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Synergistic inhibition of HIV-1 infection by combinations of soluble polyanions with other potential microbicides

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Abstract

Several polyanionic compounds with potential for use as topically applied microbicides to prevent HIV-1 sexual transmission, such as PRO 2000, are currently in phase III clinical efficacy trials. Microbicidal formulations may well comprise combinations of inhibitors to increase potency, reduce dose and minimize problems of HIV-1 resistance. We have therefore evaluated in vitro, the anti-HIV-1 activity of two leading polyanionic microbicides combined with other antiretroviral agents with microbicidal potential. Dextran sulfate (DS) and PRO 2000 were combined with the neutralizing antibody IgG1b12, the peptide-based fusion inhibitor T20, the CCR5 antagonist TAK779 and the cyanobacterial protein cyanovirin-N. Anti-HIV-1 activity was assessed in a single cycle replication assay using pseudoviruses carrying a luciferase reporter gene and the envelope glycoproteins from HIV-1 isolates JR-FL (R5) and HxB2 (X4), against both immortalized and primary CD4+ cell targets. The data were analyzed for synergy using Calcusyn™ software. Results indicate that PRO 2000 and DS can act synergistically with most inhibitors tested, although the degree of synergy depends on inhibitor concentration and combination. These data provide a rational basis for testing of microbicide combinations in vivo.

Keywords: HIV-1; Microbicide; Synergy; Antiretroviral; Polyanion

1. Introduction

The HIV-1 epidemic continues to grow at an alarming rate with an estimated 40 million people living with HIV/AIDS (AIDS, 2004). Heterosexual transmission is responsible for approximately 80% of new HIV-1 infections (AIDS, 2004). Globally, women now account for 50% of those infected with HIV-1 and in sub-Saharan Africa, where 95% of HIV-1 transmission is through heterosexual contact, and 60% of new infections occur in women (AIDS, 2004). With the development of an effective vaccine likely to be many years away there is a pressing need to develop female controlled methods of HIV-1 prevention such as topical application microbicides (Lederman et al., 2006; Stone, 2002; Turpin, 2002; Weber et al., 2005).

Many of the lead microbicide candidates target the process of HIV-1 attachment and entry into the target cell (Lederman et al., 2006; Stone, 2002; Turpin, 2002; Weber et al., 2005). Viral entry is mediated by the envelope glycoprotein (Env), which is composed of trimers of heterodimers of the surface glycoprotein gp120, non-covalently associated with the transmembrane protein gp41 (Wyatt and Sodroski, 1998). Receptor engagement on the target cell is mediated by the surface glycoprotein, gp120, which interacts sequentially with CD4 and a coreceptor (Wyatt and Sodroski, 1998). HIV-1 tropism is classified on the basis of coreceptor usage: CXCR4 (X4) tropic, CCR5 (R5) tropic or dual (R5/X4) tropic (Berger et al., 1998). HIV-1 transmission appears to be limited almost exclusively to R5 viruses, whereas X4 viruses are associated with increased pathogenicity and progression to AIDS in approximately 50% of individuals (Moore et al., 2004).

Polyanionic compounds represent some of the most promising microbicide candidates, with PRO 2000, cellulose sulfate (CS (Ushercell™)) and carrageenan (Carraguard™) currently in Phase III clinical trials (Anderson et al., 2002; http://www.microbicides.org; Lederman et al., 2006; Mayer et al., 2003; Stone, 2002; Turpin, 2002; Van Damme et al., 2000; Weber et al., 2005). Inhibition of HIV-1 attachment by soluble polyanions is thought to be mediated via electrostatic

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interactions between the negatively charged groups on the polyanion and basic residues present within the gp120 chemokine receptor binding surface, comprised of the V3 loop and CD4-induced (CD4i) site (Bagasra and Lischner, 1988; Callahan et al., 1991; de Parseval et al., 2005; Harrop et al., 1994; Harrop and Rider, 1998; McClure et al., 1992; Moulard et al., 2000; Vives et al., 2005). Despite the importance of R5 virus in HIV-1 transmission, most studies on in vitro polyanion inhibition of HIV-1 infection (Baba et al., 1988a,b; Bagasra and Lischner, 1988; Dezzutti et al., 2004; Konlee, 1998; McClure et al., 1992; Neurath et al., 2002a,b; Rusconi et al., 1996; Shaunak et al., 1994, 2003) and transmission in the macaque model (Weber et al., 2001) have been carried out using X4 or R5X4 (dual tropic) viruses. Only a few studies have analyzed polyanion inhibition of R5 isolates (Neurath et al., 2001, 2002b; Scordi-Bello et al., 2005; Shaunak et al., 2003). Concerns have been raised with respect to the possibility that polyanions will be less effective against R5 viruses as a consequence of the reduced positive charge on the V3 loop (Shattock and Doms, 2002).

The advent of highly active antiretroviral therapy (HAART) has demonstrated the need to combine different types of antiretroviral drugs when combating HIV-1 infection, and this approach has had a major impact on the life expectancy of HIV-1 infected individuals in the developed world (Palella et al., 1998). Although therapeutic intervention with HAART differs from prophylactic intervention with microbicides in that prophylaxis applies no selective pressure on pre-existing viral infection within a host, the risk remains that incoming virus may already contain mutations rendering it resistant. Indeed, in vitro escape mutants of HIV-1 to polyanions have been described (Bobardt et al., 2004; Esté et al., 1997). To combat the risk of encountering resistant HIV-1 isolates, and with the potential to reduce the dose of agents that have synergistic antiviral activity, the principle of combining two (or more) compounds that target separate viral structures or functions is applicable to microbicide formulation. Finally, the outcome of recent studies using the SHIV macaque model indicating that combinations of antiviral compounds found to work in synergy in vitro are also likely to display synergistic activity in vivo (Veazey et al., 2005), highlights the need to characterize the in vitro ability of lead microbicide compounds to work in combination with other known inhibitors of HIV-1 infection.

In the work presented here, PRO 2000 and DS were tested in combination with the following inhibitors: IgG1b12, a HIV-1 neutralizing antibody with in vivo microbicidal activity (Burton et al., 1994; Veazey et al., 2003b); T20 (Enfuvirtide^{TM}), a gp41-derived, peptide-based fusion inhibitor (Furuta et al., 1998; Matthews et al., 2004); the CCR5 antagonist TAK779 (Baba et al., 1999; Dragic et al., 2000) and the bacterial lectin cyanovirin-N (CV-N) (Bolmstedt et al., 2001; Boyd et al., 1997; Dey et al., 2000; Esser et al., 1999; Mori and Boyd, 2001; Tsai et al., 2003). All of these anti-HIV-1 agents, with the exception of TAK779 which only inhibits R5 tropic viruses, interfere with the infectivity of a broad spectrum of R5 and X4 viral isolates and are generally active in the nanomolar range. To study the antiviral effects of these combinations, we used a previously described luciferase reporter pseudovirus system (Chen et al., 1994; Connor et al., 1995). This system allows analysis of multiple Env glycoproteins in the context of a single proviral backbone. Moreover, since these pseudoviruses are limited to a single replication cycle, analysis of inhibition is more precise because the instantaneous inhibition factor of a drug is measured rather than its cumulative effect (Ferguson et al., 2001). This is particularly important for the analysis of data from drug combinations because assays which measure the cumulative effects of inhibitors over multiple cycles can substantially overestimate synergy (Ferguson et al., 2001). Since an effective microbicide will need to inhibit infection by R5 viruses, our studies were carried out principally using pseudoviruses carrying Env from the primary isolate (PI) R5 HIV-1_{JR-FL} isolate. All combinations were performed using U87.CD4.CCR5/CXCR4 as target cells. Combinations that were found to be strongly synergistic in this system were then re-tested using activated peripheral blood mononuclear cells (PBMC) as more physiologically relevant targets for inhibition of HIV-1 infection.

