Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 73 (2007) 60–68
Antiviral Research
www.elsevier.com/locate/antiviral

Parameters of inhibition of HIV-1 infection by small anionic microbicides

Andrei N. Vzorov [a], Jadranka Bozja [a], Dabney W. Dixon [b], Luigi G. Marzilli [c], Richard W. Compans [a,*]

[a] *Department of Microbiology and Immunology, Emory University, 1510 Clifton Road, Room 3001, Atlanta, GA 30322, United States*
[b] *Department of Chemistry, Georgia State University, Atlanta, GA 30303, United States*
[c] *Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, United States*

Received 7 April 2006; accepted 25 July 2006

Abstract

Sulfonated porphyrins and phthalocyanines have been shown to have anti-HIV activity and are under consideration as microbicides. Both categories of compounds are small negatively charged molecules and both were previously shown to inhibit cell fusion induced by the HIV Env protein and to block binding of gp120 to the CD4 receptor. In the present study we show that these compounds inhibit transmission of cell-associated HIV, inactivate a broad range of HIV-1 primary isolates and are active against DS polyanion-resistant virus. The compounds tested are active over a range of pH values, and possess no detectable activity against normal bacterial flora. These results support the conclusion that anionic tetrapyrroles are promising candidates as microbicides for HIV prevention.

Keywords: HIV; Topical microbicide; Normal vaginal flora

1. Introduction

Sexual transmission plays a dominant role in the spread of HIV infection, and approaches to prevent such transmission are urgently needed. One promising approach receiving increasing attention is the development of vaginal microbicides which, when applied topically, can prevent viral infection (Harrison et al., 2003; Keller et al., 2003; Stone, 2002). HIV isolates are classified as X4 or R5 viruses, depending on the coreceptor which is used for viral entry (Hoffman et al., 2002). In previous reports we compared the anti-HIV activity of a series of sulfonated porphyrins and phthalocyanines and investigated detailed structure–activity relationships (Vzorov et al., 2002, 2003; Dixon et al., 2005). We showed that several small anions (sulfonated porphyrins and phthalocyanines) could potentially be used as microbicides to provide a defense against infection by sexually transmitted virus. Our results demonstrated that the anti-HIV activity of the sulfonated small polyanionic molecules is relatively independent of the degree of sulfonation and molecular charge distribution. Based on these results and structure analysis we have used the term "small anionic tetrapyrroles" for these sulfonated compounds. Sulfated and sulfonated polymers are two other closely related classes of compounds that also exhibit microbicidal potential. The difference between a sulfate and a sulfonate is probably minor with respect to charge density; the number and position of the negative charges overall may be important, but have been very hard to define. For example, the diagram shown in Fig. 1 for dextran sulfate has three sulfates per ring, but it is unknown if that is a consistent feature of the polymer. Candidate polymers that have been studied include T-PSS[poly]sodium-4-styrene sulfonate (Herold et al., 2000), CarraGuard, a sulfated carrageenan (Maguire et al., 1998), PRO 2000, a naphthalene sulfonate polymer formulation (Profy et al., 1998), Ushercell, a sodium cellulose sulfate-based gel (Anderson et al., 2002) and highly sulfated polyanions derived from the K5 loysaccharide of *Escherichia coli* (Vicenzi et al., 2003). Some of the polyanions are in phase I–III clinical trails for use as microbicides: Ushercell (cellulose sulfate); Cellacefate (cellulose acetate 1,2-benzenedicarboxylate); CarraGuard; PRO 2000; and Vivagel (Lederman et al., 2006).

HIV-1 R5 and X4 isolates can enter cells by two different mechanisms: membrane fusion and endocytosis (Smith and Helenius, 2004). Membrane fusion, which is a specific process for HIV-1, is associated with a critical role of the V3 loop

* Corresponding author. Tel.: +1 404 727 5947; fax: +1 404 727 8250.
E-mail address: compans@microbio.emory.edu (R.W. Compans).

