against HIV-1.

A microbicide candidate must remain stable in the presence of sperm and vaginal fluid, which will be present during sexual intercourse. The healthy human vaginal environment is acidic (pH 3.5-4.5) and contains proteolytic enzymes that may inactivate anti-HIV microbicides. Similarly, the antiviral activity of polyanionic compounds and several candidate gels (cellulose sulfate and PRO 2000) was negatively affected by the presence of semen/seminal plasma [13-15,36-38]. We investigated the stability and bioactivity of P3 in the presence of SP and VFS and found that its anti-HIV-1 activity was not affected in the presence of these human fluids, even after a month of exposition. P3 peptide also remained stable in a pH range of 4–8, even after 24 hours of incubation. Remarkably, at pH=4 which is the pH of the healthy vagina, the anti-HIV-1 activity of P3 was even better than at more alkaline pH. Bacterial vaginosis is a human vaginal infection during which the vaginal pH is increased towards alkaline values (pH>4.5) [39]; such pH changes may contribute for spontaneous hydrolysis and degradation of anti-HIV compounds [40]. In our case the antiviral activity of P3 was not affected at pH 6 and 8. Taken together, these results suggest that the antiviral activity of P3 should not be affected in the vaginal lumen even after intercourse, and that it could also be active in the setting of bacterial vaginosis.

Microbicides must be stable at 37ºC for a long period of time since it is the body temperature, and at 65ºC since some manufacturing processes may require high temperatures [29]. Stability at room temperature or at 4ºC is also important since the storage at these temperatures is easier for the user and more affordable than at -20ºC. No statistically significant differences were observed in the anti-HIV-1 activity of P3 peptide stored at 25, 37 and 65ºC for a week. The stability of P3 under different temperatures is much better than other peptide-based microbicide candidates or drugs; for example, retrocyclin RC-101 is susceptible to degradation at 65º C [29] and Spantide II is not stable at temperatures higher than 40ºC [41]. The stability of P3 under different temperatures makes it an ideal microbicide to be used in places with high ambient temperature and with limited access to cold storage chains.

Vaginal microbicide products will be in contact with the healthy vaginal microbiota, especially with *Lactobacillus* species that produce hydrogen peroxide (H_2O_2) which could oxidize P3 [31]. The effect of H_2O_2 at biologically relevant concentrations on P3 was assess and it was found that it maintained full antiviral activity suggesting that it will not be oxidized in the healthy vaginal environment.

Vaginal microbicides should not cause lesions to the vaginal epithelium, which are associated with increased rates of HIV-1 acquisition, and should not be spermicidal [15,42-45]. The P3 peptide neither altered the sperm's motility or vitality nor caused significant alterations in the vaginal epithelium or vaginal irritation in BALB/C mice at 0.06 and 0.2 mg/day. Major injuries in the vaginal epithelium were found only in two mice treated with 0.4 mg/day of P3, a dose that is ~8300-fold higher than the IC_{90}. Finally, P3 did not affect bacteria growth up to a concentration of 228.4 µM (~600-fold higher than the IC_{90}). This is important because a vaginal microbicide should preserve the normal vaginal microbiota, especially the *Lactobacillus* species that crucially contribute to the health of the vagina by producing lactic acid [46,47]. Collectively, these findings indicate that P3 can be safely used as a vaginal microbicide.

In conclusion, the high anti-HIV potency of P3 in a HEC-gel, its high stability in genital fluids and in a wide range of pH and temperatures and in the presence of hydrogen peroxide, as well as the low or null cellular and bacterial toxicity makes P3 an excellent candidate for the development of a vaginal microbicide gel for the prevention of HIV transmission in women.

Acknowledgements

Financial support for this research was provided by the Fundação para a Ciência e a Tecnologia (FCT), Portugal (project VIH/SAU/0029/2011), part of the EDCTP2 program supported by the European Union. Inês Bártolo is supported by a post-doc fellowship (SFRH/BPD/76225/2011) from Fundação para a Ciência e Tecnologia (FCT), Portugal. Ana Rita Diniz is supported by a PhD grant (SFRH/BD/89140/2012) from Fundação para a Ciência e Tecnologia (FCT), Portugal.

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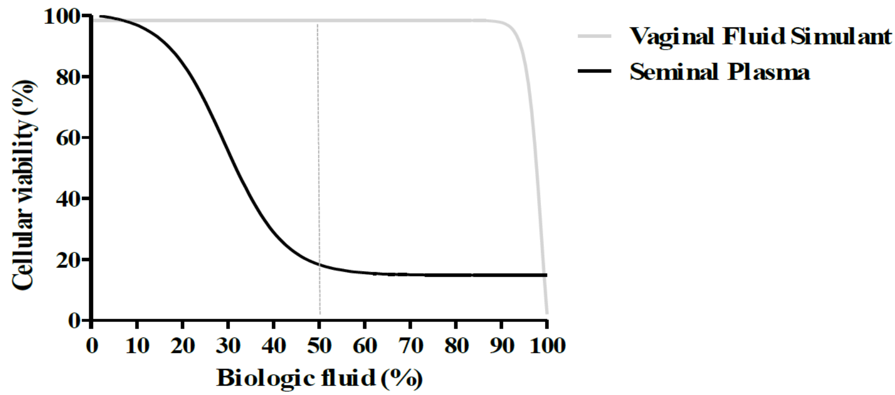
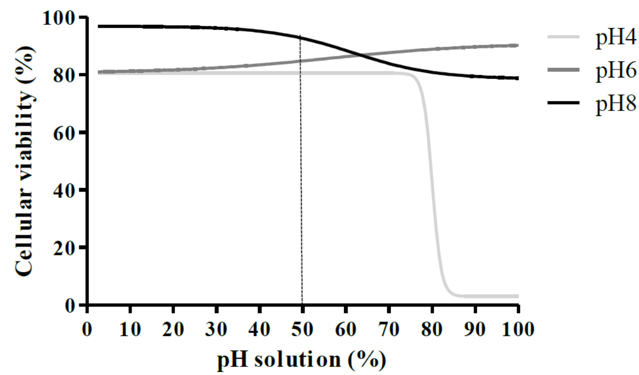
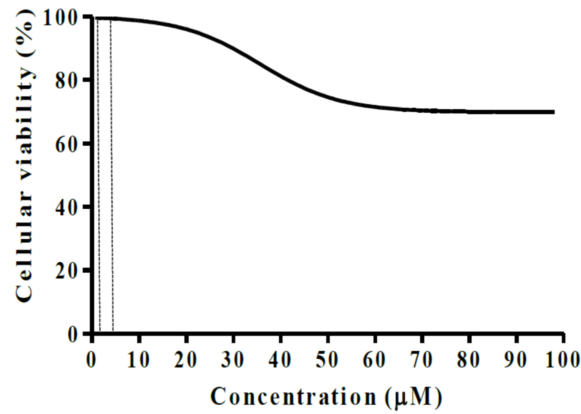
Supplementary Material



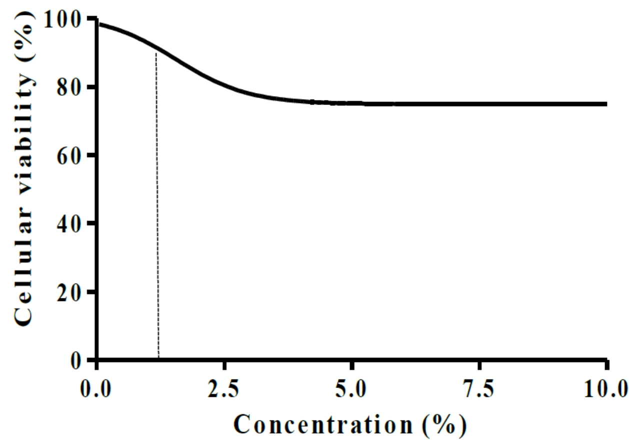
Supplementary Figure S1 – *Cellular viability of TZM-bl cells in the presence of biologic fluids. Cells were cultured in the presence of serial-fold dilutions of the biologic fluid and cellular viability was investigated using the alamarBlue assay. The grey dotted line corresponds to the concentration of VFS used in the assays and the black ones to SP concentrations.*