2. Materials and methods

2.1. Cells and inhibitors

U87.CD4.CCR5/CXCR4 cells (Centre for AIDS Reagents (CFAR), NIBSC, Potters Bar, UK) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO plc) supplemented with 10% heat inactivated fetal calf serum (HI-FCS), 100 U penicillin, 100 μg/ml streptomycin (pen/strep), 300 μg/ml G418 and 2 μg/ml puromycin. The 293T cells (ATCC) were maintained in DMEM supplemented with 10% HI-FCS and 100 U pen/strep. PBMCs were isolated from buffy coats by Ficoll-Hypaque (Sigma–Aldrich, UK) gradient centrifugation. PBMCs were activated by incubation in growth medium (GM) consisting of RPMI 1640 containing 15% HI-FCS, 100 U pen/strep, and supplemented with interleukin-2 (IL-2, from CFAR, UK) and 1 μg/ml phytohemagglutinin (PHA, from CFAR, UK) for 3–4 days before removal of PHA and culture in GM containing 10 U/ml recombinant IL-2. PRO 2000 and DS were obtained from Indevus Pharmaceuticals Inc., Lexington, MA and ML Laboratories Plc., UK, respectively. Other antiretroviral agents used in this study were IgG1b12 (D. Burton, Scripps Institute, La Jolla, CA), the gp41 peptide fusion inhibitor T20 (Enfuvirtide^{TM}, from T. Matthews, Duke University, Durham, NC), the small molecule CCR5 antagonist TAK779 (NIH Repository), and the bacterial lectin CV-N (M. Boyd, USA Cancer Research Institute, University of South Alabama, Mobile, AL). In all experiments molar concentrations were calculated for the inhibitors: in the case of DS and PRO 2000, which contain a mixture of polymers with different molecular weights, average molecular weights of 20 and 5 kDa, respectively, were used in these calculations.

2.2. Pseudovirus production

Pseudovirus was produced by co-transfecting the pNL4-3 Env (−) Luc (+) vector (Dr N. Landau, Gladstone Research

Institute, Stanford, CA), containing the proviral backbone but lacking *env* and with a luciferase reporter gene inserted into *nef* (Chen et al., 1994; Connor et al., 1995), by calcium phosphate transfection (Promega) into 293T cells along with a pcDNA3.1/Zeo(+) plasmid (Invitrogen) containing a 3 kb *env* gene insert for HIV-$1_{JR\text{-}FL}$ or HIV-1_{HxB2} (NIH AIDS reagent program). Recombinant virus was harvested from the supernatant on Day 4, aliquoted and stored at −80 °C. Viral titre was determined using both U87.CD4.CCR5/CXCR4 and activated PBMCs. U87.CD4.CCR5/CXCR4 cells were seeded at a density of 2×10^5 ml^{-1} in 96 well plates and incubated overnight at 37 °C. The following day recombinant virus was five-fold serially diluted in quadruplicate and added to the U87.CD4 cells bearing the appropriate coreceptor. PBMC cultures were used 7–10 days after initiation of mitogen activation. PBMCs were seeded in 96 well μ-titre plates at a density of 1×10^6 cells/well in 50 μl of HIV-$1_{JR\text{-}FL}$ pseudovirus stock that was either undiluted, or diluted 1:2, 1:3, 1:4 or 1:5 into IL-2 supplemented GM. After 3–5 days incubation at 37 °C all supernatants were removed, the cells washed in PBS and lysed with cell lysis buffer (Promega) and a luciferase assay was performed according to the Manufacturer's protocol (Promega). Light emission was measured using an Anthos Lucy 2 microplate luminometer (Jencons Ltd.). Results are expressed as Cell Culture Infectious $Dose_{50}$/ml ($CCID_{50}$) for each virus.

2.3. Pseudovirus neutralization assays using a single inhibitor

Initial neutralization assays in which pseudoviruses were challenged with a single inhibitor were used to define the 50% and 90% inhibitory doses (ID_{50} and ID_{90}) of each individual inhibitory agent. The results of these assays were later used to determine the optimum starting concentrations of each agent for experiments in which PRO 2000 and DS were combined with one of the following: IgG1b12, TAK779, T20 or CV-N. All neutralization experiments were first performed using U87.CD4.CCR5/CXCR4 target cells. In these neutralization experiments each agent was serially diluted from a starting concentration of 20 μM for PRO 2000 and DS and 667 nM, 22 μM, 100 nM and 1 μM for IgG1b12, T20, TAK779 and CV-N, respectively, and then added to 100 $TCID_{50}$ of each pseudovirus for 1 h at 37 °C. After this pre-incubation step the virus/inhibitor mixture was added to the U87 cells prepared as above. Infection without inhibitor and a cell only control were also set up. The cells were incubated for 3–5 days at 37 °C, without removal of either virus or inhibitor, after which a luciferase assay was performed on cell lysates as described above. In experiments where activated PBMCs were used as target cells, starting concentrations were 100 μM for PRO 2000 and TAK779, and 667 nM for IgG1b12. Inhibitors were serially diluted as above and then added to an equal volume of pseudoviral stock for 1 h at 37 °C to give a 1:2 dilution of pseudovirus. Controls were also set up as above. After this pre-incubation step 1×10^6 PBMCs/well in a 96 well μ-titre plate were resuspended in the pseudovirus/inhibitor mixture. PBMCs were then incubated for 5 days without removal of either virus or inhibitor, and a luciferase assay was performed on the cell lysates as described above.

2.4. Pseudovirus neutralization by PRO 2000 or DS in combination with other antiretroviral agents

After defining the optimum starting concentrations for each inhibitor we went on to study PRO 2000 and DS in combination with IgG1b12, T20, TAK779 and CV-N. Inhibition assays were set up as described above using both U87.CD4.CCR5/CXCR4 and PBMCs as target cells. In all assays inhibitors were two-fold serially diluted as a mixture so as to maintain a constant ratio between them, the appropriate method for synergy measurement. The initial concentrations of each inhibitor were chosen so as to ensure a minimum of three datum points between 20% and 80% inhibition. The assay was then performed as described above.

2.5. Analysis of synergy

Assays were performed in triplicate for U87.CD4.CCR5/CXCR4 cells and sextuplicate for PBMCs. The percentage inhibition for each concentration of each drug was calculated according to the formula: (relative light units test well − cell control)/(relative light units no inhibitor control − cell control) × 100. ID_{50} and ID_{90} values were determined using the Calcusyn™ software. Results of combination studies were processed using the Calcusyn™ software package (Chou, 1976; Chou and Talalay, 1981) that uses the multiple-drug effect analysis of Chou to analyze the effects of drug combinations. Using the median effect equation this programme plots dose-effect curves for each drug and combinations of drugs. The *x* intercept of the median effect equation gives the ID_{50} for each drug; the median effect plot also gives information on the slope of the dose-effect curve. Together this information is used to calculate the combination index (CI): a CI of <0.9 represents synergy, a CI of 0.9–1.1 represents additive effects, whilst a CI of >1.1 represents antagonism.

2.6. Analysis of cellular toxicity

U87.CD4.CCR5, U87.CD4.CXCR4 and PBMCs were exposed to PRO 2000, DS, IgG1b12, T20, TAK779 and CV-N singly for 5 days. All compounds were tested at the highest starting concentrations used in the initial individual antiviral activity assays, as detailed in Sections 2.3 and 3.1. After 5 days the cytotoxicity of each compound was measured using the tetrazolium assay (CellTiter96, Promega). Alternatively, the same cell types were exposed to PRO 2000 and DS in combination with IgG1b12, T20, TAK779 and CV-N for 5 days. All compounds were tested at the highest starting concentrations used in the combination assays detailed in Section 3.2. The assay was run as for the compounds tested individually.

