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Prevention of HIV-1 infection by phthalocyanines

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Abstract

The ability of selected phthalocyanines and metallophthalocyanines to block HIV infection has been evaluated in an epithelial HeLa–CD4 cell line with an integrated LTR-β-galactosidase gene. Sulfonated phthalocyanine itself (PcS), as well as its copper, nickel, and vanadyl chelates, were the most effective in blocking viral infection. These compounds were also very effective in blocking the fusion activity of the viral Env proteins. All of these compounds are expected to bind axial ligands weakly or not at all. In contrast, sulfonated phthalocyanines bearing metals expected to bind axial ligands more tightly (aluminum, cobalt, chromium, iron, silicon, and zinc) were less effective in blocking HIV infection and also less effective at inhibiting fusion. A number of active compounds were found to block binding of gp120 to CD4. Selected cationic and carboxy phthalocyanines, as well as porphyrazines, were also evaluated. Our results indicate that at least some of the compounds render the virus noninfectious, i.e. that they are virucidal. These compounds have potential as microbicides that might be used to provide protection against sexually transmitted HIV.

Keywords: Phthalocyanine; Porphyrazine; HIV; Human immunodeficiency virus

1. Introduction

The human immunodeficiency (HIV) epidemic continues to have enormous human health consequences. New approaches are critically needed, particularly ones that are therapeutically effective, safe, cost-effective and accepted across a range of social cultures. Microbicides, which serve to prevent the initial entry of the virus into the cell, offer the possibility of protection against transmission of the virus (Hammett et al., 2000; Bentley et al., 2000; Harrison, 2000; Isaacs et al., 2001; van de Wijgert and Coggins, 2002).

Herein we report recent studies on phthalocyanines (PcS) and metallophthalocyanines (MPcS) as potential microbicides. There have been a number of previous reports of the antiviral activity of phthalocyanines against HIV (Neurath et al., 1992; Dixon et al., 1992, 1994; Neurath et al., 1994, 1995). There has also been work on the photoinactivation of HIV with phthalocyanines (Horowitz et al., 1991, 1992; Rywkin et al., 1994; Margolis-Nunno et al., 1996; Zmudzka et al., 1997; Ben-Hur et al., 1997, 2000). In addition to studies on HIV, there have been many studies of photoinactivation of other viruses with phthalocyanines, largely directed toward making the blood supply safer for transfusions. Studies have been performed on vesicular stomatitis virus (VSV) (Moor et al., 1999; Ben-Hur et al., 2000), Sindbis virus (Rywkin et al., 1994), pseudorabies virus (PRV) (Ben-Hur et al., 2000), bovine viral diarrhea virus (BVDV) (Ben-Hur et al., 2000), and herpes simplex virus (HSV) (Smetana et al., 1994; Rywkin et al., 1994). All of these studies involve photoactivation of the phthalocyanine to produce species (singlet oxygen or free radical) that kill the virus. However, in at least one instance, a Pc has been shown to have antiviral activity in the absence of light. The tetracationic ZnPc(3-MeO-Py)$_4$ was found to protect Vero cells against human rhinovirus type 5 (RV-5) infection when added to the cultures before virus inoculation, with or without subsequent photoactivation (Gaspard et al., 1995). ZnPc(3-MeO-Py)$_4$ did not photoinactivate free RV-5, however. In the current study, we have investigated inactivation of HIV by phthalocyanines via processes that do not involve exposure to light.