0166-3542/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2006.07.008

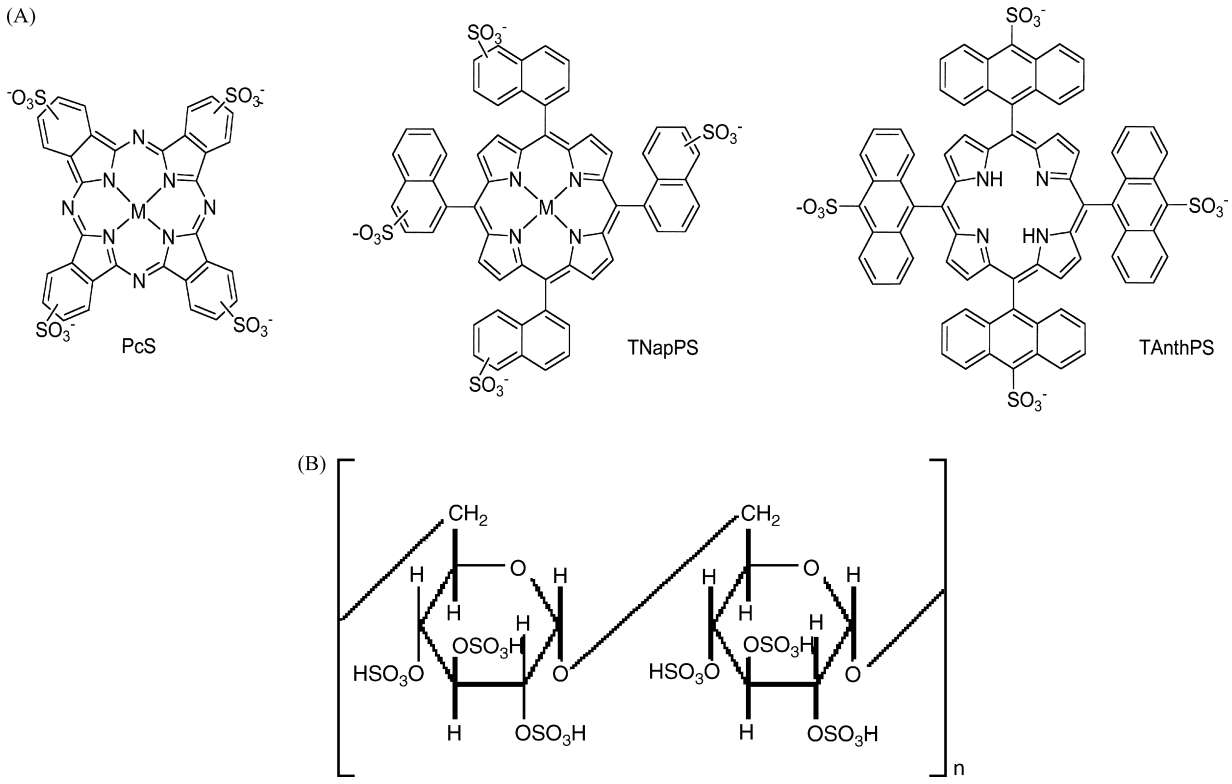

Fig. 1. Structures of porphyrins and phthalocyanines studied. (A) NiPcS and CuPcS are the nickel (M = Ni) and copper (M = Cu) chelates of PcS, respectively. These sulfonated phthalocyanines are mixtures with varying numbers and positions of sulfonate groups. TNapS (M = 2H) is also a mixture with varying numbers of sulfonates and positions of the sulfonate groups. T1Nap20S, Cu is a more highly sulfonated copper chelate (M = Cu) of the structure shown. The TAnthPS structure is largely that shown, although there may be some additional components with varying numbers of sulfonates and positions of the sulfonate groups. (B) Carrageenans are linear polymers of about 25,000 galactose derivatives. Lambda carrageenan has three sulfates per two galactose moieties in the repeating unit: -(1 → 3)-β-D-galactopyranose-2-sulfate-(1 → 4)-α-D-galactopyranose-2,6-disulfate-(1 → 3). Dextran sulfate is a mixture of a linear α-(1,6)-linked D-glucose polymer with three sulfates per glucose. Dextran is significantly more negatively charged per unit molecular weight than is the carrageenan. Sulfur connected to molecule through an oxygen atom in dextran sulfate.

of gp120 and cell receptor and coreceptors CD4, CXCR4 and CCR5 (Ray and Doms, 2006). For endocytosis, the V3 loop is not critical and multiple attachment factors can play a role (Smith and Helenius, 2004; Bobardt et al., 2004; Vzorov et al., 2005). Interaction of polymeric polyanions with the V3 loop can prevent virus attachment to the host cells through the interaction of negatively charged species with the positively charged amino acids in the V3 loop, which has been suggested as a possible mechanism for inhibition of HIV (Debnath et al., 1994; Neurath et al., 1995; Song et al., 1997). Viruses resistant to polymeric polyanions exhibited a reduction of positively charged amino acids in the V3 loop which led to reduced infectivity and fusogenicity (Bobardt et al., 2004). It is very important for a microbicide to inhibit both possible HIV entry pathways. Polyanions have been highly effective in inhibiting infection by X4 viruses. It was demonstrated by several approaches that monomeric X4 and R5X4 gp120 molecules strongly bind to polymeric polyanions, whereas gp120 from R5 viruses binds to polymeric polyanions relatively weakly ($gp120_{Bal}$) or not at all ($gp120_{JRFL}$) (Moulard et al., 2000). The positive charge on the gp120 protein of CXCR4 using X4 viruses is significantly greater than that of R5 viruses, which use the CCR5 coreceptor for entry (Moulard et al., 2000). Sulfonated and sulfated polyanions were also reported to inhibit R5 viruses when target cells were exposed to the virus in the presence of compounds (Greenhead et al., 2000; Vicenzi et al., 2003; Dezzutti et al., 2004; Scordi-Bello et al., 2005). These data may indicate that anionic polymers do not interact as effectively with positively charged sequences of R5 viruses, and R5 virus particles may be able to escape more readily from inhibition by some polymers.

We have recently demonstrated that two classes of tetrapyrrole compounds, sulfonated porphyrins and phthalocyanines, exhibit several desirable characteristics as microbicides: inhibition of binding of the HIV type 1 gp120 to CD4; inhibition of the ability of HIV Env proteins to induce cell fusion with receptor-bearing target cells; rapid interaction of the compounds with HIV and irreversible inactivation of infectivity (Vzorov et al., 2002, 2003; Dixon et al., 2005). The most active compounds in each class showed no detectable cytotoxic effect. Microbicides must protect against R5 viruses, as most HIV isolates involved in sexual transmission appear to utilize the CCR5 coreceptor (Shattock and Doms, 2002). In addition, it is important for microbicides to protect against cell-associated virus transmission, and to be active over the range of pH values found in the female genital tract. It is also desirable for candidate microbicides to lack activity against normal bacterial flora. In the present study, we have investigated these parameters for several of the most active porphyrins as well as phthalocyanines.

2. Materials and methods

2.1. Compounds

Sulfonated porphyrins and phthalocyanines were described previously (Vzorov et al., 2002, 2003). Polymers: dextran sulfate M_r 5000 (DS 5000), the sulfated polymer carrageenan lambda and DEAE–dextran (a cationic polymer) were obtained from Sigma–Aldrich (Milwaukee, WI, USA).

2.2. Cell lines

The recombinant human epithelial cell lines MAGI, MAGI-CCR5 (indicator cell lines) and human T cell lines CEMx174, HUT78 and MT-4 (HTLV-1-transformed) were obtained through the NIH AIDS Research and Reference Reagent Program. The recombinant epithelial human cell line JC53-BL (indicator cell line), which is a derivative of HeLa cells that expresses high levels of CD4 and the HIV-1 coreceptors CCR5 and CXCR4 (Derdeyn et al., 2000), was obtained from Dr. J. Kappes (University of Alabama, Birmingham). JC53-BL cells contain a reporter cassette of β-galactosidase that is expressed from an HIV-1 LTR. MAGI, MAGI-CCR5 and JC53-BL cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). HUT78 and CEMx174 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Human peripheral blood lymphocytes (hPBL) were isolated from normal human blood by stimulating for 3 days in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 5 μg of concanavalin A (ConA) per ml, 5% interleukin 2 (IL-2) and antibiotics.

2.3. Viruses

HIV-1 UG92037 (A/A subtype), HIV-1 IIIB, HIV-1 89.6, HIV-1 JR-FL (B subtypes); HIV-1 BR92 025-RE2 (C subtype); HIV-1 THA(92)001 (A/EA) subtype) were obtained through the NIH AIDS Research and Reference Reagent Program. Viruses were grown as previously described (Vzorov and Compans, 2000).