3. Results

3.1. Inhibition of HIV-1 infection by individual antiretroviral agents

To obtain reliable data for synergy analysis we defined the starting concentration for each drug yielding the largest number of datum points between 80% and 20% inhibition of pseudoviral infection. The two inhibitors under investigation were then combined over this predetermined range of concentrations to allow analysis of the data using the Calcusyn™ software (Chou, 1976; Chou and Talalay, 1981). Thus, HIV-1 inhibition assays were initially performed with each agent used alone against either HIV-1_{JR-FL} or HIV-1_{HxB2}. Since R5 viruses are the dominant transmitted phenotype most experiments were carried out using HIV-1_{JR-FL}, but we also analyzed HIV-1_{HxB2} to investigate potential differences between viruses of different coreceptor tropism. We chose to introduce the inhibitors in with the infected cells prior to mixing with target cells: in other studies we have pre-mixed the inhibitors with the target cells first then added the infected cells, or added inhibitor, target and infected cells together. Since there was no statistically significant difference between these different protocols (results not shown), we decided to continue with the method used here.

Because DS did not inhibit infection of U87.CD4.CCR5 cells by HIV-1_{JR-FL} by more than 70% in any experiment (data not shown), only the ID_{50} values are shown for this inhibitor. By contrast, PRO 2000 inhibited up to 100% on both viruses, and so both ID_{50} and ID_{90} results for PRO 2000 combinations on both HIV-1_{JR-FL} and HIV-1_{HxB2} are given. Dose-reduction index (DRI) values represent the reduction in concentration of one inhibitor in the presence of the second inhibitor, as compared to each used alone, to achieve the same level of inhibition. As can be seen in Tables 1–3, all compounds were inhibitory in the low nanomolar to low micromolar range against both HIV-1_{JR-FL} and HIV-1_{HxB2} pseudoviruses, with TAK779 being the most potent inhibitor against the HIV-1_{JR-FL} pseudovirus at both the ID_{50} and ID_{90}. The inhibitors generally had lower ID_{50} and ID_{90} values against HIV-1_{HXB2} than against HIV-1_{JR-FL}: for example, ID_{90} values for IgG1b12, T20, CV-N and PRO 2000 were approximately 4-, 26-, 24- and 4-fold lower, respectively, against HIV-1_{HxB2} than against HIV-1_{JR-FL} (Table 2). It is evident from Tables 1 and 2 that there is substantial variation in the ID_{90} and ID_{50} values for PRO 2000 and DS, respectively, when used singly. As the data presented in these tables are the combined results of at least two independent experiments for each inhibitor alone or in combination (with the exceptions indicated in the table legends), this variation is therefore indicative of an expected degree of inter-assay variability.

3.2. Inhibition of HIV-1 infection by PRO 2000 and DS in combination with other antiretroviral agents

Having defined the starting concentration for each agent when used alone, we went on to study the effect of combining PRO 2000 and DS with one of IgG1b12, T20, TAK779 or CV-N. In these assays, each chosen combination of inhibitors was

Table 1
ID_{50} and DRI values for inhibitors in combination with PRO 2000

	ID_{50}[a]	ID_{50}[b]	DRI[c]
JR-FL			
PRO 2000[d]	78.7	24.03	3.3
+IgG1b12	1.04	0.4	2.6
PRO 2000	1221.0 ± 1142.4	92.5 ± 5.2	12.6 ± 11.7
+T20	427.0 ± 287.0	154.4 ± 8.7	2.9 ± 2.0
PRO 2000	230.4 ± 123.9	88.9 ± 53.6	2.8 ± 0.3
+TAK779	0.24 ± 0.2	0.04 ± 0.02	6.9 ± 1.1
PRO 2000	242.8 ± 125.4	92.3 ± 30.4	3.5 ± 2.5
+CV-N	25.3 ± 4.94	11.5 ± 3.8	2.6 ± 1.3
HxB2			
PRO 2000[d]	86.1	6.8	12.6
+IgG1b12	0.2	0.2	0.9
PRO 2000	89.7 ± 3.5	6.8 ± 2.0	14.3 ± 3.7
+T20	19.7 ± 16.3	7.6 ± 2.2	2.2 ± 1.5
PRO 2000	52.5. ± 36.6	13.5 ± 3.5	3.4 ± 1.8
+CV-N	3.5 ± 1.8	3.4 ± 0.9	1.0 ± 0.3

All values calculated by Calcusyn™ from the combined data sets of at least two independent experiments performed in triplicate.
[a] ID_{50} (nM) for each inhibitor when used individually.
[b] ID_{50} (nM) for each inhibitor in a respective combination.
[c] The dose reduction index for each inhibitor in a respective combination.
[d] Data represent the values calculated by Calcusyn™ from a single representative experiment performed in triplicate.

Table 2
ID_{90} and DRI values for inhibitors in combination with PRO 2000

	ID_{90}[a]	ID_{90}[b]	DRI[c]
JR-FL			
PRO 2000[d]	1313.2	170.2	7.7
+IgG1b12	8.6	2.8	3.2
PRO 2000	2710.91 ± 1397.8	497.6 ± 63.4	5.9 ± 3.6
+T20	6895.1 ± 5759.3	831.0 ± 105.9	10.2 ± 7.2
PRO 2000	3116.6 ± 1580.9	594.7 ± 364.7	5.8 ± 0.89
+TAK779	2.85 ± 1.25	0.2 ± 0.1	14.2 ± 3.2
PRO 2000	7151.5 ± 2380.5	424.3 ± 123.7	16.6 ± 0.8
+CV-N	1834.8 ± 1140.2	53.0 ± 15.5	31.0 ± 12.5
HxB2			
PRO 2000[d]	974.5	35.2	27.7
+IgG1b12	2.2	1.2	1.9
PRO 2000	1276.3 ± 301.8	88.0 ± 5.8	14.2 ± 2.6
+T20	265.5 ± 88.0	98.1 ± 6.5	2.8 ± 1.1
PRO 2000	196.8. ± 127.7	214.9 ± 101.2	0.8 ± 0.2
+CV-N	76.5 ± 32.6	53.7 ± 25.3	1.5 ± 0.1

All values calculated by Calcusyn™ from the combined data sets of at least two independent experiments performed in triplicate.
[a] ID_{90} (nM) for each inhibitor when used individually.
[b] ID_{90} (nM) for each inhibitor in a respective combination.
[c] The dose reduction index for each inhibitor in a respective combination.
[d] Data represent the values calculated by Calcusyn™ from a single representative experiment performed in triplicate.

Table 3
ID_{50} and DRI values for inhibitors in combination with DS

	ID_{50}[a]	ID_{50}[b]	DRI[c]
JR-FL			
DS	2.3 ± 1.2	0.3 ± 0.1	12.5 ± 9.5
+IgG1b12	1.0 ± 0.3	0.6 ± 0.2	1.7 ± 0.2
DS	7.0 ± 5.8	6.3 ± 6.1	2.8 ± 1.7
+T20	5438.2 ± 5351.8	1318.9 ± 1264.3	2.9 ± 1.3
DS	5.32 ± 0.8	1.0 ± 0.4	5.7 ± 1.4
+TAK779	365.1 ± 56.5	50.1 ± 19.1	9.0 ± 4.6
DS	3.8 ± 0.4	0.6 ± 0.2	6.1 ± 0.8
+CV-N	25.3 ± 4.9	10.0 ± 2.3	2.8 ± 1.1

All values calculated by Calcusyn™ from the combined data sets of at least two independent experiments performed in triplicate.
[a] The ID_{50} (nM) for each inhibitor when used individually.
[b] ID_{50} (nM) for each inhibitor in a respective combination.
[c] The dose reduction index for each inhibitor in a respective combination.