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2. Materials and methods

2.1. Phthalocyanines

The cationic phthalocyanines tested were Alec Blue (Aldrich, St. Louis, MO), PcCat(1) and Alcian Blue 8GX (Kodak), PcCat(2). The Al and Co carboxyphthalocyanines (AlPcC and CoPcC) were from Midcentury Chemicals (Chicago, IL). The Co and Fe sulfonated phthalocyanines (Midcentury Chemicals) were made via the Weber–Busch synthesis (Weber and Busch, 1965), and thus are expected to be tetrasulfonates. The other commercial phthalocyanines are apparently mixtures with different extents of sulfonation as well as different positions of the sulfonate on the periphery of the Pc skeleton. There was no obvious correlation between the activity of a given phthalocyanine and the number of components in the mixture. CuPcS(3,4′,4″,4‴) (Aldrich) is designated as CuPcS(1) and CuPcS(4,4′,4″,4‴) (Fluka, St. Louis, MO) is designated as CuPcS(2). The free acid [CuPcS(H+)] and lithium salt [CuPcS(Li+)] of CuPcS were from Frontier Scientific (Logan, Utah). NiPcS was from Aldrich; the rest of the phthalocyanines were from Midcentury Chemicals. The aluminum and zinc phthalocyanines were available in two sulfonation levels. AlPcS(1) and ZnPcS(1) are the more highly sulfonated forms (presumably largely the tetrasulfonates). AlPcS(2) and ZnPcS(2) are less sulfonated (listed as the trisulfonated forms). The porphyrazines DX11 and DX18 were a kind gift of Dr. Brian Hoffman (Northwestern University).

2.2. Cell lines

The mouse NIH/3T3 and human HEp2 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The recombinant cell lines human MAGI, monkey sMAGI, mouse 3T3.T4, 3T3.T4.CCR5, 3T3.T4.CXCR4, and human T-cell lines CEMx174 and HUT78 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS (NIH). The human 293T cell line was kindly provided by Dr. S.L. Lydy (Emory University, Atlanta, GA). NIH/3T3, HEp2, 3T3.T4, 3T3.T4.CCR5, 3T3.T4.CXCR4, MAGI, sMAGI, and 293T 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.

2.3. Viruses and plasmids

For construction of recombinant vaccinia viruses, plasmids pRB21 and vRB12 were kindly provided by Drs. Bernard Moss (National Institutes of Health, Bethesda, MD) and David Steinhauer (National Institute for Medical Research, London, UK). The 3′SHIV-89.6 plasmid was obtained from Dr. J. Sodroski (Harvard Medical School, Boston, MA). Recombinant vaccinia viruses expressing full length (VV-239env) and truncated (VV-239T) SIVmac239 Env proteins were previously described by Ritter et al. (1993), and VVenv1 expressing the BH10 Env protein was described by Owens and Compans (1989). A recombinant vaccinia virus encoding a truncated Env protein of HIV-1 89.6 was constructed as follows. The HIV-1 89.6 truncated env gene was obtained by polymerase chain reaction (PCR) amplification from the HIV-1 89.6 plasmid with the following primers: the 5′-primer introducing an EcoRI site 5′-GAGAAGAATTCAGTGGCAATGAGAG TGAAGG-3′; the 3′ primer introducing an Nhe I site and a premature stop codon after the codon for amino acid (aa)17 in the cytoplasmic domain 5′-CCTGTCGGCTAGC CTCGATCATGGGAGG AGGGTCTGAAACGATAATG. The PCR product was then digested by EcoR I and Nhe I and ligated into EcoR I and Nhe I—predigested pRB21 as a donor plasmid for vaccinia recombination. The recombinant vaccinia virus was obtained by a plaque selection system using a recipient vaccinia virus vRB12 described by Blasco and Moss (1995). The plasmid pIIIenv3-1 encoding the Env protein of the HXB2 strain of HIV-1 was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS (NIH). The Tat-responsive HIV-LTR in pIIIenv3-1 was used to promote expression of HXB2 Rev and Env. The helper plasmid pCMVtat was kindly provided by Dr. Steven Bartz (Fred Hutchinson Cancer Research Center, Seattle, WA). Virus-infected H9/HTLV-IIIB NIH 1983 cells were obtained from the AIDS Research and Reference Reagent Program, and the supernatant was used to infect HUT78 cells. HIV-1 IIIB virus was produced by continued passage of infected HUT78 cells and virus stock was prepared as described previously (Vzorov and Compans, 2000). To prepare HIV-1 89.6 virus, 293T cells were transfected with p89.6 (from the NIH AIDS Research and Reference Reagent Program). At 48 h post-transfection, DMEM was removed and the cells were washed once in RPMI. Then 2×10^6 CEMx174 cells were added to a plate in 5 ml of RPMI containing 10% fetal calf serum and cocultured overnight. The following day, CEMx174 cells were removed from virus-producing 293T cells and placed in T-25 flasks for continued passage. SIVmac1A11 virus stock was described previously (Vzorov and Compans, 2000).