2.4. Plasmid construction

For construction of mutant virus, plasmid pNL4-3 was provided by the NIH AIDS Research and Reference Reagent Program. Vector pSP72 was obtained from Promega Corporation (Madison, WI, USA). The plasmid containing the HIV-1 NL4-3 *env* gene was prepared with insertion of the *Eco*RI to *Xho*I fragment of pNL4-3 into *Eco*RI and *Xho*I restriction sites of pSP72. Oligodeoxynucleotides were designed to produce a fragment containing the desired mutations. The 5′ oligo, containing a *Pvu*II site was 5′-CATAATAGTACAGCTGAACACATCTGTAGAAATTAATTGTACAAGACCCAACTACGAGACAAGAAAAAGTATCCGTATCCACAGGGGACCAGGGAGAGCATTTGTTACAATAGG-3′; the 3′ oligo, containing a *Nhe*I site was 5′-CTTAATTTGCTAGCTATCTGTTTTAAAGTGGCATTCCATTTTGCTCTACTAATGTTACAATGTGCTTGTCTCATATCTCCTATTTTTCCTATTGTAACAAATGCTCTCCCTGGTC-3′. First round PCR amplification was carried out by using the 5′ and 3′ oligos as template and primers and five cycles with steps of 1 min at 95 °C, 2 min at 58 °C and 3 min at 72 °C. Second round PCR amplification was carried out by adding new primers, the 5′ primer CTTAATTTGCTAGCTATCTG and the 3′ primer CATAATAGTACGACTGAACAC, and 25 cycles with steps of 1 min at 95 °C, 2 min at 58 °C and 3 min at 72 °C. Before the 25 cycles, a denaturing step of 5 min at 95 °C and after 25 cycles, an extension step of 10 min at 72 °C were used. The PCR fragment was inserted into the *Pvu*II to *Nhe*I site of pEnvNL4-3 and then transferred to the *Eco*RI to *Xho*I site of pNL4-3 using *Eco*RI and *Xho*I restriction sites of pEnvNL4-3. The resulting plasmid was designated pNL4-3 + 6. To recover infectious HIV, plasmids were transfected into 293T cells using calcium phosphate precipitation (Bartz and Vodicka, 1997).

2.5. Removal of unbound compound

To separate virus from unbound compounds we used a filtration–dilution method described previously (Vzorov et al., 2003). This method was selected by comparison with two other methods: centrifugation and dialysis. With centrifugation we found that infectivity was reduced, which indicated that it was destructive for the virus, whereas dialysis was inconvenient for the small volume of samples used. We used a maximum concentration of 25 μg/ml of compounds in the filtration–dilution method, which allowed the sulfonated tetrapyrroles to pass through the membrane readily.

2.6. Screening of compounds for activity against R5 and X4 HIV-1

Compound stock solutions were adjusted to a concentration of 500 μg/ml with growth medium (DMEM with FCS), diluted 10-fold in growth medium, and mixed with 5×10^2 virus particles. Samples were left in the dark at room temperature for 1 h. For indicator cell line assays, 25 μl of virus/compound mixture was mixed with 225 μl of growth medium containing DEAE–dextran (15 μg/ml) and 50 μl added to wells with confluent monolayers of cells on a 96-well plate. The samples were tested in duplicate. At 2 h post-infection, an additional 200 μl of complete DMEM was added. After 3 days activity was measured by determining virus titers using an indicator cell line assay (Chackerian et al., 1997). Comparison of the number of β-gal expressing cells in wells infected with compound-treated virus to the number found in wells infected with untreated virus was used to determine residual viral infectivity (expressed in percentage).

2.7. Cell-associated virus transmission

The fraction of HUT78 cells chronically infected by HIV-1 IIIB was standardized by fluorescent activated cell sorting (FACS) analysis using anti-p24. Various numbers of HUT78

cells (3×10^2, 3×10^3 and 3×10^4) infected with HIV-1 IIIB were exposed to test compounds at a concentration of 50 μg/ml in the dark for 10 min at 37 °C, diluted 10-fold and used to inoculate MAGI cells. After 3 days virus titers were determined using assays in MAGI cells (Kimpton and Emerman, 1992). We found that HIV-infected HUT78 cells resulted in the following levels of transmission to MAGI cells (per well of 96-well plates): 3×10^2 HUT78+ HIV-1 IIIB ≈10 infected MAGI cells; 3×10^3 HUT78+ HIV-1 IIIB ≈100 infected MAGI cells; 3×10^4 HUT78+ HIV-1 IIIB >100 infected MAGI cells.

2.8. pH dependence of activity

To determine the pH dependence of the virucidal activity we used two virus strains: HIV-1 IIIB and HIV-1 JR-FL. Virus particles were mixed with test compounds at a concentration of 50 μg/ml in HEPES–saline buffered with sodium citrate and citric acid using a range of pH values from 4.0 to 8.0, at a standard incubation period of 60 min. Control samples contained virus inocula incubated at similar pH values in the absence of the compounds. The residual infectivity was measured by a highly sensitive β-galactosidase assay in JC53-BL cells (Derdeyn et al., 2000).

2.9. Pretreatment of cells

To determine if infection is inhibited when cells are pretreated with the compounds, MAGI cells were exposed to various concentrations of the compounds (0.05, 0.5, 5 and 50 μg/ml) in 50 μl of growth medium in 96-well plate for 2 h prior to addition of HIV-1 in 50 μl of growth medium without removal of compounds. The samples were tested in duplicate. At 2 h post-infection, an additional 150 μl of complete DMEM was added. After 3 days virus titers were measured using an indicator cell line assay as described above.

2.10. Inhibition of HIV-1 infection in human PBL

To determine the effects of compounds on virus replication, the compounds at a final concentration of 0.5, 5 or 50 μg/ml were mixed with 2×10^2 cell-free infectious particles of HIV-1 IIIB or JR-FL and added immediately to 96-well plates with 3×10^4 per well of PBL in medium containing 15 μg/ml DEAE–dextran. After 2 h incubation the unbound virus was removed by three washes and new medium with a corresponding amount of compound was added. After 3 days, culture supernatant was harvested and tested for p24 content by ELISA core antigen assay (Coulter Corporation).

2.11. Cytotoxicity test

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Pauwels et al., 1988) was performed using the HeLa epithelial cell line. For the MTT assay, compounds at varying concentrations (1000, 200, 40 and 8 μg/ml) in growth medium were added to 96-well plates with HeLa cells. Following 72 h incubation, cells were washed with Hanks' balanced salt solution to removed colored compounds and 100 μl of growth medium with 10 μl of MTT (10 mg/ml) reagent was added to each well. After 4–12 h incubation at 37 °C, 100 μl acidic isopropanol (0.04 M HCl in absolute isopropanol) was added. The absorbance was read in a computer-controlled photometer. The absorbance at 690 nm was automatically subtracted from the absorbance at 540 nm to eliminate the effects of non-specific absorption.