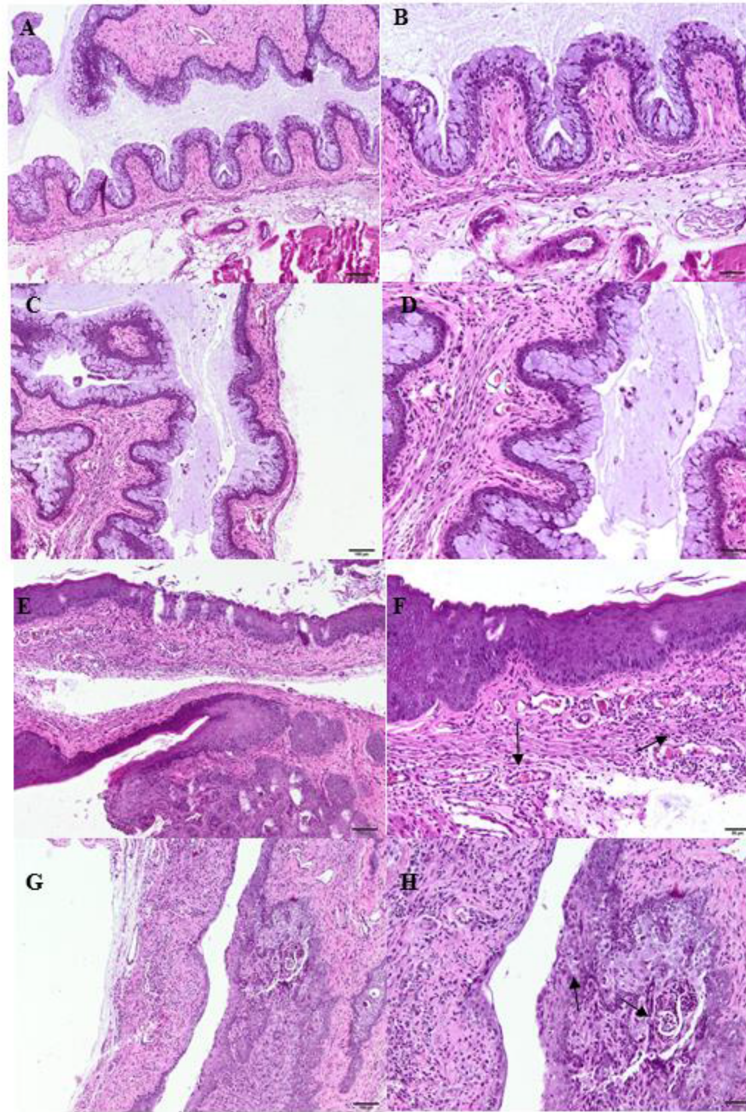
Supplementary Figure S2 – *Cellular viability of TZM-bl cells in the presence of solutions with different pHs. Cells were cultured in the presence of serial-fold dilutions of growth medium solutions at different pHs and cellular viability was investigated using the alamarBlue assay. The dotted line corresponds to the concentration of P3 pH solution used in the assays.*



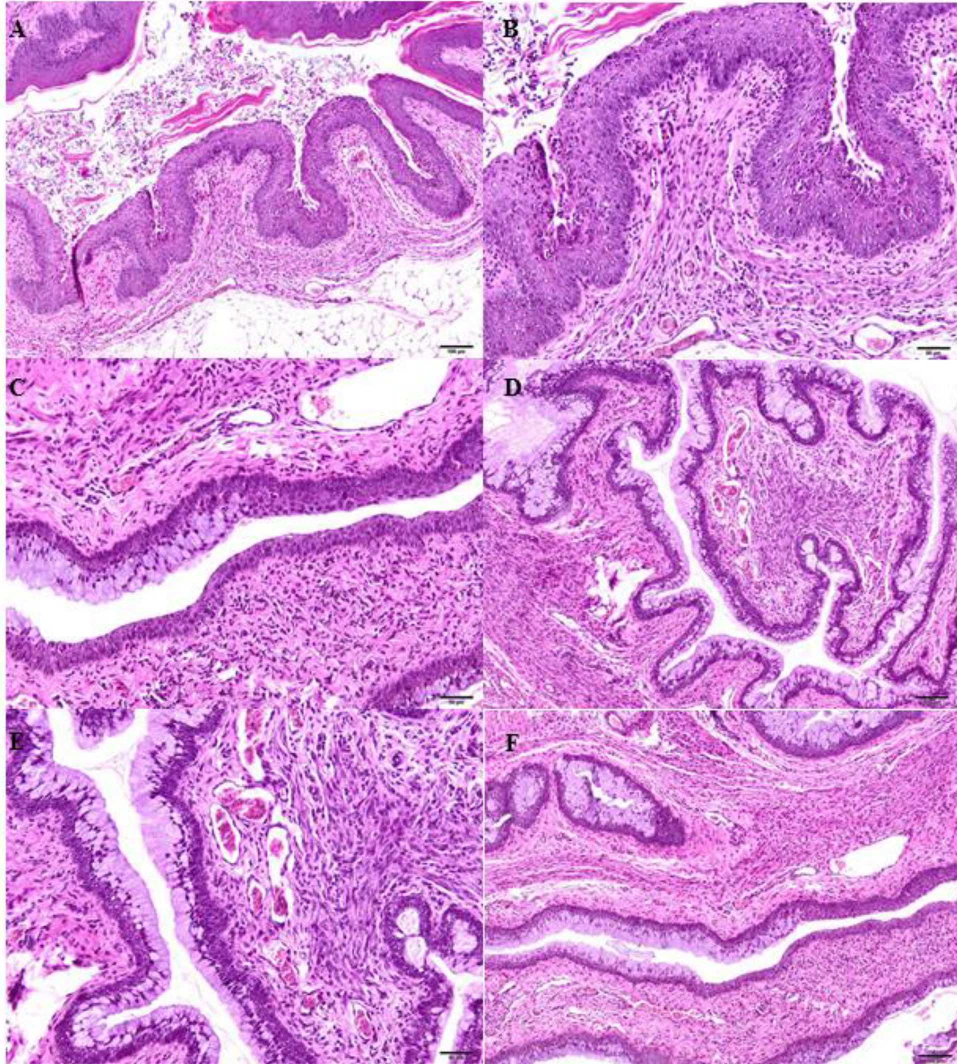
Supplementary Figure S3 – *Cellular viability of TZM-bl cells in the presence of different concentrations of H_2O_2. Cells were cultured in the presence of serial-fold dilutions of H_2O_2 and cellular viability was investigated using the alamarBlue assay. The dotted lines correspond to the concentration of H_2O_2 used in the assays (1.2µM and 5µM).*