diluted as a mixture so as to maintain a constant ratio between them, which allowed subsequent analysis of synergy. A summary of the synergy analysis of the combination studies using U87.CD4.CCR5/CXCR4 target cells is presented in Table 4, with the ID_{50} and ID_{90} concentration for drugs in combination with PRO 2000 shown in Tables 1 and 2, respectively, and the ID_{50} concentrations for inhibitors in combination with DS in Table 3. Table 4 illustrates that in general the inhibitor combinations synergize against both HIV-$1_{JR\text{-}FL}$ and HIV-1_{HxB2} pseudoviruses, with some exceptions. PRO 2000 with CV-N against HIV-1_{HxB2} was antagonistic at all concentrations tested, PRO 2000 with IgG1b12 against HIV-1_{HxB2} was antagonistic at the IC_{50}, PRO 2000 with T20 and CV-N were weakly antagonistic or additive for HIV-$1_{JR\text{-}FL}$ infection at the IC_{50} and DS with T20 was antagonistic at the IC_{50} and additive at other concentrations (Table 4). With many combinations there was a trend for increasing synergy with increasing dose. At the ID_{90}, the strongest synergy with PRO 2000 was seen with CV-N (CI = 0.1) and TAK779 (CI = 0.26). At the theoretical ID_{90} for DS, the strongest synergy was seen with TAK779 (CI = 0.05), IgG1b12 (CI = 0.2) and CV-N (CI = 0.22)

Table 4
CI values for PRO 2000 and DS in combination with other inhibitors

Inhibitors	CI		
	50%	75%	90%
JR-FL			
PRO2000:IgG1b12	0.59 ± 0.11[a]	0.54 ± 0.03	0.50 ± 0.04
PRO2000:T20	1.26 ± 0.06	0.75 ± 0.06	0.46 ± 0.02
PRO2000:TAK779	0.52 ± 0.02	0.36 ± 0.04	0.26 ± 0.05
PRO2000:CV-N	1.11 ± 0.69	0.32 ± 0.14	0.10 ± 0.02
DS:IgG1b12	0.78 ± 0.22	0.39 ± 0.11	0.20 ± 0.06
DS:T20	3.9 ± 3.1	0.7 ± 0.48	0.93 ± 0.83
DS:TAK779	0.34 ± 0.12	0.11 ± 0.00	0.05 ± 0.02
DS: CV-N	0.60 ± 0.20	0.33 ± 0.15	0.22 ± 0.13
HxB_2			
PRO2000:IgG1b12	1.38 ± 0.19	0.97 ± 0.15	0.69 ± 0.12
PRO2000:T20	0.98 ± 0.61	0.56 ± 0.14	0.47 ± 0.13
PRO2000:CV-N	1.56 ± 0.55	1.60 ± 0.40	2.00 ± 0.30
DS:IgG1b12	ND[b]	ND	ND
DS:T20	ND	ND	ND
DS:CV-N	ND	ND	ND

[a] Combination index (CI) < 0.9 indicates synergy; CI 0.9–1.1 indicates addition; CI > 1.1 indicates antagonism. Values show the mean of at least two independent experiments performed in triplicate ± S.E.M.
[b] ND represents not done.

Tables 1–3 also show the dose reduction index (DRI) for each drug in a given combination. The DRI is the ratio of drug inhibition alone:drug inhibition in combination. Thus a DRI of >1 indicates a decrease in the concentration of drug required to achieve the same level of inhibition as that achieved when used alone. From the ID_{90} DRI values in Table 2 it can be seen that the most successful combinations against HIV-$1_{JR\text{-}FL}$ for PRO 2000 were CV-N and IgG1b12, yielding 31- and 7.7-fold reductions respectively for PRO 2000. Similarly, the DRI for DS at the ID_{50} was 12.5, 6.1 and 5.7 for IgG1b12, CV-N and TAK779, respectively (Table 3). Thus there was good concordance between strong synergy and strong dose reduction for PRO 2000 with CV-N, and for DS with TAK779, IgG1b12 and CV-N. When these experiments were repeated using the HIV-1_{HxB2} pseudovirus, the greatest DRI values at ID_{90} for PRO 2000 were achieved when PRO 2000 was combined with IgG1b12 (27.7) and T20 (14.2).

3.3. Inhibition of HIV-1 infection of activated PBMCs by PRO 2000 in combination with other antiretroviral agents

Having demonstrated that PRO 2000 and DS could work in synergy with other antiretroviral agents to inhibit infection of U87.CD4.CCR5/CXCR4 target cells by HIV-1 pseudoviruses, it was important to investigate synergy in a more physiologically relevant target cell. For this reason we went on to develop a combination assay in which activated PBMCs were the target cells for infection by HIV-$1_{JR\text{-}FL}$ pseudovirus, and performed experiments where PRO 2000 was combined with either IgG1b12 or TAK779. These two combinations were chosen as they yielded the strongest and most reproducible synergy across the concentrations tested in U87.CD4.CCR5. PBMC were activated for 7–10 days prior to infection to maximize CCR5 expression (data not shown). As above, ID_{90} values and therefore initial starting concentrations of each drug for combination studies were defined by carrying out inhibition assays in which each agent was used individually. The results of these experiments are shown in Tables 5 (ID_{50}) and 6 (ID_{90}), and reveal that for all inhibitors there was a significant decrease in potency in PBMC, an effect most marked for TAK779.

Having established optimum starting concentrations for each drug, combination assays were set up in exactly the same manner as described previously and the resulting data were analyzed (Tables 6 and 7), and CI values calculated (Table 7) using Calcusyn™. Consistent with experiments in which U87.CD4.CCR5 target cells were used, PRO 2000 synergized with TAK779 in this primary cell system and showed a trend for increasing synergy with increasing dose, with CI and DRI values that were similar to those achieved in the U87.CD4.CCR5 system (Tables 5 and 6). However, as previously noted there was a marked decrease in the potency of both

Table 5
ID_{50} concentrations and DRI values for inhibitors in combination with PRO 2000 against HIV-1_{JR-FL} infection of PBMC cultures

	ID_{50}[a]	ID_{50}[b]	DRI[c]
PRO 2000	2011.0 ± 1395.8	496.9 ± 240.6	3.5 ± 1.1
+IgG1b12	8.9 ± 3.9	3.5 ± 3.1	8.3 ± 6.4
PRO 2000	2282.7 ± 1124.1	686.7 ± 403.3	3.6 ± 0.5
+TAK-799	870.0 ± 711.8	228.9 ± 134.4	3.0 ± 1.3

All values calculated by Calcusyn™ from the combined data sets of at least two independent experiments performed in triplicate.
[a] ID_{50} (nM) for each inhibitor when used individually.
[b] ID_{50} (nM) for each inhibitor in a respective combination.
[c] The dose reduction index for each inhibitor in a respective combination.

Table 6
ID_{90} concentrations and DRI values for inhibitors in combination with PRO 2000 against HIV-1_{JR-FL} infection of PBMC cultures

	ID_{90}[a]	ID_{90}[b]	DRI[c]
PRO 2000	9352.5 ± 5957.6	5758.1 ± 738.5	1.8 ± 1.3
+IgG1b12	69.2 ± 14.2	26.7 ± 18.0	5.4 ± 4.2
PRO 2000	10585.0 ± 4722.7	1535.3 ± 872.8	7.6 ± 1.2
+TAK-799	8796.0 ± 795.1	511.8 ± 290.9	26.7 ± 16.7

All values calculated by Calcusyn™ from the combined data sets of at least two independent experiments performed in triplicate.
[a] ID_{90} (nM) for each inhibitor when used individually.
[b] ID_{90} (nM) for each inhibitor in a respective combination.
[c] The dose reduction index for each inhibitor in a respective combination.

agents when used in primary cells. Thus although the CI values for the two target cell systems are very similar, the absolute concentrations of both agents in combination needed to inhibit 90% of infection by HIV-1_{JR-FL} in PBMC are higher (Tables 5 and 6).