2.12. Minimal growth inhibitory concentration (MGIC) assay

The bacterial strains *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus fermentum*, *Lactobacillus reuteri* and *Lactobacillus acidophilus* were obtained from the American Type Culture Collection (Manassas, VA, USA). The minimal growth inhibition concentration test as described previously (Shafer et al., 1998; Bozja et al., 2004) was used to identify the percentage of bacteria that retain colony forming ability after incubation with the compounds. Briefly, logarithmically grown bacteria were diluted to approximately 5×10^6 colony forming units (cfu) per ml and various concentrations of compounds were added. The mixture was incubated for 1 h at 37 °C, with 5% CO_2. Bacterial survival/killing was measured by placing bacterial dilutions onto MRS plates (Lactobacilli MRS broth, Difco, Becton Dickinson).

3. Results

3.1. Inactivation of viruses

To determine the spectrum of their virucidal activity, we tested the activity of selected porphyrins or phthalocyanines against primary HIV-1 isolates of different subtypes which exhibit CCR5 or CCR5/CXCR4 coreceptor usage (Table 1). To evaluate the virucidal activity, we used an epithelial HeLa-CD4 cell line bearing the CCR5 coreceptor with an integrated LTR-β-galactosidase gene. The compounds tested include the sulfonated tetranaphthyl (TNapPS) and tetraanthracenyl (TAnthPS) porphyrin as well as the sulfonated phthalocyanine without a central metal atom (PcS) and its nickel (NiPcS) and copper (CuPcS) insertion compounds (Fig. 1). The compounds tested were found to exhibit high activity against the primary HIV-1 R5 isolates. Treatment with NiPcS, CuPcS or TAnthPS

Table 1
HIV isolates tested

Virus (HIV-1)[a]	Subtype Gag/Env	Coreceptor usage
IIIB	B/B	CXCR4
89.6	B/B	CCR5/CXCR4
JR-FL	B/B	CCR5
BR92 025-RE2	C/C	CCR5
UG92037	A/A	CCR5
THA(92)001	A/EA	CCR5

[a] Viruses were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS (NIAID, NIH).

Table 2
EC_{50}, CC_{50} and therapeutic indices for selected compounds

Compound	Inactivation HIV-1 IIIB EC_{50} (μg/ml)	Inactivation HIV-1 JR-FL EC_{50} (μg/ml)	CC_{50} (μg/ml)[a]	Therapeutic index (HIV-1 IIIB)[b]	Therapeutic index (HIV-1 JR-FL)[b]
NiPcS	0.5	20	40	80	2
PcS	0.5	3	750	1500	250
CuPcS	0.5	16	1000	2000	63
TNapPS	2	>50	150	75	<3
TAnthPS	5	12	600	120	50
T1Nap20S,Cu	0.5	8	900	1800	113

[a] Results obtained by MTT assay using HeLa cells.
[b] The therapeutic index value was defined as the CC_{50}/EC_{50}.

inactivated about 93% of HIV-1 JR-FL, as compared with 95% of HIV-1 IIIB and 94% of HIV-1 89.6 (B subtypes). The compounds also displayed activity against HIV-1 BR92 025-RE2 (C subtype), blocking 91% of infection; HIV-1 UG92037 (A/A subtype), blocking 88% of infection; and HIV-1 THA(92)001 (A/EA) subtype), blocking 86% of infection. To determine the EC_{50} of the compounds we used HIV-1 JR-FL, which is among the most resistant of the HIV-1 R5 isolates to neutralization by antibodies and with the lowest positively charged regions on the gp120 protein (Moulard et al., 2000). For comparison we used HIV-1 IIIB (X4) with significantly greater positively charged regions in the gp120 protein than found in R5 viruses. Generally, the compounds exhibited a lower EC_{50} with HIV-1 IIIB than with HIV-1 JR-FL (Table 2) and the EC_{50} values were about the same against the laboratory-derived X4 virus HIV-1 IIIB and another X4 virus that deviates from the consensus sequence, NL4-3 (not shown).

To evaluate cytotoxicity, we used a colorimetric MTT toxicity assay which provides an indication of the mitochondrial integrity and activity as a measure of cell viability and/or number. Most compounds showed no detectable cytotoxic effect at a concentration of 200 μg/ml. The therapeutic indices of leading compounds for R5 viruses were still high, in the range of 100–200. These results indicate that porphyrins or phthalocyanines are able to block infection by primary HIV-1 isolates of several distinct HIV antigenic subtypes which utilize the CCR5 coreceptor, and that the leading compounds tested exhibited low toxicity.

3.2. Activity after removal of anionic tetrapyrroles

Virucidal activity against HIV-1 X4 of three compounds NiPcS, PcS and ZnPcS(1) was previously shown by a filtration–dilution method (Vzorov et al., 2003). In addition, we investigated the virucidal activity of CuPcS against HIV-1 IIIB and determined the EC_{50}. We observed no increase in infectivity after removal of compounds by the filtration–dilution method, and the EC_{50} was 0.5 μg/ml. We also investigated virucidal activity against HIV-1 R5 (HIV-1 JR-FL). A partial recovery of virus infectivity of about 50% was observed. For CuPcS, the EC_{50} with HIV-1 JR-FL after removal of compound was about 25 μg/ml. Therefore, the anionic tetrapyrrole exhibits virucidal activity against both X4 and R5 HIV-1 viruses.