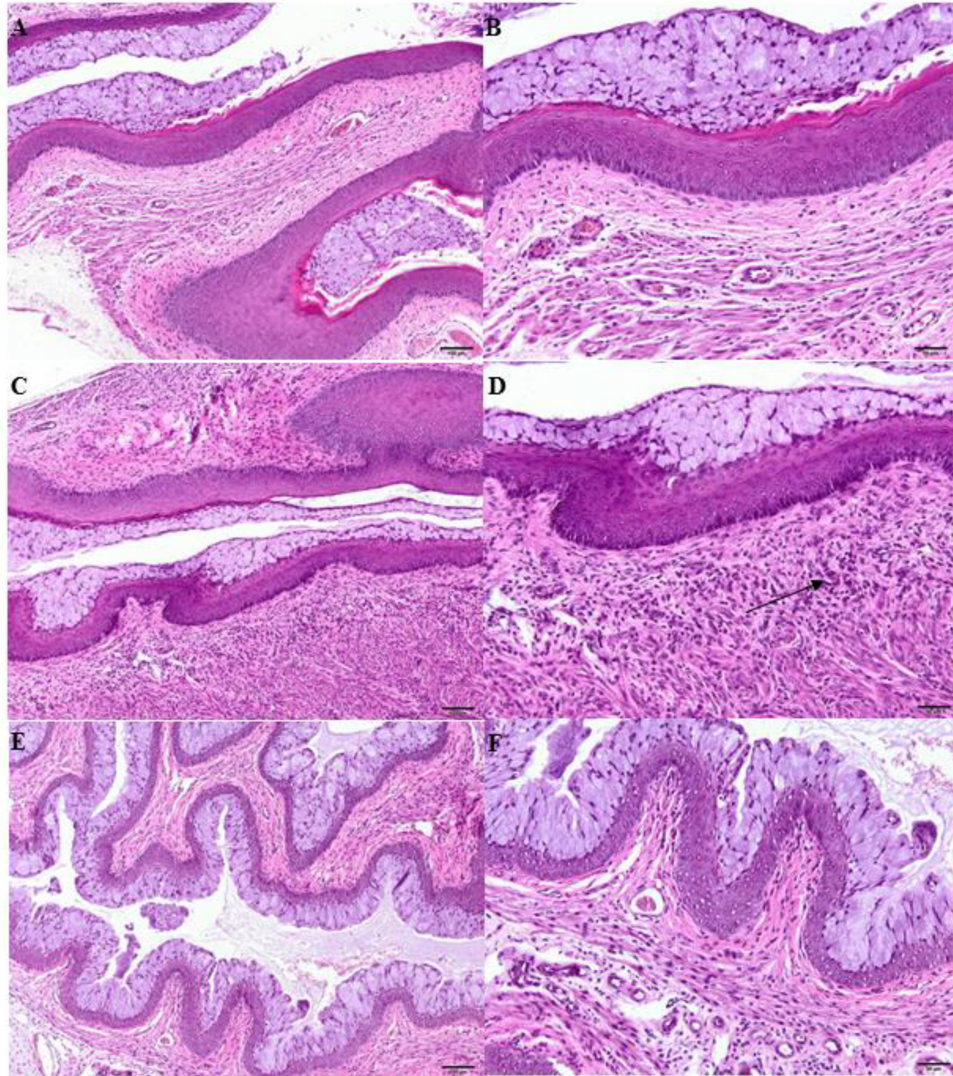
Supplementary Figure S4 – *Cellular viability of TZM-bl cells in the presence of different concentrations of HEC-gel. Cells were cultured in the presence of serial-fold dilutions of HEC-gel and cellular viability was investigated using the alamarBlue assay. The dotted line corresponds to the concentration of HEC-gel used in the assays (1.5%)*



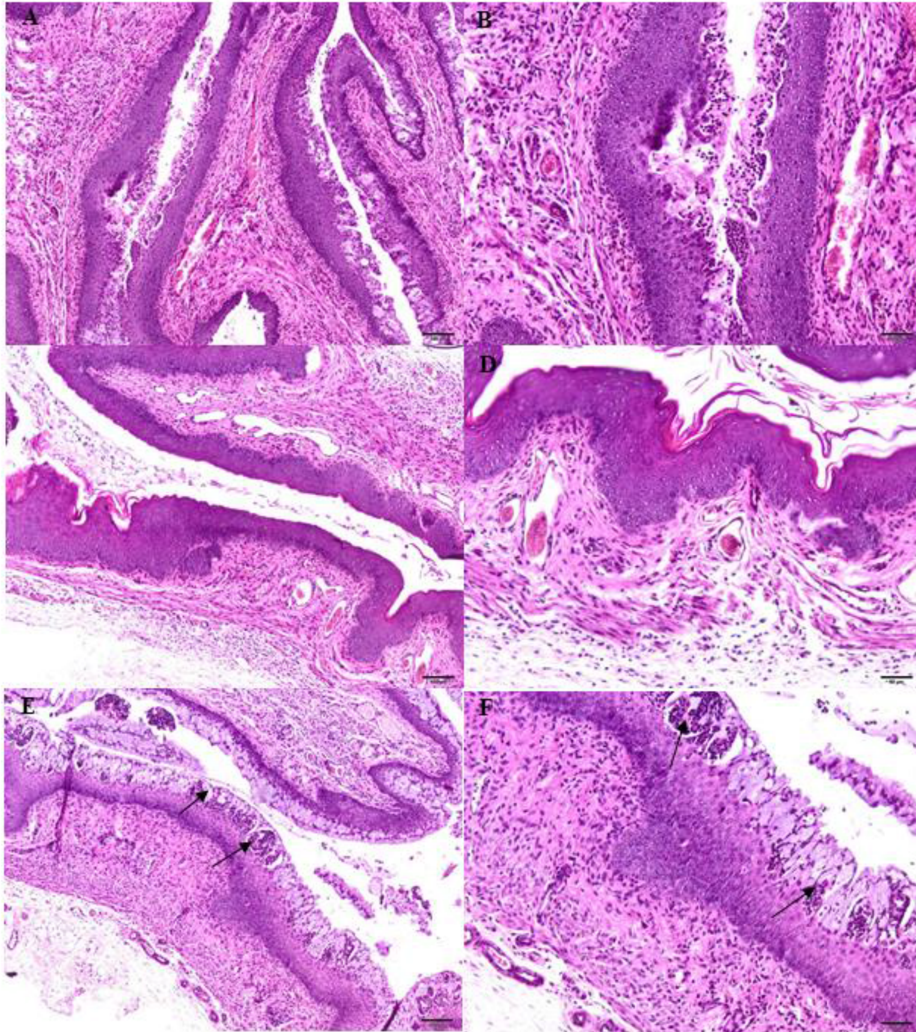
Supplementary Figure S5 – *Immunohistochemical analyses of vaginal epithelium in BALB/c mice of control groups after intravaginal administration of PBS (placebo group) or 3% N9 (irritation control group) during 7 consecutive days. A, B: PBS-mouse 1; C, D: PBS-mouse 3. In neither case histological lesions were observed. E, F: mouse 13 treated with 3% N9. Signs of epithelial hyperplasia and inflammatory infiltrate in the submucosa and congestion (arrows) were found. G, H: mouse 15 treated with 3% N9 showed hyperplasia and presence of inflammatory cells in the mucosal epithelium (arrows).*



Supplementary Figure S6 *– Immunohistochemical analyses of vaginal epithelium in BALB/c mice after intravaginal administration of 0.06mg/day of P3 (A group) during 7 consecutive days. A, B: mouse 4 showed minimal epithelial hyperplasia and vascular congestion; C, D: mouse 5 presented epithelial hyperplasia and a slight inflammatory infiltrate in the submucosa (arrow). E, F: mouse 6 without significant histological lesions.*



Supplementary Figure S7 – *Immunohistochemical analyses of vaginal epithelium in BALB/c mice after intravaginal administration of 0.2mg/day of P3 (B group) during 7 consecutive days. A, B: mouse 7 showed minimal inflammatory infiltrate in the epithelium and limited congestion in the submucosa; C, D: mouse 8 presented scarce congestion in submucosa; E, F: mouse 9 showed minimal inflammatory infiltrate and congestion in the submucosa.*



Supplementary Figure S8 – *Immunohistochemical analyses of vaginal epithelium in BALB/c mice after intravaginal administration of 0.4mg/day of P3 (C group) during 7 consecutive days. A, B: mouse 10 showed severe injury in the epithelium with hyperplasia and inflammatory infiltrate, as well as moderate congestion and inflammatory infiltrate in the submucosa; C, D: mouse 11 showed minimal vascular congestion and minimal hyperplasia in the epithelium. E, F: mouse 12 presented severe lesions in the epithelium with inflammatory infiltrates (arrows) extending throughout the submucosa although less evident here.*

Supplementary Methods

Method S1 – Cell lines and virus

TZM-bl cells (AIDS Research and Reference Reagent Program, National Institutes of Health, USA) were cultured in complete growth medium that consists of Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin-streptomycin (Gibco/Invitrogen, USA), 1mM of sodium pyruvate (Gibco/Invitrogen, USA), 2mM of L-glutamine (Gibco/Invitrogen, USA) and 1mM of non-essential amino acids (Gibco/Invitrogen, USA).

Peripheral blood mononuclear cells (PBMCs) from healthy individuals (blood donors) were separated by Ficoll-Paque PLUS (GE Healthcare, Waukesha, WI, USA) density gradient centrifugation and stimulated for 3 days with 5 µg/ml of phytohemaglutinin (PHA; Sigma-Aldrich, St. Louis, MO, USA). PBMCs cultures were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml of penicillin-streptomycin, 2mM of L-glutamine (Gibco/Invitrogen, USA) 0.3 mg/ml of gentamicin (Gibco/Invitrogen, Carlsbad, CA, USA), 5µg/ml of polybrene (Sigma-Aldrich, St. Louis, MO, USA) and 20U/ml units of recombinant interleukin-2 (Roche, Basel, Switzerland). All cell cultures were maintained at 37ºC in 5% CO_2.