When PRO 2000 was combined with IgG1b12, moderate synergy was seen at low concentrations of both drugs (CI at $ID_{50} = 0.607$). However, unlike the results obtained in U87 cells, antagonism was seen at higher concentrations of both agents (CI at $ID_{90} = 1.579$). Consistent with experiments using this combination in the U87 system, potency was lower against HIV-1_{JR-FL} infection in PBMC, showing ∼34- and ∼10-fold reductions in ID_{90} values for PRO 2000 and IgG1b12, respectively (Table 6).

3.4. Toxicity of the inhibitors

All of the inhibitors used here have been tested for toxicity in vitro or for safety in vivo singly, or in limited combinations,

Table 7
CI values for PRO 2000 in combination with IgG1b12 and TAK-799 against HIV-1_{JR-FL} infection of PBMCs

Inhibitors	CI		
	50%	75%	90%
PRO 2000:IgG1b12	0.61 ± 0.17[a]	0.94 ± 0.05	1.58 ± 0.62
PRO 2000:TAK-799	0.69 ± 0.21	0.33 ± 0.07	0.20 ± 0.09

[a] Combination index (CI) < 0.9 indicates synergy; CI 0.9–1.1 indicates addition; CI > 1.1 indicates antagonism. Values show the mean of at least two independent experiments performed in triplicate ± 1 S.D.

Table 8
Metabolic activity of cells exposed to individual inhibitors for 5 days

	U87.CD4.R5	U87.CD4.X4	PBMC
PRO2000	71.3 ± 5.0[a]	87.1 ± 5.2	97.4 ± 28.9
DS	81.8 ± 7.1	104.5 ± 2.5	NA
IgG1b12	116.2 ± 11.6	111.0 ± 8.1	122.5 ± 11.5
T20	74.3 ± 2.6	103.0 ± 5.1	NA
TAK779	84.0 ± 5.1	NA[b]	87.5 ± 1.5
CV-N	47.4 ± 14.4	41.9 ± 11.9	NA

[a] Cell viability is represented as a % of the value for cells not treated with inhibitor. Results represent the mean of triplicate wells ± 1 S.D.
[b] Not applicable.

Table 9
Metabolic activity of cells exposed to combinations of inhibitors for 5 days

	U87.CD4.R5	U87.CD4.X4	PBMC
PRO2000:IgG1b12	107.8 ± 6.2[a]	109.3 ± 5.5	90.3 ± 14.6
PRO2000:T20	96.2 ± 2.1	111.7 ± 3.6	NA[b]
PRO2000:TAK779	78.3 ± 2.3	NA	94.9 ± 7.5
PRO2000:CV-N	78.3 ± 8.8	85.5 ± 9.7	NA
DS:IgG1b12	114.8 ± 19.9	NA	NA
DS:T20	106.7 ± 5.2	NA	NA
DS:TAK779	77.2 ± 0.5	NA	NA
DS:CV-N	47.4 ± 14.4	NA	NA

[a] Cell viability is represented as a % of the value for cells not treated with inhibitor. Results represent the mean of triplicate wells ± 1 S.D.
[b] Not applicable.

in previous studies (Boyd et al., 1997; Veazey et al., 2005; Lederman et al., 2006; Mauck et al., 2001; Mayer et al., 2003; Stone, 2002; Turpin, 2002; Van Damme et al., 2000, 2002; Weber et al., 2001, 2005; Weber and Lacey, 2001). Nevertheless, to exclude the possibility of toxicity in the tissue culture systems used here, and to analyze toxicity in combinations, we tested the inhibitors for their effect on cell metabolism in U87 and activated PBMC. Inhibitors were tested at the highest concentrations used in the inhibition studies above, whether used singly (Table 8) or in combinations (Table 9), and were left on the cells for 5 days, the maximum time used in any of the assays. Although there was some expected small variation, this stringent test nevertheless failed to demonstrate any significant reduction in cell metabolic function under any conditions as demonstrated by ANOVA: no group of test samples was significantly different from the control sample without inhibitor (P values all >0.05). We therefore confirm that under the conditions of the studies carried out here, toxicity is probably not influencing the outcome of the observed inhibitory effects.

4. Discussion

Our results demonstrate that combinations of the candidate microbicides PRO 2000 and DS synergize with several other known inhibitors of HIV-1 in vitro, providing a rationale for further development of combination microbicide formulations using these agents. We chose to study PRO 2000 and DS, as polyanionic compounds represent two of the most clinically advanced candidate microbicides that are relatively cheap and easy to manufacture, highly stable, and non-toxic (Lederman et

al., 2006; Mauck et al., 2001; Mayer et al., 2003; Stone, 2002; Turpin, 2002; Van Damme et al., 2000, 2002; Weber et al., 2001, 2005; Weber and Lacey, 2001). IgG1b12 and CV-N are antiviral when used singly, as shown by the partial or complete protection of macaques from SHIV challenge after topical administration (Tachibana et al., 1998; Tsai et al., 2003, 2004; Veazey et al., 2003b). The concept of using small molecule inhibitors targeted to the CCR5 receptor (such as TAK779) as microbicides, has been demonstrated in macaque challenge experiments using the RANTES analogue PSC-RANTES and CMPD167, another small molecule CCR5 antagonist (Lederman et al., 2004; Veazey et al., 2003a, 2005). Although these inhibitors were non-toxic, they were used at very high concentrations; between 10^3- and 10^6-fold higher than the ID_{50} concentration in vitro. The recent demonstration that combinations of HIV-1 entry inhibitors found to be synergistic in vitro, also appear to work in synergy in vivo, suggests that it may be possible to achieve substantial dose reductions through the combination of synergistic agents (Veazey et al., 2005). CV-N has good potential as far as use as a potential microbicide: it interacts directly with the virus, can be synthesized relatively cheaply as a recombinant protein in *E. coli*, in *Lactobacillus* spp and in transgenic plants (Colleluori et al., 2005; Pusch et al., 2005; Sexton et al., 2006), is non-toxic at relatively high concentrations and is very stable (Boyd et al., 1997). However, although not statistically significant in our analysis, there was a clear trend towards toxicity with CV-N, suggesting that caution should be exercised when using this compound at high concentrations. By contrast with the above-mentioned agents, IgG1b12, TAK779 and other CCR5 inhibitors are relatively expensive to produce; therefore, reducing the amount of either compound by using them in synergistic combinations would be advantageous when considering their use as microbicides. However, our data showing that the greatest synergy is obtained at the highest doses of each agent used (with the exceptions of PRO 2000 + CV-N against HxB2 in Table 4 and PRO 2000 + IgG1b12 against JR-FL in Table 7), imply that the highest feasible doses of each inhibitor should be tested in vivo.

Our data showing that the inhibitors used singly inhibit both an R5 PI and an X4 T cell line adapted (TCLA) pseudovirus in the picomolar to nanomolar range are in accord with previous work using infectious virus in multicycle assays and syncytium inhibition assays (Baba et al., 1999; Boyd et al., 1997; Burton et al., 1994; Dragic et al., 2000; Rusconi et al., 1996; Scordi-Bello et al., 2005; Shaunak et al., 1994, 2003; Wild et al., 1994). This validates our use of a single cycle assay for these studies with its inherent advantages for studying inhibition and synergy (Ferguson et al., 2001). We demonstrate that all of the inhibitors (with the exception of TAK779 that was not tested on HIV-1_{HXB2}) are more active against HIV-1_{HXB2} than HIV-$1_{JR\text{-}FL}$. This is in accord with several studies demonstrating that X4 T cell line adapted (TCLA) viruses are more sensitive to polyanions (Moulard et al., 2000), to neutralizing antibodies (Moore and Ho, 1995), and to T20 (Derdeyn et al., 2000, 2001). Greater inhibition by polyanions of X4 TCLA viruses compared to R5 viruses is most likely due to the greater positive charge of X4 gp120, allowing stronger binding to polyanions (Moulard et al., 2000). Although not fully elucidated, neutralizing antibodies are likely to be more active against TCLA viruses because the epitopes are better exposed on the Env trimer (Sattentau and Moore, 1995). To date, the greater sensitivity of X4 TCLA viruses to T20 is not understood, but may result from greater accessibility of the agent to sites on gp41. However, in the context of topical microbicide use, X4 virus sensitivity is somewhat academic as, with very rare exceptions, R5 viruses are the transmitted phenotype (Moore et al., 2004; Shattock and Doms, 2002).