3.3. Effects of DEAE–dextran

It was previously reported that anionic polymers exhibit higher antiviral activity against X4 than R5 HIV strains (Shattock and Doms, 2002). To investigate possible differences in activity between anionic polymers and small sulfonated anions, we used several approaches. We incubated mixtures of HIV-1 IIIB with CuPcS, dextran sulfate M_r 5000 (DS 5000) or the sulfated polymer carrageenan lambda at a concentration of 50, 5 or 0.5 μg/ml. After 1 h incubation mixtures were diluted 10-fold and added to JC53-BL cells in the presence of DEAE–dextran. All three compounds were effective in blocking HIV-1 IIIB infection without DEAE–dextran and had an EC_{50} about 1 μg/ml (Fig. 2). However, in the presence of DEAE–dextran, they demonstrated differences in activity: CuPcS remained highly active with an EC_{50} of 1 μg/ml whereas DS 5000 and carrageenan lambda dramatically lost

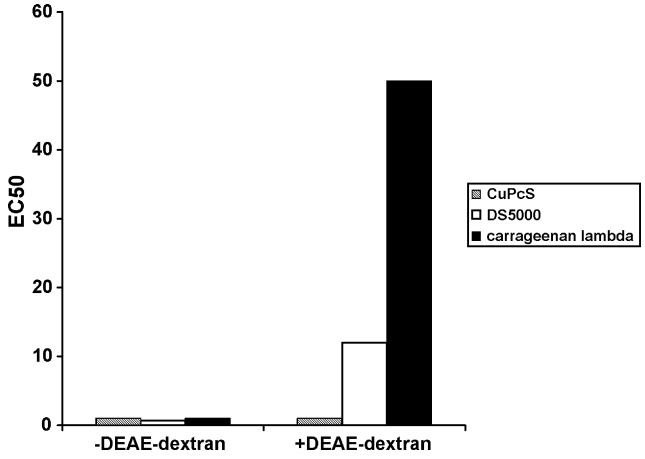

Fig. 2. Effect of DEAE–dextran on anti-HIV activity of test compounds. Mixtures of HIV-1 IIIB with CuPcS, dextran sulfate M_r 5000 (DS 5000) or the sulfated polymer carrageenan lambda were incubated at a concentration of compounds of 50, 5 and 0.5 μg/ml. After 1 h incubation, mixtures were diluted 10-fold and added to JC53-BL cells in the presence or absence of 15 μg/ml of DEAE–dextran. Inhibition of HIV infection was measured by removal of the media after 3 days, fixation and staining of cells with X-gal (Vzorov et al., 2002). The percentage of residual infectivity was determined by dividing the number of infected cells in wells inoculated with compound-treated virus by the number in wells inoculated with untreated virus. Numerical data (EC_{50}) reported are the averages of three experiments, each run in duplicate. All studies were performed at neutral pH (7.2).

virucidal activity with an EC_{50} of 12 μg/ml and an EC_{50} >50 μg/ml, respectively. The EC_{50} values of sulfated polymers, but not of CuPcS, were also more variable in the presence of DEAE–dextran. The results indicate that the cationic polymer DEAE–dextran blocks the anti-HIV effect of negatively charged macromolecules; in contrast, a small sulfonated anion was not sensitive to such inhibition.

3.4. Inhibition of a DS polyanion-resistant virus

To further investigate the possible differences between sulfated polyanions and small sulfonated anions we constructed a polyanion-resistant mutant virus. Mutations were introduced in the V3 loop of gp120 in NL4-3 virus to change the amino acid net charge from +9 to +6 to obtain a polyanion-resistant virus as described by Bobardt et al. (2004). We found that the resulting NL4-3 + 6 virus was able to grow in MT-4 cells, but the level of replication was significantly lower than the parental NL4-3 virus (>20-fold lower). The mutant virus was found to be completely resistant to inhibition by DS 5000. However, all three small anionic compounds tested (T1Nap20S,Cu, CuPcS and PcS) exhibited activity against the NL4-3 + 6 mutant. The most active compounds T1Nap20S,Cu and CuPcS had an EC_{50} of 10 μg/ml (Fig. 3). These results demonstrate that small sulfonated anions exhibit microbicidal activity against an HIV-1 mutant that is resistant to inhibition by DS 5000.

3.5. pH dependence of activity

It is important to determine the possible pH dependence of the virucidal or antiviral effect because the compounds need to be biologically active at the physiological pH of the target sites in the body, and because two possible entry routes may be used by HIV, pH-independent membrane fusion and pH-dependent entry or endocytosis. The pH ranges from 4 to 4.4 vaginally and 6 to 7 cervically (Hillier, 1996). We determined the pH dependence of the antiviral activity by preparing virus–compound mixtures in HEPES–saline buffered with sodium citrate and citric acid at various pH values, using a range of pH values from 4.0 to 8.0. The activity was measured by assay on the indicator cell line JC53-BL. With HIV-1 IIIB, we observed a three-fold reduction of viral infectivity in control samples treated at pH 4, but full retention of infectivity with buffers of other pH values. The sulfonated phthalocyanine PcS exhibited pH-dependent activity, blocking only 15% of infection at pH 4 and about 70% at pH 8. However, the copper sulfonated phthalocyanine CuPcS and porphyrin TAnthPS showed full activity at all pH ranges: CuPcS blocked infection by 93% at pH 4 and 90% at pH 8; TAnthPS blocked infection by 94% at pH 4 and 94% at pH 8 (Fig. 4). Manganese deuteroporphyrin MnDP, used as a negative control, exhibited low anti-HIV activity at all tested pH values. To extend our results we also compared the effect on HIV-1 JR-FL. We observed that all compounds showed activity against HIV-1 JR-FL at various pH levels. The most active compounds (T1Nap20S,Cu and CuPcS) blocked 89% of virus infection at pH 4-5 (EC_{50} = 10 μg/ml), and 96–100% of virus infection at pH 6 and higher (not shown). These results demonstrate that the most

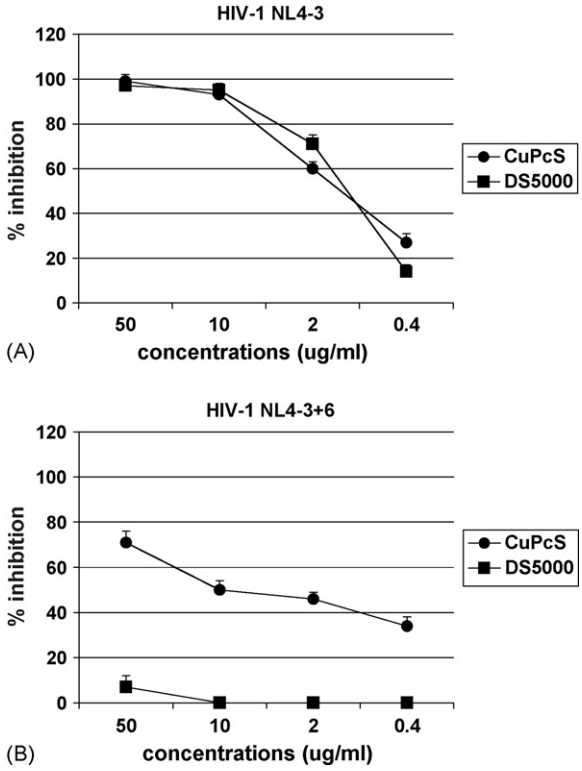

Fig. 3. Inhibition of a polyanion-resistant mutant virus. Viruses HIV-1 NL4-3 (A) or the HIV-1NL4-3 + 6 mutant (B) were mixed with varying concentrations of CuPcS or DS 5000 (0.4, 2, 10 and 50 μg/ml) and added to 96-well plates with 3×10^4 per well of MT-4 cells. After 2 h incubation the unbound virus and residual compound were removed by three washes. After 3 days, the culture supernatant was harvested from each well and used to determine p24 content by ELISA core antigen assay (Coulter Corporation). Comparison of the amount of Gag antigen in wells with samples of compound-treated virus to the amount found in wells with samples of untreated virus was used quantitate viral replication (expressed as percentage).