The clinical isolate 93AOHDC50 used in this study was previously isolated, titrated and characterized for co-receptor usage [1]. The 50% tissue culture infectious dose (TCID50) of the virus was determined in a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells [1,2] and calculated using the statistical method of Reed and Muench [3].

Method S2 – Seminal plasma and vaginal fluid simulant

Semen samples were collected in a sterile container from seven HIV-1-seronegative healthy individuals by masturbation. The volunteers had no recent history of sexual transmitted infections, no urogenital abnormalities, and abstained from sexual intercourse for 48h prior to collection. The semen samples were allowed to liquefy for 30–45 min at room temperature and seminal plasma (SP) was obtained by centrifuging pooled whole semen at 1200 g for 10 min at 4ºC. The SP was filtered and stored in aliquots at -80°C until use.

Vaginal fluid simulant (VFS) was prepared as described elsewhere [4]. 1 L of VFS contained the following reagents: 3.51g of NaCl; 1.400g of KOH; 0.222g of $Ca(OH)_2$; 0.018g of bovine serum albumin; 2.0g of lactic acid; 1.00g of acetic acid; 0.16g of glycerol; 0.4g of urea and 5.0g of glucose. The pH was adjusted to 4.2 using HCl (1M).

SP and VFS cytotoxicity was assessed in TZM-bl cells. Cells were cultured in serial-fold dilutions of the biologic fluids and cellular viability was investigated using the alamarBlue assay [5].

Method S3 – Liquid chromatography and mass spectrometry (LC-MS/MS) analysis

Individual standard solutions (0.25-250 ppm) of P3 were prepared in acetonitrile (99.9% LC–MS) (J.T.Baker, Deventer, the Netherlands) in the presence of SP (0.5 or 1.0%), VFS or growth medium (GM) and ana-

lyzed after 24h, 48h and 1 week. In order to precipitate the proteins in the biological fluids, 1 ml of acetonitrile (1:1) was added to 1 ml of sample and samples were centrifuged at 13,000 rpm for 10 min and filtered (0.20 mm PVDF membrane; Chromafil®Xtra from Macherey-Nagel®) just before analysis. All samples were analyzed as triplicates. Experiments were performed to confirm that this procedure did not interfere with the concentration of the analyte in the standard solutions.

Liquid chromatography (LC) – The LC system was a Waters® Alliance 2695 (Waters®) equipped with a quaternary pump, solvent degasser, auto sampler and column oven, coupled to a Photodiode Array Detector Waters 996 PDA (Waters®) scanning from 210 to 600 nm. The separation was performed on a reversed phase column LiChrospher® 100 RP-18 (10 x 4.0mm; Merck®, Germany) operating at 35 °C, using an injection volume of 10 µl. The mobile phase consisted of water [Ultra-pure water (18.2MΩ.cm) from a Millipore-Direct Q3 UV system (Millipore, USA)] with TFA 0.1% (v/v) (98% p.a.; Merck®, Germany) (A) and acetonitrile with TFA 0.1% (v/v) (B) at a flow rate of 0.30 ml/min. The following gradient was used: 70% of eluente A as initial conditions, 70% A to 30% A for 7 min. A cleaning step was performed using 100% of acetonitrile for 1 min and finally the column was re-equilibrated with 70% of eluent A for 5 min.

Tandem mass spectrometry – A triple quadrupole mass spectrometer MicroMass® Quattro micro (Micromass®, Waters®) with an electrospray ionization source (ESI) was coupled to the LC system, described above. The ion source conditions were optimized using a standard solution directly injected in the ion source: temperature 120 ºC, capillary voltage 3.0 kV and cone voltage 50 V. The compound was ionized in positive ion mode and the spectra, in Full Scan, were recorded in the range m/z 100–1700. All analyses were performed in multiple reactions monitoring (MRM) mode in order to achieve a high selectivity and sensitivity. For the MS/MS experiments, collision energy was optimized (55 eV) to maximize [precursor ion > product ion] the transitions signal. Three transitions were considered: [1460>229]; [1460>258]; [1460>486]. The optimized conditions were confirmed when performing the LC–MS/MS analysis of the standard solutions.

High purity nitrogen (N_2) was used both as drying and nebulizing gas. Ultrahigh-purity Argon (Ar) was used as collision gas. MassLynxTM Software (Waters®) was used for data acquisition and processing.

Method S4 – Anti-bacterial activity

Anti-bacterial activity of P3 was determined according to CLSI guidelines [7]. The following bacteria were used: reference strains *E. coli* ATCC 10536, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633 and *Enterococcus faecalis* ATCC 29212; clinical strains of *E. coli* and *Pseudomonas aeruginosa* isolated in our laboratory; and strains of *Lactobacillus rhamnosus* and *Lactobacillus plantarum* also isolated in our laboratory. The Minimum Inhibitory Concentration (MIC) was determined by the agar diffusion method, in plates of Mueller-Hinton agar or Rogosa agar (for Lactobacilli) [8]. Briefly, 10^8cfu/ml bacterial suspensions were prepared in sterile water and spread in the culture media. Sterile disks containing different concentrations of P3 were placed on the inoculated surface. Plates were incubated at 37ºC for 24h or 48h. Lactobacilli were incubated at microaerophilic conditions. A negative control made of sterile water was used. Disks of amoxicillin and imipenem were used as positive controls. The maximum concentration of P3 tested was 228.4 µM (~600-fold higher concentration than the IC_{90}).

Method S5 – Histological studies in BALB/c mice

The presence of histological lesions in mouse's vaginas was evaluated with hematoxylin-eosin staining. Samples were embedded in paraffin by submersion in increasing concentrations of ethanol in water (Rectapur, VWR, England), two baths of xylene (Analar (VWR), England) and another of paraffin, before being placed in a paraffin mold. Subsequently, they were cut using a semimotorized microtome (RM2145 Leica, Germany) and processed for staining. For dewaxing, samples were submersed into two baths of xylene solution (10 min) and three baths in descending order of ethanol (100%, 90% and 70%) (5 min), before being stained with hematoxylin (Merck, Germany) for 5 min and eosin (Merck, Germany) for another 5 min. Post-eosin staining dehydration was performed with passages in increasing concentrations of ethanol (70%, 90% and 100%) and a bath of xylene solution. Finally, they were mounted with D.P.X. (Prolabo, VWR, Spain).

The existence of injury in vaginal epithelium, inflammatory infiltrate, vascular congestion and/or edema in the submucosa was evaluated in each biological sample. The values (score) assigned for each of these lesions were: 0 (no change) when no injury or the observed changes were within normal range; 1 (minimum) when changes were sparse but exceeded those considered normal; 2 (light) when injuries were identifiable but with no severity; 3 (moderate) for significant injury that could increase in severity; 4 (very serious) for very serious injuries that occupy most of the analyzed tissue. These values were added up and determined the level of vaginal irritation as minimum 1-4, average 5-8, moderate 9-11 and severe 12-16 [9].

Supplementary References

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CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

General discussion and conclusions

In this thesis, some prevention and treatment strategies were studied with the goal of contributing to the reduction of the global spread of HIV. In the first experimental study (Chapter 2) we proved that DTG is potently active against INSTI-naïve and resistant HIV-2 viral isolates. Primary resistance to this drug was detected for the first time in one ARV-naïve patient and key mutations associated to INSTI resistance were identified. Since ARV therapy is not able to eliminate the virus and a vaccine is still not available, prevention of HIV infection is of crucial importance. In the second and third experimental studies of this thesis (Chapters 3 and 4) we have contributed for the development of an effective microbicide against HIV-1 and HIV-2 sexual transmission. In Chapter 3, we tested the mechanism of action of new anti-HIV polyanionic carbosilane dendrimers (G2-S16, G2-NS16, and G3-Sh16) and also tested their toxicity in mice. In Chapter 4, we analyzed the stability and mice toxicity of the fusion inhibitor peptide P3. Both studies were done in preparation for trials of infection prevention in humanized mice.