One concern with the use of CCR5 inhibitors such as TAK779 as microbicides is that ex vivo studies have found that while CCR5 inhibitors prevent localized infection of cervical explants, they are not capable of preventing the uptake of HIV-1 by dendritic cells (DCs) and their subsequent migration out of the tissue (Hu et al., 2004). The administration of synergistic mixtures of CCR5 antagonists with agents such as PRO 2000 that bind virus directly and inhibit gp120 interacting with molecules such as DC-SIGN on the surface of DCs may be a strategy to overcome these limitations. Finally, the synergy observed between the polyanions and IgG1b12 suggests the idea that anionic microbicides might synergize with neutralizing antibodies raised actively by immunization.

It is presently unclear what the molecular mechanisms are that determine the synergy that we have observed. However, a general mechanism may be that the binding of polyanions to the Env spike alters its conformation, potentially increasing exposure of binding sites for IgG1b12, CV-N and possibly, although less likely, T20. In support of this concept, dextran sulfate induces the shedding of gp120 from HIV-1 virions (Bugelski et al., 1991 and our unpublished results), confirming that polyanions can induce conformational changes in Env. Moreover polyanions have been shown to alter gp120 V2 loop conformation (Jagodzinski et al., 1999), potentially increasing exposure of the CD4 binding surface. Alternatively, the polymeric polyanions might interact with both Env and the other inhibitors, forming a 'bridge' between them that would increase access of the inhibitors to the Env surface. A further possibility that we cannot exclude is that the polyanions act directly on the other inhibitors to increase their anti-HIV-1 activity, perhaps by altering their conformation or by multimerizing them.

On a more specific level, the synergy between PRO 2000/DS and TAK779 against HIV-$1_{JR\text{-}FL}$ appears to have a greater effect on TAK779 (reducing the ID_{90} or ID_{50} values by 19.2- and 4-fold, respectively) than on the polyanions. We have previously shown that dextran sulfate interferes with the Env-chemokine receptor interaction (Moulard et al., 2000). Thus the binding of PRO 2000 or DS to HIV-1 Env may render gp120 less able to bind CCR5, which would lead to a reduction in the ID_{50} values for TAK779. Synergy between a CCR5 antagonist (SCH-C) and T20 in vitro was demonstrated previously, with a therapeutic rather than prophylactic objective (Tremblay et al., 2002). They proposed similarly that synergy might be explained by the CCR5 antagonist interfering with Env-coreceptor-induced conformational changes thus allowing greater access of T20 to its complementary binding surface on gp41. Such an explanation may also apply to the synergy observed here in combinations of polyanions and T20. Although beyond the scope of the present study, such hypotheses can and should be tested experimentally

to gain further insight into the molecular mechanisms underlying synergy between candidate HIV-1 microbicides.

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References

AIDS, U., 2004. AIDS Epidemic Update (2004).

Anderson, R.A., Feathergill, K.A., Diao, X.H., Cooper, M.D., Kirkpatrick, R., Herold, B.C., Doncel, G.F., Chany, C.J., Waller, D.P., Rencher, W.F., Zaneveld, L.J., 2002. Preclinical evaluation of sodium cellulose sulfate (Ushercell) as a contraceptive antimicrobial agent. J. Androl. 23, 426–438.

Baba, M., Pauwels, R., Balzarini, J., Arnout, J., Desmyter, J., De Clercq, E., 1988a. Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus in vitro. Proc. Natl. Acad. Sci. U.S.A. 85, 6132–6136.

Baba, M., Snoeck, R., Pauwels, R., De Clercq, E., 1988b. Sulfated polysaccharides are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and human immunodeficiency virus. Antimicrob. Agents Chemother. 32, 1742–1745.

Baba, M., Nishimura, O., Kanzaki, N., Okamoto, M., Sawada, H., Iizawa, Y., Shiraishi, M., Aramaki, Y., Okonogi, K., Ogawa, Y., Meguro, K., Fujino, M., 1999. A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. Proc. Natl. Acad. Sci. U.S.A. 96, 5698–5703.

Bagasra, O., Lischner, H.W., 1988. Activity of dextran sulfate and other polyanionic polysaccharides against human immunodeficiency virus. J. Infect. Dis. 158, 1084–1087.

Berger, E.A., Doms, R.W., Fenyo, E.M., Korber, B.T., Littman, D.R., Moore, J.P., Sattentau, Q.J., Schuitemaker, H., Sodroski, J., Weiss, R.A., 1998. A new classification for HIV-1. Nature 391, 240.

Bobardt, M.D., Armand-Ugon, M., Clotet, I., Zhang, Z., David, G., Este, J.A., Gallay, P.A., 2004. Effect of polyanion-resistance on HIV-1 infection. Virology 325, 389–398.

Bolmstedt, A.J., O'Keefe, B.R., Shenoy, S.R., McMahon, J.B., Boyd, M.R., 2001. Cyanovirin-N defines a new class of antiviral agent targeting N-linked, high-mannose glycans in an oligosaccharide-specific manner. Mol. Pharmacol. 59, 949–954.

Boyd, M.R., Gustafson, K.R., McMahon, J.B., Shoemaker, R.H., O'Keefe, B.R., Mori, T., Gulakowski, R.J., Wu, L., Rivera, M.I., Laurencot, C.M., Currens, M.J., Cardellina II., J.H., Buckheit Jr., R.W., Nara, P.L., Pannell, L.K., Sowder II, R.C., Henderson, L.E., 1997. Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development. Antimicrob. Agents Chemother. 41, 1521–1530.

Bugelski, P.J., Ellens, H., Hart, T.K., Kirsh, R.L., 1991. Soluble CD4 and dextran sulfate mediate release of gp120 from HIV-1: implications for clinical trials. J. Acquir. Immune Defic. Syndr. 4, 923–924.

Burton, D.R., Pyati, J., Koduri, R., Sharp, S.J., Thornton, G.B., Parren, P.W., Sawyer, L.S., Hendry, R.M., Dunlop, N., Nara, P.L., et al., 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 266, 1024–1027.

Callahan, L.N., Phelan, M., Mallinson, M., Norcross, M.A., 1991. Dextran sulfate blocks antibody binding to the principal neutralizing domain of human immunodeficiency virus type 1 without interfering with gp120–CD4 interactions. J. Virol. 65, 1543–1550.

Chen, B.K., Saksela, K., Andino, R., Baltimore, D., 1994. Distinct modes of human immunodeficiency virus type 1 proviral latency revealed by superinfection of nonproductively infected cell lines with recombinant luciferase-encoding viruses. J. Virol. 68, 654–660.

Chou, T.C., 1976. Derivation and properties of Michaelis–Menten type and Hill type equations for reference ligands. J. Theor. Biol. 59, 253–276.

Chou, T.C., Talalay, P., 1981. Generalized equations for the analysis of inhibitions of Michaelis–Menten and higher-order kinetic systems with two or more mutually exclusive and nonexclusive inhibitors. Eur. J. Biochem. 115, 207–216.