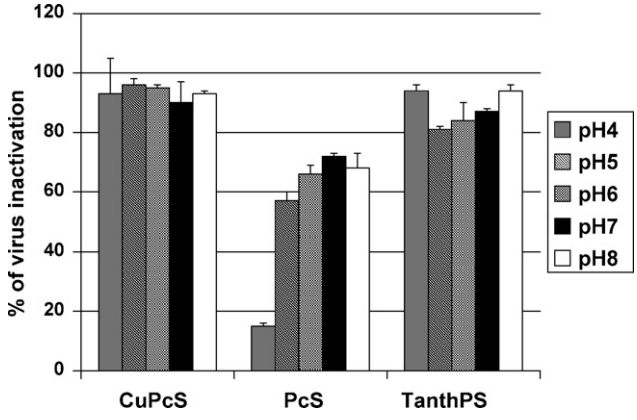

Fig. 4. pH dependence of activity. Compounds at a concentration of 50 μg/ml were incubated with HIV-1 in HEPES buffer using a range of pH values from 4.0 to 8.0 in the dark for 60 min, diluted 10-fold and used to inoculate JC53-BL cells. Control samples contained similar virus inocula incubated at similar pH values in the absence of the compounds. After 3 days, the number of infected cells was determined. The percentage of residual infectivity was determined by dividing the number of infected cells in wells inoculated with compound-treated virus by the number in wells inoculated with untreated virus. Data are plotted as the mean of three experiments, each replicated twice. Error bars represent standard deviations.

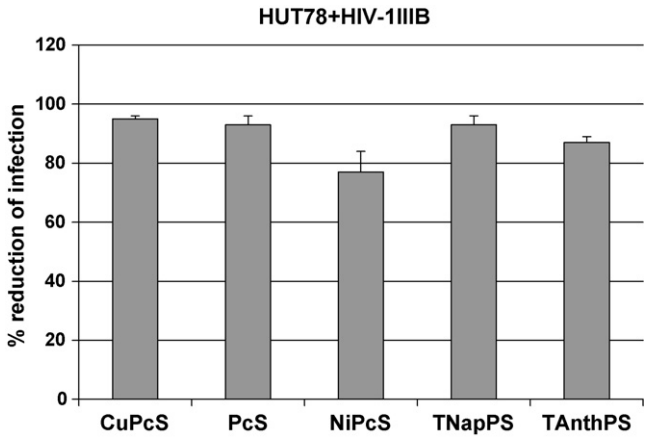

Fig. 5. Prevention of cell-associated virus transmission. Various amounts of HUT78 cells (3×10^2, 3×10^3 and 3×10^4) infected with HIV-1 IIIB were exposed to test compounds at a concentration of 50 μg/ml in the dark for 10 min at 37 °C, diluted 10-fold and used to inoculate MAGI cells; residual infectivity titers were determined as in Fig. 2. The data shown were obtained with 3×10^3 infected HUT78 cells.

active phthalocyanine and porphyrin compounds tested inhibit HIV-1 infection at a broad range of pH values.

3.6. Prevention of cell-associated virus transmission

To evaluate the potential for blocking cell-associated HIV transmission, we investigated the effects of test compounds on virus transmission from HUT78 cells infected with HIV-1 IIIB to MAGI cells. We found that the porphyrins TNapPS and TAnthPS exhibited high activity against cell-associated virus transmission, blocking about 90–95% of HIV infection (Fig. 5). The phthalocyanines PcS and CuPcS also blocked about 95% of HIV-1 infection by cell-associated virus, and NiPcS blocked about 80% of cell-associated HIV infection. These results indicate that small sulfonated anions efficiently inhibit transmission of cell-associated HIV-1.

3.7. Pretreatment of cells blocks infection

To determine if infection is inhibited when cells are pretreated with the phthalocyanine or porphyrin compounds, MAGI cells were exposed to various concentrations of the compounds (0.05, 0.5, 5 and 50 μg/ml) for 2 h prior to exposure to IIIB virions in the presence of compounds. Pretreatment of cells with compounds resulted in similar inhibition of infection to that observed when virus was pretreated with compounds (Vzorov et al., 2002, 2003). The EC_{50} value for NiPcS was 3 μg/ml, PcS was 1 μg/ml, CuPcS was 1 μg/ml, TNapPS was 2 μg/ml and TAnthPS was 3 μg/ml. Therefore, the compounds tested are able to prevent infection when used to pretreat target cells in culture.

3.8. Inhibition of HIV-1 infection in primary human T cells

We further evaluated the effects of compounds on virus infection in primary human blood lymphocytes (PBL) isolated from normal human blood (Table 3). We tested three active compounds, CuPcS, NiPcS and TNapPS for inhibition of HIV-1 IIIB or JR-FL infection in the presence of DEAE–dextran. The p24 content in the supernatant was determined by ELISA core antigen assay at 3 days post-infection in the presence or absence of compounds. We found that all three compounds at 25 μg/ml inhibited p24 production by HIV-1 IIIB or JR-FL by about 95%. CuPcS had an $EC_{50} = 1$ μg/ml with HIV-1 IIIB and an $EC_{50} = 12$ μg/ml with HIV-1 JR-FL. The results indicate that these compounds have antiviral activity in primary human T cells against both X4 and R5 isolates. This activity was observed against HIV-1 in PBL cultures, despite the use of the cationic polymer DEAE–dextran to promote attachment and entry of HIV-1 to the cells.