In Chapter 2, we have characterized the activity of DTG on a panel of 16 HIV-2 primary isolates obtained from INSTI-naïve and RAL-failing HIV-2-infected patients from Portugal. As expected, DTG and RAL were very effective against HIV-2 primary isolates obtained from RAL-naïve patients; however, viral isolates from RAL-failing patients were highly resistant to RAL confirming that RAL-based therapy of HIV-2 infected patients selects for highly resistant viruses [1-3]. Importantly, DTG showed potent activity against all RAL-resistant isolates, indicating that it is useful as second line therapy for patients failing therapy with RAL [1-5].

Primary isolate 10PTHSJIG carried the E92Q and T97A mutations. Thus far the role of these mutations in HIV-2 was still unclear. Ni et al. demonstrated *in vitro* that mutation E92Q did not confer significant resistance to RAL, unless it was in the presence of the major mutation Y143C [6]. However, E92Q mutation was associated to low-level resistance to RAL [7] and DTG [4], in other studies. Cavaco-Silva et al. identified the combination of mutations E92Q + T97A in two patients with resistance to RAL, suggesting that this combination may be responsible for development of resistance to RAL in HIV-2. In our work, mutations E92Q and T97A were found in a patient that was highly resistant to RAL but susceptible to DTG, confirming that the combination of these two mutations is enough to reduce viral susceptibility to RAL but not to DTG.

The major RAL-resistance mutation Q148K was found in combination with the minor mutation E92A in primary isolate 15PTHCEC. 15PTHCEC primary isolate was obtained from one INI-experienced patient and was highly resistant to RAL. E92A is a mutation less frequently described at this residue and its role in INI susceptibility is not yet clear [6, 8]. Q148K is a major mutation strongly related to resistance to RAL in HIV-1 and HIV-2, especially when in association with other minor mutations in the integrase (e.g., E92Q, T97A, G140S) [2, 8-11]. However, its role in HIV-2 susceptibility to DTG was unclear, as the results of the small number of studies available showed different conclusions. Descamps et al. performed one study in HIV-2 infected patients that were failing RAL-based regimens and were receiving DTG and suggested that mutations in codon 148 plus one or two secondary mutations could also compromise the efficacy of DTG (59% and 24% viral inhibition, respectively) [2]. However, *in vitro* studies performed by Smith et al. with site directed mutants showed that Q148K had only moderate effect on the reference isolate HIV-2ROD9 susceptibility to DTG [4]. Our results show that primary isolate 15PTHCEC was still highly sensitive to DTG despite the presence of Q148K, proving that this mutation is not enough to confer resistance to DTG.

Overall, we prove that Q148K and the double E92Q + T97A mutations are sufficient to confer HIV-2 high-level resistance to RAL but do not significantly affect HIV-2 susceptibility to DTG. Possible reasons for such different results may be the type of viruses tested (molecular clones [4], mutated HIV-2 integrases [6, 7] or HIV-2 primary isolates [2]), different cell lines and also different methods used to measure resistance. Future studies should be performed using site-directed mutagenesis for Q148K, E92Q and T97A integrase mutations, alone or in combinations, in order to determine the extent of resistance associated with each mutation.

Our IC_{50} values for RAL and DTG are similar to those obtained by Bercoff et al. [3] but significantly lower when compared to other publications (Table 1). For example, Smith et al. (2015) [4] and Charpentier et al. [5] report values that are 13.8- and 12.0-fold higher than ours for inhibition of HIV-2ROD by DTG. Similarly, Roquebert et al. [9] obtained a mean IC_{50} of 2.4nM for RAL inhibition of HIV-2 isolates form treatment naïve patients, while we obtained a median IC_{50} value that was 14.9-fold lower (of 0.161nM). These differences in IC_{50} are probably a consequence of the different cells and assays used by the different authors (Table 1). In fact, studies reporting higher IC_{50} levels were done in PBMCs and used RT-PCR or p24 quantifications to measure viral replication, while we performed single-cycle infectivity assays in TZM-bl cells and the luciferase reporter gene protocol. On the other hand, these results demonstrate that our assay is much more sensitive than the others, especially for primary isolates. Single-cycle infection assays are time-saving, cheaper, highly reproducible, biologically safe and have the potential to be used in high-throughput protocols in 96-well plates [12]. Most importantly, our method measures the amount of viral infection which is a result of viable and infectious viral particles, whether RT-PCR and p24 quantifications quantify all viral particles, infectious or not [13, 14]. This is even more important when working with HIV-2 viral isolates that are naturally less infectious and have lower replication rates than HIV-1 viral isolates [15].

Table 1 – Comparison of RAL and DTG inhibition values described in the literature.

Reference	Virus	RAL	DTG	Cells	Assay
Diniz et al. 2018 "Differences in susceptibility patterns of isolates from HIV-2 infected patients to raltegravir and dolutegravir"	HIV-2 ROD10	IC_{50}=5.786nM	IC_{50}=0.167nM	TZM-bl	Single-cycle infectivity assay + luciferase reporter assay
	HIV-2 RAL-experienced viral isolates	IC_{50}=53.86nM	IC_{50}=0.718nM		
	HIV-2 RAL-naïve viral isolates	IC_{50}=0.161nM	IC_{50}=0.078nM		
Smith et al. 2015 "*In vitro* activity of dolutegravir against wild-type and integrase inhibitor-resistant HIV-2"	HIV-2 group A		EC_{50}=1.9nM	MAGIC-5A	Single-cycle assay MAGIC+x-gal (light microscopy)
	HIV-2 goup B		EC_{50}=2.6nM		
	HIV-1 isolates from INSTI naive patients		EC_{50}=1.3nM		
	HIV-2 ROD9		EC_{50}=2.3nM		
	HIV-1 NL4.3		EC_{50}=1.5nM		
Smith et al. 2011 "Phenotypic susceptibility of HIV-2 to raltegravir: integrase mutations Q148R and N155H confer raltegravir resistance"	HIV-2 ROD9	EC_{50}=9.4nM		MAGIC-5A	Single-cycle assay MAGIC+x-gal (light microscopy)
	HIV-1 NL4.3	EC_{50}=4.9nM			
	wild-type HIV-2 strains (CBL20-H9, CDC77618, 7924A, CBL23-H9 and EHO)	EC_{50}=2.6-20nM			

Table 1 – Comparison of RAL and DTG inhibition values described in the literature (cont.).

Reference	Virus	RAL	DTG	Cells	Assay
Bercoff et al. 2010 "Polymorphisms of HIV-2 integrase and selection of resistance to ralte-gravir"	HIV-2ROD	IC_{50}=0.00395nM		MT-4	MTT assay (Absorbance)
	HIV-2ROD Q91R+I175M	IC_{50}=0.0424nM			
Charpentier 2010 "In-vitro phenotypic suscep-tibility of HIV-2 clinical isolates to the integrase inhibitor S/GSK1349572"	HIV-2 INSTI naive iso-lates		EC_{50}=0.8nM	PBMC	HIV-2 RNA copies in the su-pernatant quantified with a real-time quantitative reverse transcription-PCR assay
	HIV-2ROD		EC_{50}=2nM		
	HIV-1BRU		EC_{50}=4nM		
	HIV-2 G140S+Q148R	EC_{50}=165nM			
	HIV-2 G140T+Q148R +N155H	EC_{50}=24nM			
	HIV-2 T97A+Y143C	EC_{50}=11nM			
Malet et al. 2018 "Muta-tions Located outside the Integrase Gene Can Confer Resistance to HIV-1 Integrase Strand Transfer Inhibitors"	HIV-1Lai wt	EC_{50}=10nM	EC_{50}=3nM	HeLa P4 PBMC	DTG and RAL – β-galactosidase DTG – HIV-1 RNA copies in the supernatant quantified with a real-time quantitative reverse transcription-PCR assay
Roquebert et al. 2008 "HIV-2 integrase gene polymorphism and phenotypic susceptibility of HIV-2 clinical isolates to the integrase in-hibitors raltegravir and elvitegravir *in vitro*"	HIV-2 naive isolates	IC_{50}=2.4nM		PBMC	HIV-2 RNA copies in the su-pernatant quantified with a real-time quantitative reverse transcription-PCR assay
	HIV-2ROD	IC_{50}=4.0nM			
	HIV-1BRU	IC_{50}=3.0nM			
Salgado et al. 2009 "Mutation N155H in HIV-2 integrase confers high phenotypic resistance to raltegravir and impairs replication capacity"	HIV-2ROD	IC_{50}=3.33nM		PBMC	p24 assay
Smith et al. 2014 "Ralte-gravir-associated Muta-tions in HIV-2 Confer Cross-resistance to Dolutegravir *In Vitro*" (poster)	HIV-1 NL4-3	EC_{50}=0.9nM		HeLa-CD4+	Single cycle assay using HeLa-CD4 indicator cells (MAGIC-5A)
	HIV-1 Lai	EC_{50}=1.3nM			
	HIV-2ROD	EC_{50}=2.5nM			
	HIV-2EHO	EC_{50}=2.6nM			