Colleluori, D.M., Tien, D., Kang, F., Pagliei, T., Kuss, R., McCormick, T., Watson, K., McFadden, K., Chaiken, I., Buckheit Jr., R.W., Romano, J.W., 2005. Expression, purification, and characterization of recombinant cyanovirin-N for vaginal anti-HIV microbicide development. Protein Expr. Purif. 39, 229–236.

Connor, R.I., Chen, B.K., Choe, S., Landau, N.R., 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology 206, 935–944.

de Parseval, A., Bobardt, M.D., Chatterji, A., Chatterji, U., Elder, J.H., David, G., Zolla-Pazner, S., Farzan, M., Lee, T.H., Gallay, P.A., 2005. A highly conserved arginine in gp120 governs HIV-1 binding to both syndecans and CCR5 via sulfated motifs. J. Biol. Chem. 280, 39493–39504.

Derdeyn, C.A., Decker, J.M., Sfakianos, J.N., Wu, X., O'Brien, W.A., Ratner, L., Kappes, J.C., Shaw, G.M., Hunter, E., 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J. Virol. 74, 8358–8367.

Derdeyn, C.A., Decker, J.M., Sfakianos, J.N., Zhang, Z., O'Brien, W.A., Ratner, L., Shaw, G.M., Hunter, E., 2001. Sensitivity of human immunodeficiency virus type 1 to fusion inhibitors targeted to the gp41 first heptad repeat involves distinct regions of gp41 and is consistently modulated by gp120 interactions with the coreceptor. J. Virol. 75, 8605–8614.

Dey, B., Lerner, D.L., Lusso, P., Boyd, M.R., Elder, J.H., Berger, E.A., 2000. Multiple antiviral activities of cyanovirin-N: blocking of human immunodeficiency virus type 1 gp120 interaction with CD4 and coreceptor and inhibition of diverse enveloped viruses. J. Virol. 74, 4562–4569.

Dezzutti, C.S., James, V.N., Ramos, A., Sullivan, S.T., Siddig, A., Bush, T.J., Grohskopf, L.A., Paxton, L., Subbarao, S., Hart, C.E., 2004. In vitro comparison of topical microbicides for prevention of human immunodeficiency virus type 1 transmission. Antimicrob. Agents Chemother. 48, 3834–3844.

Dragic, T., Trkola, A., Thompson, D.A., Cormier, E.G., Kajumo, F.A., Maxwell, E., Lin, S.W., Ying, W., Smith, S.O., Sakmar, T.P., Moore, J.P., 2000. A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. Proc. Natl. Acad. Sci. U.S.A. 97, 5639–5644.

Esser, M.T., Mori, T., Mondor, I., Sattentau, Q.J., Dey, B., Berger, E.A., Boyd, M.R., Lifson, J.D., 1999. Cyanovirin-N binds to gp120 to interfere with CD4-dependent human immunodeficiency virus type 1 virion binding, fusion, and infectivity but does not affect the CD4 binding site on gp120 or soluble CD4-induced conformational changes in gp120. J. Virol. 73, 4360–4371.

Esté, J.A., Schols, D., De Vreese, K., Van Laethem, K., Vandamme, A.M., Desmyter, J., De Clercq, E., 1997. Development of resistance of human immunodeficiency virus type 1 to dextran sulfate associated with the emergence of specific mutations in the envelope gp120 glycoprotein. Mol. Pharmacol. 52, 98–104.

Ferguson, N.M., Fraser, C., Anderson, R.M., 2001. Viral dynamics and anti-viral pharmacodynamics: rethinking in vitro measures of drug potency. Trends Pharmacol. Sci. 22, 97–100.

Furuta, R.A., Wild, C.T., Weng, Y., Weiss, C.D., 1998. Capture of an early fusion-active conformation of HIV-1 gp41. Nat. Struct. Biol. 5, 276–279.

Harrop, H.A., Rider, C.C., 1998. Heparin and its derivatives bind to HIV-1 recombinant envelope glycoproteins, rather than to recombinant HIV-1 receptor, CD4. Glycobiology 8, 131–137.

Harrop, H.A., Coombe, D.R., Rider, C.C., 1994. Heparin specifically inhibits binding of V3 loop antibodies to HIV-1 gp120, an effect potentiated by CD4 binding. AIDS 8, 183–192.

Hu, Q., Frank, I., Williams, V., Santos, J.J., Watts, P., Griffin, G.E., Moore, J.P., Pope, M., Shattock, R.J., 2004. Blockade of attachment and fusion receptors inhibits HIV-1 infection of human cervical tissue. J. Exp. Med. 199, 1065–1075.

Jagodzinski, P.P., Wierzbicki, A., Wustner, J., Kaneko, Y., Kozbor, D., 1999. Enhanced human immunodeficiency virus infection in macrophages by high-molecular-weight dextran sulfate is associated with conformational changes of gp120 and expression of the CCR5 receptor. Viral Immunol. 12, 23–33.

Konlee, M., 1998. Sulfated polysaccharides (chondroitin sulfate and carrageenan) plus glucosamine sulfate are potent inhibitors of HIV. Posit. Health News 17, 4–7.

Lederman, M.M., Veazey, R.S., Offord, R., Mosier, D.E., Dufour, J., Mefford, M., Piatak Jr., M., Lifson, J.D., Salkowitz, J.R., Rodriguez, B., Blauvelt, A., Hartley, O., 2004. Prevention of vaginal SHIV transmission in rhesus macaques through inhibition of CCR5. Science 306, 485–487.

Lederman, M.M., Offord, R.E., Hartley, O., 2006. Microbicides and other topical strategies to prevent vaginal transmission of HIV. Nat. Rev. Immunol. 6, 371–382.

Matthews, T., Salgo, M., Greenberg, M., Chung, J., DeMasi, R., Bolognesi, D., 2004. Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. Nat. Rev. Drug Discov. 3, 215–225.

Mauck, C., Weiner, D.H., Ballagh, S., Creinin, M., Archer, D.F., Schwartz, J., Pymar, H., Lai, J.J., Callahan, M., 2001. Single and multiple exposure tolerance study of cellulose sulfate gel: a phase I safety and colposcopy study. Contraception 64, 383–391.

Mayer, K.H., Karim, S.A., Kelly, C., Maslankowski, L., Rees, H., Profy, A.T., Day, J., Welch, J., Rosenberg, Z., 2003. Safety and tolerability of vaginal PRO 2000 gel in sexually active HIV-uninfected and abstinent HIV-infected women. AIDS 17, 321–329.

McClure, M.O., Moore, J.P., Blanc, D.F., Scotting, P., Cook, G.M., Keynes, R.J., Weber, J.N., Davies, D., Weiss, R.A., 1992. Investigations into the mechanism by which sulfated polysaccharides inhibit HIV infection in vitro. AIDS Res. Hum. Retroviruses 8, 19–26.

Moore, J.P., Ho, D.D., 1995. HIV-1 neutralization: the consequences of viral adaptation to growth on transformed T cells. AIDS 9 (Suppl. A), S117–S136.

Moore, J.P., Kitchen, S.G., Pugach, P., Zack, J.A., 2004. The CCR5 and CXCR4 coreceptors—central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res. Hum. Retroviruses 20, 111–126.

Mori, T., Boyd, M.R., 2001. Cyanovirin-N, a potent human immunodeficiency virus-inactivating protein, blocks both CD4-dependent and CD4-independent binding of soluble gp120 (sgp120) to target cells, inhibits sCD4-induced binding of sgp120 to cell-associated CXCR4, and dissociates bound sgp120 from target cells. Antimicrob. Agents Chemother. 45, 664–672.

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

Neurath, A.R., Strick, N., Li, Y.Y., Debnath, A.K., 2001. Cellulose acetate phthalate, a common pharmaceutical excipient, inactivates HIV-1 and blocks the coreceptor binding site on the virus envelope glycoprotein gp120. BMC Infect. Dis. 1, 17.