Table 3
Inhibition of HIV-1 infection in primary human PBL

Compound	Antiviral activity (compounds present during infection)			
	Percentage inhibition, HIV-1 IIIB		Percentage inhibition, HIV-1 JR-FL	
	25 μg/ml	2.5 μg/ml	25 μg/ml	2.5 μg/ml
CuPcS	97	74	95	3
NiPcS	95	6	94	11
TNapPS	93	17	94	0

Data shown are the mean of two experiments, each replicated twice.

3.9. Lack of effects on normal vaginal flora

We also carried out studies to determine whether the active compounds exhibit microbicidal activity versus several types of Lactobacilli which are found as normal vaginal flora (Antonio et al., 1999; McLean and Rosenstein, 2000; Vasquez et al., 2002). The MGIC test was used to identify the percentage of bacteria that retain colony forming ability after incubation with the compounds (Shafer et al., 1998; Bozja et al., 2004). None of the candidate microbicides tested was observed to have a detectable effect on viability of Lactobacilli in vitro (not shown).

4. Discussion

The present results show that small polyanionic molecules are able to interact with Env proteins and inactivate a range of viruses with either CXCR4 or CCR5 tropism. The results show that the anti-HIV activity of the sulfonated small polyanionic molecules is relatively independent of the degree of sulfonation and the nature of the central metal atom, indicating that a range of structures may be effective for viral inhibition. However, both these characteristics can affect anti-HIV activity of the compounds. The leading compounds showed little decrease in activity against R5 and equivalent activity against X4 viruses at low pH. One compound, PcS (without the central metal atom), which has virucidal activity but does not block fusion or binding of gp120 to CD4 (Vzorov et al., 2003), showed reduction of activity against both viruses at low pH levels. We observed no effect of pH on the spectra of the PcS, indicating that the pH dependence of this compound was not due to a change of its chemical properties. The compounds tested were found to

exhibit EC_{50} values against X4 viruses about 10-fold higher than against R5 viruses. These data may suggest that the mechanism of inhibition is based on a charge interaction. However, the therapeutic indices of leading compounds for R5 viruses were high, in the range of 100–200. Although we did not observe complete prevention of infection in some cases, the probability of mucosal transmission for HIV-1 virions is quite small (about 0.001) and is related, in part, to dose (Pope and Haase, 2003). Because of the low efficiency of transmission, even compounds which block 80–90% of virus infection could play a significant role in reducing HIV transmission in vivo.

Interestingly, we found that DEAE–dextran, a positively charged polymer, dramatically reduced the activity against HIV-1 of the sulfated polymers DS (M_r 5000) or carrageenan lambda, but did not exhibit any blocking effect on anti-HIV-activity of the low mol. wt. anionic compounds under similar conditions. We also did not observe reduced activity of our compounds against HIV-1 in PBL culture, despite the use of DEAE–dextran to promote attachment and entry of HIV-1 to the cells. These results further confirm that there are significant differences between the activities of small polyanionic molecules and large polymers, and indicate that small anionic tetrapyrroles more effectively block HIV infection than polymers under such conditions. The compounds tested also exhibited activity against a mutant virus having an Env protein with mutations in a conserved V3 loop region. This virus was completely resistant to the polyanion DS5000, but remained sensitive to the small polyanionic molecules tested. This sensitivity may be due to the small size of the sulfonated molecules and their access to multiple HIV target sequences. The observation of a sharp dose response of HIV infection to our compounds may also suggest that effective inhibition may require interaction with multiple target sites on virus particles.

We also investigated the virucidal activity of our compounds. This represents the irreversible inactivation of infectivity after removal of unbound compound from the sample. In previous studies we did not find any differences in activity against HIV-1 X4 virus after removal of anionic compounds by a filtration–dilution method (Vzorov et al., 2002, 2003). With R5 (HIV-1 JR-FL) we observed partial recovery of virus infectivity after removal of compound, although a virucidal effect was still observed. These results may indicate that the interaction of compounds is weaker, and the filtration–dilution method may result in some dissociation of compounds from R5 virus particles.

Candidate microbicides should also be evaluated for their effects on vaginal microflora because changes in the microflora, such as a loss of *Lactobacillus*, may result in an increase in vaginal pH and consequently a decrease in the host microbial defenses against HIV, which are less active at acid pH (Clarke et al., 2002). Our results demonstrate that the sulfonated porphyrins and phthalocyanines tested do not have a detectable effect on viability of Lactobacilli in vitro. A series of porphyrin-based compounds was previously shown to have potent bactericidal action in vitro against the sexually transmitted pathogens *Neisseria gonorrhoeae* and *Haemophilus ducreyi* but did not show bactericidal activity against five species of Lactobacilli (Bozja et al., 2004).

In summary, the present data show that sulfonated porphyrins and phthalocyanines inhibit transmission of cell-associated HIV, and inactivate a broad range of primary HIV-1 virus isolates. We used several different R5 primary isolates: HIV-1 JR-FL, BR92 025-RE2, UG92037 and THA(92)001. HIV-1 JR-FL is among the most resistant HIV-1 R5 isolates to neutralization by antibodies and has low fusogenicity. We also used a dual tropic virus HIV-1 89.6 and HIV-1 NL4-3 that deviates from the consensus sequence of X4 primary clinical isolates. These viruses represent a spectrum of frequently sexually transmitted viruses, and anionic tetrapyrroles were active against all of them. The small anionic compounds exhibit both virucidal and antiviral activity against X4 and R5 HIV-1 viruses, are active at a range of pH values, and in primary human T cells. Their activity at low pH, as well as activity against a non-fusogenic virus, suggest that they may also inhibit infection via the endocytic pathway. They lack activity against normal bacterial flora, and are able to prevent infection when used to pretreat target cells. All of these properties support the conclusion that small anionic tetrapyrroles are promising candidates for further development as microbicides.

Acknowledgements

This study was supported by NIH grant AI45883 from the National Institute of Allergy and Infectious Diseases. The authors thank Dahnide Taylor for technical assistance and Tanya Cassingham for assistance in preparing the manuscript.

References

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.

Antonio, M.A., Hawes, S.E., Hillier, S.L., 1999. The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. J. Infect. Dis. 180, 1950–1956.

Bartz, S.R., Vodicka, M.A., 1997. Production of high-titer human immunodeficiency virus type 1 pseudotyped with vesicular stomatitis virus glycoprotein. Methods 12, 337–342.

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.

Bozja, J., Yi, K., Shafer, W.M., Stojiljkovic, I., 2004. Porphyrin-based compounds exert antibacterial action against the sexually transmitted pathogens *Neisseria gonorrhoeae* and *Haemophilus ducreyi*. Int. J. Antimicrob. Agents 24, 578–584.