Importantly, we demonstrated for the first time primary resistance to DTG but not to RAL in two isolates of a patient that was infected by his mother at birth. This is the first time that primary resistance to DTG is reported in a treatment naïve HIV infected patient. Primary resistance to DTG evolved independently in this patient as his mother was fully sensitive to DTG and RAL. K221Q and D222K substitutions in the integrase carboxi-terminal domain (CTD) were found to be the most likely determinants of the natural resistance to DTG.

Studies performed with HIV-1 have shown that IN binds RT and facilitates the early steps of reverse transcription, enhancing the synthesis of cDNA [16, 17]. Furthermore, amino acid residues 220 to 270 within the CTD of HIV-1 integrase are of great importance to IN-RT binding [18, 19]. Tekeste et al. showed that HIV-1 IN substitutions at amino acid residues 220 to 270, sgnificantly decreased reverse transcription and consequently viral replication *in vitro*, proving that the RT-IN interaction is biologically significant for proper viral function [20]. We propose that, in analogy with HIV-1, mutations at codons 221 and 222 in the CTD of HIV-2 integrase may improve IN-RT binding, thus increasing reverse transcription and viral replication rates, resulting in an indirect mechanism of natural resistance to DTG (Figure 1). This hypothesis is consistent with the fact that viral replication fitness was higher for viral isolates belonging to the son ($TCID_{50}$/ml for 00PTHDECT was 1.4 x 10^5 and for 03PTHDECT was 3.5 x 10^4) in comparison with the viral isolate from the mother ($TCID_{50}$/ml=2.8 x 10^4).

To better understand the amino acid variability at positions 221 and 222 of the integrase gene of HIV-2, we analyzed 572 HIV-2 integrase sequences from the HIV sequence database available at https://www.hiv.lanl.gov/content/index and concluded that the presence of amino acid Q at position 221 and amino acid K at position 222 is 10.5% and 5.4%, respectively. The presence of both mutations in the same sequence was 2.45%. Therefore, we conclude that these positions are highly polymorphic in HIV-2 integrase and that mutations K221Q and D222K are relatively frequent in HIV-2 isolates.

Future work should include reverse mutation of these codons to determine if sensitivity to DTG appears and determination of the resistance mechanisms involved, through the quantification of RT replicative capacity and determination of the binding of DTG to the IN-RT complex. Considering the growing knowledge of the importance of the IN-RT complex, we believe that this interaction can be a potential therapeutic target. The development of new drugs with the ability to prevent the allosteric binding of these two enzymes could be an additional resource to block HIV replication.

Another interesting possibility that can explain the resistance to DTG observed in patient 1 is the presence of mutations outside the IN gene. For HIV-1, most IN resistance mutations are selected within the catalytic site of the enzyme [21, 22], however, a significant proportion of patients failing therapy with INSTIs do not have mutations in the IN gene [23, 24], probably acquiring resistance through a different mechanism.

Cultivating HIV-1 Lai in the presence of high and constant concentrations of DTG, Malet et al. obtained a virus that was highly resistance to RAL, EVG and DTG, with resistance mutations selected outside the IN gene [25]. The mutations found were located in the 3' polypurine tract (3' PPT) of the *nef* gene, in a highly conserved region [25]. Similarly, Lungu et al. compared the nucleotide sequences of *nef*/long terminal repeat (LTR) regions from 10 HIV-1-infected patients failing monotherapy with DTG and found that one patient had two mutations in a highly conserved region of the 3' PPT, however, other IN polymorphisms not associated with resistance

were also present [26]. Additionally, Dicker et al. reported that bases at the end of the viral LTR strongly influenced the activity of IN, suggesting that mutations in the terminal four bases of the nonprocessed strand of HIV-1 LTR can cause resistance to INSTIs [27].

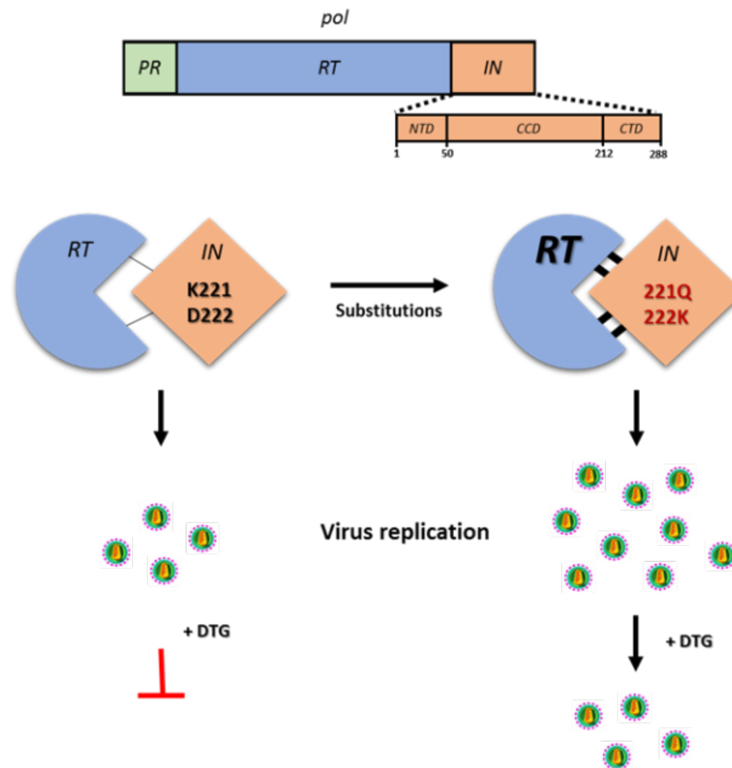

Figure 1 – *Hypothetical impact of mutations K221Q and D222K on the resistance of HIV-2 to DTG. Mutations K221Q and D222K in the CTD of IN increase its binding to the RT and increase the activity of RT. The higher level of viral replication results in an indirect mechanism of resistance to DTG.*

Currently, we are sequencing the *nef* gene and LTR of our HIV-2 viral isolates to determine if resistance mutations were selected in these regions, in particular for viral isolates naturally resistant to DTG and for the RAL resistant isolate that did not present resistance mutations.

All studies have limitations. The main limitation of this study is the small sample size, since we obtained samples from only 14 patients. Similarly, we could only obtain longitudinal samples from two patients. Collecting longitudinal data is important for this type of studies but is not always possible for many reasons, including death, and the loss of some patients to follow-up. Regarding resistance mutation analysis, the main limitations were the scarcity of prior studies and, as mentioned previously, the use of different methods to measure antiviral activity by the different authors which impedes the direct comparison of results.

Overall, our findings prove that DTG has a highly potent activity against most primary HIV-2 isolates including those that have acquired resistance to RAL, being an important therapeutic option for both RAL-naïve and RAL-failing HIV-2 infected patients. However, the fact that primary resistance to DTG was found in one patient without known IN resistance mutations points to possible limitations to the use of DTG as first line ARV for HIV-2 infection, unlike RAL, for which we did not find primary resistance. Clinical trials that are currently ongoing with large number of patients will allow a better understanding of the mechanisms of resistance to DTG,

determination of the frequency of primary resistance in ARV-naïve and INSTI-naïve patients, the extent of resistance caused by IN mutations already described but with contradictory reports and an overall safer use of these INSTIs.