Neurath, A.R., Strick, N., Jiang, S., Li, Y.Y., Debnath, A.K., 2002a. Anti-HIV-1 activity of cellulose acetate phthalate: synergy with soluble CD4 and induction of "dead-end" gp41 six-helix bundles. BMC Infect. Dis. 2, 6.

Neurath, A.R., Strick, N., Li, Y.Y., 2002b. Anti-HIV-1 activity of anionic polymers: a comparative study of candidate microbicides. BMC Infect. Dis. 2, 27.

Palella Jr., F.J., Delaney, K.M., Moorman, A.C., Loveless, M.O., Fuhrer, J., Satten, G.A., Aschman, D.J., Holmberg, S.D., 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection HIV Outpatient Study Investigators. N. Engl. J. Med. 338, 853–860.

Pusch, O., Boden, D., Hannify, S., Lee, F., Tucker, L.D., Boyd, M.R., Wells, J.M., Ramratnam, B., 2005. Bioengineering lactic acid bacteria to secrete the HIV-1 virucide cyanovirin. J Acquir. Immune Defic. Syndr. 40, 512–520.

Rusconi, S., Moonis, M., Merrill, D.P., Pallai, P.V., Neidhardt, E.A., Singh, S.K., Willis, K.J., Osburne, M.S., Profy, A.T., Jenson, J.C., Hirsch, M.S., 1996. Naphthalene sulfonate polymers with CD4-blocking and anti-human immunodeficiency virus type 1 activities. Antimicrob. Agents Chemother. 40, 234–236.

Sattentau, Q.J., Moore, J.P., 1995. Human immunodeficiency virus type 1 neutralization is determined by epitope exposure on the gp120 oligomer. J. Exp. Med. 182, 185–196.

Scordi-Bello, I., Mosoian, A., He, C., Chen, Y., Cheng, Y., Jarvis, G., Keller, M., Hogarty, K., Waller, D.P., Profy, A., Herold, B.C., Klotman, M.E., 2005. Candidate sulfonated and sulfated topical microbicides: comparison of anti-human immunodeficiency virus activities and mechanisms of action. Antimicrob. Agents Chemother. 49, 3607–3615.

Sexton, A., Drake, P.M., Mahmood, N., Harman, S.J., Shattock, R.J., Ma, J.K., 2006. Transgenic plant production of cyanovirin-N, an HIV microbicide. FASEB J. 20, 356–358.

Shattock, R.J., Doms, R.W., 2002. AIDS models: microbicides could learn from vaccines. Nat. Med. 8, 425.

Shaunak, S., Gooderham, N.J., Edwards, R.J., Payvandi, N., Javan, C.M., Baggett, N., MacDermot, J., Weber, J.N., Davies, D.S., 1994. Infection by HIV-1 blocked by binding of dextrin 2-sulphate to the cell surface of activated human peripheral blood mononuclear cells and cultured T-cells. Br. J. Pharmacol. 113, 151–158.

Shaunak, S., Thornton, M., Teo, I., Chandler, B., Jones, M., Steel, S., 2003. Optimisation of the degree of sulfation of a polymer based construct to block the entry of HIV-1 into cells. J. Drug Target. 11, 443–448.

Stone, A., 2002. Microbicides: a new approach to preventing HIV and other sexually transmitted infections. Nat. Rev. Drug Discov. 1, 977–985.

Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., Kishimoto, T., Nagasawa, T., 1998. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature 393, 591–594.

Tremblay, C.L., Giguel, F., Kollmann, C., Guan, Y., Chou, T.C., Baroudy, B.M., Hirsch, M.S., 2002. Anti-human immunodeficiency virus interactions of SCH-C (SCH 351125), a CCR5 antagonist, with other antiretroviral agents in vitro. Antimicrob. Agents Chemother. 46, 1336–1339.

Tsai, C.C., Emau, P., Jiang, Y., Agy, M.B., Shattock, R.J., Schmidt, A., Morton, W.R., Gustafson, K.R., Boyd, M.R., 2004. Cyanovirin-N inhibits AIDS virus infections in vaginal transmission models. AIDS Res. Hum. Retroviruses 20, 11–18.

Tsai, C.C., Emau, P., Jiang, Y., Tian, B., Morton, W.R., Gustafson, K.R., Boyd, M.R., 2003. Cyanovirin-N gel as a topical microbicide prevents rectal transmission of SHIV89 6P in macaques. AIDS Res. Hum. Retroviruses 19, 535–541.

Turpin, J.A., 2002. Considerations and development of topical microbicides to inhibit the sexual transmission of HIV. Expert Opin. Investig. Drugs 11, 1077–1097.

Van Damme, L., Wright, A., Depraetere, K., Rosenstein, I., Vandersmissen, V., Poulter, L., McKinlay, M., Van Dyck, E., Weber, J., Profy, A., Laga, M., Kitchen, V., 2000. A phase I study of a novel potential intravaginal microbicide, PRO, 2000, in healthy sexually inactive women. Sex. Transm. Infect. 76, 126–130.

Van Damme, L., Jespers, V., Van Dyck, E., Chapman, A., 2002. Penile application of dextrin sulphate gel (Emmelle). Contraception 66, 133–136.

Veazey, R.S., Klasse, P.J., Ketas, T.J., Reeves, J.D., Piatak Jr., M., Kunstman, K., Kuhmann, S.E., Marx, P.A., Lifson, J.D., Dufour, J., Mefford, M., Pandrea, I., Wolinsky, S.M., Doms, R.W., DeMartino, J.A., Siciliano, S.J., Lyons, K., Springer, M.S., Moore, J.P., 2003a. Use of a small molecule CCR5 inhibitor in macaques to treat simian immunodeficiency virus infection or prevent simian-human immunodeficiency virus infection. J. Exp. Med. 198, 1551–1562.

Veazey, R.S., Shattock, R.J., Pope, M., Kirijan, J.C., Jones, J., Hu, Q., Ketas, T., Marx, P.A., Klasse, P.J., Burton, D.R., Moore, J.P., 2003b. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat. Med. 9, 343–346.

Veazey, R.S., Klasse, P.J., Schader, S.M., Hu, Q., Ketas, T.J., Lu, M., Marx, P.A., Dufour, J., Colonno, R.J., Shattock, R.J., Springer, M.S., Moore, J.P., 2005. Protection of macaques from vaginal SHIV challenge by vaginally delivered inhibitors of virus-cell fusion. Nature 438, 99–102.

Vives, R.R., Imberty, A., Sattentau, Q.J., Lortat-Jacob, H., 2005. Heparan sulfate targets the HIV-1 envelope glycoprotein gp120 coreceptor binding site. J. Biol. Chem. 280, 21353–21357.

Weber, J.N., Lacey, C.J., 2001. The development of novel vaginal microbicides: from the bench to the clinic. AIDS 15 (Suppl. 1), S35–S37.

Weber, J., Nunn, A., O'Connor, T., Jeffries, D., Kitchen, V., McCormack, S., Stott, J., Almond, N., Stone, A., Darbyshire, J., 2001. 'Chemical condoms' for the prevention of HIV infection: evaluation of novel agents against SHIV(89, 6PD) in vitro and in vivo. AIDS 15, 1563–1568.

Weber, J., Desai, K., Darbyshire, J., 2005. The development of vaginal microbicides for the prevention of HIV transmission. PLoS Med. 2, e142.

Wild, C.T., Shugars, D.C., Greenwell, T.K., McDanal, C.B., Matthews, T.J., 1994. Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. Proc. Natl. Acad. Sci. U.S.A. 91, 9770–9774.

Wyatt, R., Sodroski, J., 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science 280, 1884–1888.