Chackerian, B., Long, E.M., Luciw, P.A., Overbaugh, J., 1997. Human immunodeficiency virus type 1 coreceptors participate in postentry stages in the virus replication cycle and function in simian immunodeficiency virus infection. J. Virol. 71, 3932–3939.

Clarke, J.G., Peipert, J.F., Hillier, S.L., Heber, W., Boardman, L., Moench, T.R., Mayer, K., 2002. Microflora changes with the use of a vaginal microbicide. Sex. Transm. Dis. 29, 288–293.

Debnath, A.K., Jiang, S., Strick, N., Lin, K., Haberfield, P., Neurath, A.R., 1994. Three-dimensional structure–activity analysis of a series of porphyrin derivatives with anti-HIV-1 activity targeted to the V3 loop of the gp120 envelope glycoprotein of the human immunodeficiency virus type 1. J. Med. Chem. 37, 1099–1108.

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 HIV 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.

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.

Dixon, D.W., Gill, A.F., Giribabu, L., Vzorov, A.N., Alam, A.B., Compans, R.W., 2005. Sulfonated naphthyl porphyrins as agents against HIV-1. J. Inorg. Biochem. 99, 813–821.

Greenhead, P., Hayes, P., Watts, P.S., Laing, K.G., Griffin, G.E., Shattock, R.J., 2000. Parameters of human immunodeficiency virus infection of human cervical tissue and inhibition by vaginal virucides. J. Virol. 74, 5577–5586.

Harrison, P.F., Rosenberg, Z., Bowcut, J., 2003. Topical microbicides for disease prevention: status and challenges. Clin. Infect. Dis. 36, 1290–1294.

Herold, B.C., Bourne, N., Marcellino, D., Kirkpatrick, R., Strauss, D.M., Zaneveld, L.J., Waller, D.P., Anderson, R.A., Chany, C.J., Barham, B.J., Stanberry, L.R., Cooper, M.D., 2000. Poly(sodium 4-styrene sulfonate): an effective candidate topical antimicrobial for the prevention of sexually transmitted diseases. J. Infect. Dis. 181, 770–773.

Hillier, S.L., 1996. Presented at the Advances in AIDS Vaccine Development, Bethesda, MD.

Hoffman, N.G., Seillier-Moiseiwitsch, F., Ahn, J., Walker, J.M., Swanstrom, R., 2002. Variability in the human immunodeficiency virus type 1 gp120 Env protein linked to phenotype-associated changes in the V3 loop. J. Virol. 76, 3852–3864.

Keller, M.J., Klotman, M.E., Herold, B.C., 2003. Development of topical microbicides for prevention of human immunodeficiency virus and herpes simplex virus. Am. J. Reprod. Immunol. 49, 279–284.

Kimpton, J., Emerman, M., 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. J. Virol. 66, 2232–2239.

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.

Maguire, R.A., Zacharopoulos, V.R., Phillips, D.M., 1998. Carrageenan-based nonoxynol-9 spermicides for prevention of sexually transmitted infections. Sex. Transm. Dis. 25, 494–500.

McLean, N.W., Rosenstein, I.J., 2000. Characterisation and selection of a *Lactobacillus* species to re-colonise the vagina of women with recurrent bacterial vaginosis. J. Med. Microbiol. 49, 543–552.

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., Debnath, A.K., 1995. Structural requirements for and consequences of an antiviral porphyrin binding to the V3 loop of the human immunodeficiency virus (HIV-1) envelope glycoprotein gp120. J. Mol. Recognit. 8, 345–357.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J., De Clercq, E., 1988. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods 20, 309–321.

Pope, M., Haase, A.T., 2003. Transmission, acute HIV-1 infection and the quest for strategies to prevent infection. Nat. Med. 9, 847–852.

Profy, A.T., Sonderfan, A.J., Bourne, N., 1998. PRO 2000 gel, a potential topical microbicide for HIV prevention, can block infection by other sexually tramsmitted disease pathogens. In: Proceedings of the 12th World AIDS Conference, Geneva, Switzerland, June 28–July 3, pp. 233–237.

Ray, N., Doms, R.W., 2006. HIV-1 coreceptors and their inhibitors. Curr. Top. Microbiol. Immunol. 303, 97–120.

Scordi-Bello, I.A., Mosoian, A., He, C., Chen, Y., Cheng, Y., Jarvis, G.A., Keller, M.J., Hogarty, K., Waller, D.P., Profy, A.T., 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.

Shafer, W.M., Qu, X., Waring, A.J., Lehrer, R.I., 1998. Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. Proc. Natl. Acad. Sci. U.S.A. 95 (4), 1829–1833.

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

Smith, A.E., Helenius, A., 2004. How viruses enter animal cells. Science 304, 237–242.

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

Song, R., Witvrouw, M., Schols, D., Robert, A., Balzarini, J., DeClercq, E., Bernadou, J., Meunier, B., 1997. Anti-HIV activities of anionic metalloporphyrins and related compounds. Antivir. Chem. Chemother. 8, 85–97.

Vasquez, A., Jakobsson, T., Ahrne, S., Forsum, U., Molin, G., 2002. Vaginal *Lactobacillus* flora of healthy Swedish women. J. Clin. Microbiol. 40, 2746–2749.

Vicenzi, E., Gatti, A., Ghezzi, S., Oreste, P., Zoppetti, G., Poli, G., 2003. Broad spectrum inhibition of HIV-1 infection by sulfated K5 *Escherichia coli* polysaccharide derivatives. AIDS 17, 177–181.

Vzorov, A.N., Compans, R.W., 2000. Effect of the cytoplasmic domain of the simian immunodeficiency virus envelope protein on incorporation of heterologous envelope proteins and sensitivity to neutralization. J. Virol. 74, 8219–8225.

Vzorov, A.N., Dixon, D.W., Trommel, J.S., Marzilli, L.G., Compans, R.W., 2002. Inactivation of human immunodeficiency virus type 1 by porphyrins. Antimicrob. Agents Chemother. 46, 3917–3925.

Vzorov, A.N., Marzilli, L.G., Compans, R.W., Dixon, D.W., 2003. Prevention of HIV-1 infection by phthalocyanines. Antivir. Res. 59, 99–109.

Vzorov, A.N., Gernert, K.M., Compans, R.W., 2005. Multiple domains of the SIV Env protein determine virus replication efficiency and neutralization sensitivity. Virology 332, 89–101.