Microbicides containing antiretroviral drugs are very promising formulations designed for application in the vagina and/or rectum to prevent the transmission of HIV during sexual intercourse [28, 29]. The objective is to act on the earliest steps of the infection process, inhibiting or blocking viral entry at the vaginal or rectal mucosa. A potent and effective microbicide can greatly empower women, because its use does not require the cooperation, consent or even knowledge of the partner. This is important because women represent nearly 60% of all people living with HIV in western and central Africa, acquiring the virus largely by heterosexual exposure [30, 31].

So far, the only successful clinical trial with microbicides to prevent HIV-1 infection was CAPRISA 004, that showed that a TDF-based gel provided 39% reduction of HIV acquisition overall for women from South Africa, and 54% for highly adherent women [28]. However, the VOICE study [32] and FACTS 001 [30] failed to prove the protective effect of the TDF gel. A TDF-based microbicide will likely be ineffective against TDF-resistant strains and its regular use may promote the selection and dissemination of resistant strains that will not respond to TDF-based therapeutic regimens [31]. Hence, one priority in this field is to find new microbicide candidates that interfere with the life cycle of HIV-1 and HIV-2 but are not based on ARV drugs in current use.

In this work, we evaluated the potential use of two different molecules as vaginal microbicides: non-HIV specific water-soluble polyanionic carbosilane dendrimers (Chapter 3) and the HIV fusion inhibitor peptide P3 (Chapter 4).

Polyanionic carbosilane dendrimers are complex nanomolecules that inhibit HIV-1 infection at the fusion and the entry step, through electrostatic interactions between their periphery functional groups and HIV-1 envelope proteins such as gp120, or proteins at the cell surface such as CD4 or CCR5 coreceptor [33-35]. Blocking gp120/CD4 interaction, dendrimers are able to inhibit binding of HIV-1 to the target cell, and subsequent infection. Dendrimers are safe and effective compounds against HIV-1 and HSV-2 *in vitro* [36]. Furthermore, dendrimers can inhibit cell-to-cell HIV-1 transmission and showed synergistic activity in combinations with ARVs against X4 and R5 tropic HIV-1 in cell lines as well as in primary human cells [36].

In our work, new dendrimers G2-S16, G2-NS16, and G3-Sh16 were tested for the first time against R5 and X4 HIV-2 primary isolates and their mechanism of action was determined.

It was shown that dendrimers G2-S16, G2-NS16 and G3-Sh16 potently inhibited both R5 and X4-HIV-2 primary isolates. The dendrimer with best inhibition values was G2-NS16, reaching >93% of viral inhibition. Dendrimers bound to target cells and perturbed the ability of the viral envelope to interact with its cell surface receptors, especially dendrimer G2-NS16, that best blocked the virus-cell attachment and viral entry in the experiments performed. Besides acting on the target cell to prevent virus binding, the dendrimers could also directly inactivate the virus, strongly decreasing the infectivity of HIV-2 particles, especially for X4-HIV-2 primary isolates. G2-NS16 was also the dendrimer that best inactivated HIV-2 virions.

We have also shown that the three dendrimers inhibit cell-associated HIV-2 infection in a dose-dependent manner, in particular G2-NS16. Cell-to-cell infection is a potent and fast way of viral

spread, that can overcome several barriers, like distance between infected and target cells, presence of neutralizing antibodies and low viral infectivity [37]. Cell-to-cell infection allows high efficiency of viral delivery, exposing uninfected cells to a large number of virions [38-40]. Inhibition of cell-to-cell infection required concentrations 4.4- to 8.4-fold higher than inhibition of cell-free HIV-2 infection, confirming that it is much harder to inhibit cell-to-cell infection than cell-free infection [41].

To decrease the risk of drug resistance, an ideal HIV microbicide should combine two or more anti-HIV drugs, including ARVs targeting different steps of the replication cycle, and also anti-HIV compounds that act in a non-specific way [42]. We found that combining dendrimers with TDF and RAL resulted in increased anti-HIV-2 activity. G2-NS16/TDF/RAL was the most potent combination regimen against HIV-2 isolates, which is consistent with the higher anti-HIV-2 potency of the G2-NS16 dendrimer when given alone, relative to the other dendrimers. Our experiments proved that all combinations of microbicides with TDF and RAL resulted in significant synergistic interactions against the primary R5- and X4-HIV-2 isolates. Similar results have been previously obtained for HIV-1 using a combination of these dendrimers with TDF and/or MVC [43].

A topical microbicide must not interfere with sperm's vitality nor with the normal vaginal flora [44, 45]. We showed that dendrimer G2-S16 did not affect sperm's motility, as was already described for dendrimers G2-NS16 and G3-Sh16 in a previous study [46]. Recently, it was also demonstrated that dendrimer G2-S16 can also prevent HIV-1 infection in the presence of semen [47], which is very important considering that semen has a negative influence on the efficacy of various classes of nanocompounds against HIV infection [46, 47], being a probable reason for the *in vivo* failure of many microbicides that showed great promise *in vitro*, such as cellulose sulfate [49, 50].

Maintaining the normal vaginal flora and acidic pH is crucial for a healthy vaginal environment [51, 52]. A successful microbicide has to be stable and biocompatible with normal vaginal flora while preventing HIV-2 transmission [44]. We showed that dendrimer G2-S16 was not toxic against several microorganisms observed in normal vaginal microbiota, and these results are consistent with the results obtained for dendrimers G2-NS16 and G3-Sh16 in previous studies [46].

One important challenge when formulating a compound in a topical vaginal microbicide gel is the possibility of disruption of the integrity of the mucosal epithelial surface, after several administrations, thus increasing the risk of HIV infection, as was observed in initial microbicide studies (surfactants and acidifying agents) [53] and with VivaGel® [54, 55]. We showed that 3%-HEC-G2-S16 displayed a good safety profile and did not cause alterations to the vaginal epithelium of mice.

Overall our results suggested that 3%- HEC gels with G2-S16 is a potential good microbicide candidate. Recent studies have shown that a topical vaginal administration of the 3%-HEC-G2-S16 gel inhibits HIV-1 infection by 84% in humanized bone marrow–liver– thymus (h-BLT) mice, while avoiding adverse symptoms like inflammation and vaginal irritation [55]. Dendrimer G2-S16 has been recently submitted to extensive experiments to prove its potential as anti-HIV-1 topical microbicide and the next step is to try it in clinical trials [57].

A limitation of this study was the fact that dendrimers were only tested in the presence of semen to determine their influence in sperm's motility and vitality and not to determine if antiviral activity was maintained. However, recent studies have shown that semen does not affect the anti-HIV-1 activity of the dendrimers [47]. Also, we did not have the chance to perform real prevention studies in animal models. These studies were done recently in humanized BLT mice and have shown that dendrimer G2-S16 is highly effective at preventing HIV-1 infection [56].

Since dendrimer G2-NS16 had the best results against HIV-2 isolates we believe that a microbicide combining G2-S16 and G2-NS16 could offer protection against both HIV-1 and HIV-2, thus greatly increasing microbicide's efficacy. Furthermore, since these dendrimers are also active against HSV-2, dual protection against HIV and HSV-2 could be obtained in a one single microbicide.

Besides combining dendrimers with one or more ARV, another interesting possibility is to modify the dendrimers to carry ARVs in their terminal groups or in the cavities inside the interior branches (Figure 2), which could protect the ARV from degradation, prolong the release of the active compound and also maximize antiviral activity due to the combination of the action of the dendrimer and the ARV at the site of HIV infection [42]. This strategy has already been performed for other dendrimers and ARVs like efavirenz [58], lamivudine [59] and zidovudine [60].

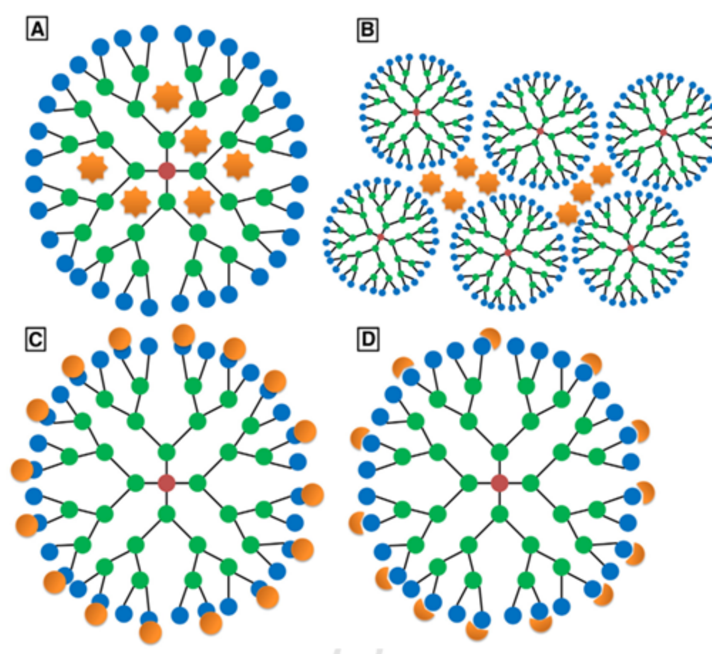

Figure 2 – *Representation of drug-dendrimer interactions. (A) Drug encapsulated dendrimer, (B) dendrimer-drug networks, (C) drug conjugated dendrimer by covalent interactions, and (D) by electrostatic interactions. (Adapted from [42])*

Peptides and proteins have great potential as therapeutics, already representing 10% of the pharmaceutical market and continuing to increase [61]. Insulin and cyclosporine (51AA and 11AA, respectively) are examples of peptide/protein therapeutics available.

The most well studied anti-HIV-1 peptide is T-20, an HIV-1 fusion inhibitor. T-20 has strong HIV-1 activity, however it is not active against HIV-2, has poor bioavailability and requires twice daily injections [62-64]. T-1249 is also a fusion inhibitor peptide active against HIV, but with difficult formulation due to its large size and associated with high costs of production [65, 66]. Re-

cently, a novel short peptide fusion inhibitor, termed 2P23, was created and exhibited potent activity against HIV-1, HIV-2 and SIV [67]. Peptides have advantages compared with other drugs: increased selectivity, fewer side effects and toxicity, and the possibility of being designed to target multiple molecules, because they have several points of contact with the target [61]. However, limitations include difficulty to cross physiological barriers and low oral bioavailability, due to degradation and poor absorption in the gastrointestinal tract, which is the reason why most peptides/proteins are formulated for intravenous or subcutaneous delivery [61, 68, 69]. Vaginal delivery of small peptides as microbicides can overcome these limitations, because the peptide is directly administered to the site of action, suffering less degradation, thus remaining present in high and protective concentrations.

In Chapter 4, the fusion inhibitor peptide P3 was evaluated as a potential microbicide to prevent vaginal HIV transmission. P3 was recently developed based on ancestral sequences of the transmembrane glycoproteins of HIV-2 and SIV, and potently inhibits HIV-1 and HIV-2 replication [63, 67]. Furthermore, P3 has high target binding affinity, predicting that it should be more resistant to degradation by proteolytic cleavage in biologic fluids than other fusion inhibitor peptides [63], and is a small-sized peptide (34 AA) with easier and cheaper production than other peptides.

When developing an anti-HIV peptide for vaginal administration, it is important to determine if the peptide retains its stability and activity in the presence of body fluids (vaginal fluid and semen) encountered during the sexual transmission event [70]. It was described that semen and female genital tract secretions impairs the antiviral activity of microbicide candidates [71], thus being a crucial factor in microbicide development. We showed that P3 is stable and active against HIV-1 in the presence of seminal plasma, vaginal fluid simulant and a combination of both.

A vaginally applied microbicide must be stable at different pH values: normal acidic pH of the vagina (3.5 – 4.5) [51] but also in the near neutral pH present after ejaculation as a result of the basic characteristics of the sperm [72]. In order to determine if P3 maintained its antiviral activity at different pH values, the antiviral activity of P3 was tested at pH 4, 6 and 8. The results showed that none of the pH values tested affected significantly the antiviral activity of P3. Remarkably, the higher antiviral activity of P3 was obtained at pH=4, which is very close to the normal pH of the vagina (3.5 – 4.5). The fact that P3 was active at pH 6 and 8 is also advantageous, because several vaginal infections, such as bacterial vaginosis, caused by excessive proliferation of indigenous microorganisms occur at pH values close to neutral or basic [73], for which P3 is still very active. Overall, the results obtained demonstrate that P3 was not affected by the vaginal environment, and can even be active in the setting of bacterial vaginosis.

Thermal stability of candidate microbicides should be tested considering that these compounds must be active at body temperature, but will also be subjected to different temperatures in the manufacturing process of the microbicide and during the shelf-life of the final product [74]. We showed that P3 was very stable at 4°C, 25°C, 37°C and 65°C, proving the very high thermal stability of this peptide. The stability of P3 under different temperatures makes it an ideal microbicide to be used in places with high ambient temperature and with limited access to cold storage chains.

Normal vaginal environment contains Lactobacillus species that produce hydrogen peroxide (H_2O_2), a powerful oxidizing agent, especially in acidic conditions [73]. P3 maintained its stabil-

ity and antiviral activity in the presence of biological concentrations of H2O2, proving that this peptide is not affected by H2O2.

Vaginal microbicides must be innocuous to the vaginal environment before, during and after intercourse, which means that they should not disrupt the vaginal epithelium, should preserve the normal vaginal microbiota and should not alter the motility or vitality of the sperm [45, 49, 50, 53, 75]. P3 did not cause significant alterations of the vaginal epithelium or vaginal irritation in BALB/C mice at 0.06 and 0.2 mg/day. Major injuries to the vaginal epithelium were found only in two mice treated with 0.4 mg/day of P3, a dose which is ~8300-fold higher than the IC90. Our results showed that P3 did not affect the growth of different species of bacteria including two common clinical species of lactobacillus, Lactobacillus rhamnosus and Lactobacillus plantarum, responsible for lactic acid production and maintenance of the normal acidic pH of the vagina [76, 77]. Bacteria growth was not affected by P3 up to a concentration of 228.4 μM (~600-fold higher than the IC90). Finally, P3 did not affect the sperm's characteristics and, therefore was not spermicidal and could be safely used as a vaginal microbicide.

Upon formulation of P3 in a 1.5%-HEC gel, we concluded that P3 at the IC90 concentration was effectively delivered to target cells and was fully active against HIV-1. Based on these results we concluded that 1.5%-HEC-P3 gel is an attractive microbicide candidate for HIV-1 prevention.

Ongoing studies in hBLT mice will determine whether P3 formulated in a HEC gel can prevent vaginal transmission of HIV-1 and HIV-2. If it is proved that antiviral activity of P3 is maintained in hBLT mice, the next step will be to push P3 into clinical trials with monkeys, because P3 is also active against SIV [67]. It would also be interesting to study if P3 as anti-HSV-2 activity, thus being able to be formulated as a microbicide with dual action.

If the antiviral activity decreases in mice experiments, it will probably be caused by peptide degradation or poor absorption and there are some modifications that can be made to P3. One option is carrier systems, such as nanoparticles (dendrimers, micelles, liposomes or nanosuspensions) which have already been studied as carriers of proteins and polypeptides [78-80]. Dendrimers are nanoparticles with the ability to encapsulate or bind drugs to its surface, as shown above (Figure 2), and we propose that the encapsulation of P3 in polyanionic carbosilane dendrimers G2-S16, G2-NS16 or G3-Sh16 would greatly enhance the bioavailability of P3, protecting it against hydrolysis and enzymatic degradation, as well as further improve the antiviral activity of the microbicide, due to the intrinsic anti-HIV activity of the dendrimers.

Like in the previous study, a potential limitation of this study was that the antiviral activity of P3 was not determined in the presence of semen. Also lacking are prevention studies in animal models like humanized mice.

In conclusion, in this thesis we have shown that DTG has a potent activity against most primary HIV-2 isolates including those that have acquired resistance to RAL, being an important therapeutic option for both RAL-naïve and RAL-failing HIV-2 infected patients. We have also shown that dendrimers G2-S16, G2-NS16, and G3-Sh16 as well as the fusion inhibitor peptide P3 are highly stable in physiologic conditions, have an excellent safety profile and have very potent antiviral activity, suggesting that they may be useful as vaginal microbicides. Prevention studies with microbicides containing these and other entry inhibitors are warrented in animal models.

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