2.4. Monoclonal antibodies, antisera, proteins

SIM.2 and SIM.4 antibodies recognizing human CD4 and recombinant soluble human CD4 were provided by the NIH AIDS Research and Reference Reagent Program (NIH). The recombinant IIIB gp120 protein (baculovirus-expressed) was obtained from Intracel (Cambridge, MA). Anti-mouse IgG peroxidase conjugate was obtained from Sigma (St. Louis, MO).

2.5. Screening of phthalocyanines for activity against HIV-1

Phthalocyanine stock solutions were prepared at concentrations of 5 mg/ml, diluted 100-fold in growth medium, and mixed with virus stock. Samples were left in the dark at room temperature for 1 h. For MAGI or sMAGI 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 MAGI or sMAGI cells (on a 96-well plate). At 2 h post-infection, an additional 200 μl of complete DMEM was added. After 3 days, virucidal activity was measured by removal of the media, fixation with 1% formaldehyde and 0.2% glutaraldehyde and staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). We observed about 50–60 separate blue nuclei per well for the positive control. Scoring of blue nuclei in a 96-well format was greatly enhanced by using a planar lens [Olympus (Japan) 4×] to visualize the entire well. For determining virus titers we used RT (Roche), MAGI (Kimpton and Emerman, 1992) or sMAGI (Chackerian et al., 1995) assays. Comparison of the number of blue 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).

2.6. Removal of unbound compound

To separate virus from unbound compounds, 50 μl of the virus/compound mixture was mixed with 450 μl of PBS and loaded into a reservoir with a filter (Microcon YM-100, Millipore). The sample reservoir was placed into an Eppendorf tube and spun at 10,000 rpm for 3 min. To collect the sample, the reservoir was inverted into a new Eppendorf tube and spun again (recovery spin). The volume of the sample after the recovery spin (about 50 μl) was readjusted to 500 μl with PBS and spun using a new filter. The procedure was repeated a total of four times. The final volume was adjusted up to 100 μl using PBS. To this was added 100 μl of 2 × DMEM containing 20% of FCS and 30 μg/ml DEAE-Dextran; 50 μl of the resulting solution was added to the MAGI cells. In control experiments in which the concentrations of compounds were measured spectroscopically, this procedure removed approximately 99% of carboxylated tetrapyrroles. Carboxylated tetrapyrroles gave the calculated dilutions, but sulfonated tetrapyrroles did not go through the membrane readily.

2.7. gp120–CD4 binding assay

To investigate the possible effect of phthalocyanine compounds on binding of HIV-1 IIIB gp120 to CD4, we developed a gp120–CD4 binding assay. The assay was developed as a modification of a capture gp120 ELISA kit (Intracel Corporation). Briefly, a 96-well plate was coated with soluble CD4 and 0.5 μg of HIV-1 IIIB gp120 per well was incubated in the presence or absence of test compounds for 1 h at room temperature. After four washes with buffer to remove unbound proteins, the bound gp120 was detected by anti-gp120 peroxidase-conjugated antibodies and quantitated by the protocol provided by the manufacturer.

2.8. Cell fusion assays

For cell fusion assays, we compared three different expression systems: (1) a recombinant vaccinia expression system, which is able to express high levels of Env; (2) a plasmid expression system which is able to express Env proteins in the absence of other HIV proteins or vaccinia proteins; and (3) cells persistently infected with HIV-1 IIIB or HIV-1 89.6. In the first system, HEp2 cells were infected at a m.o.i. of 5. After 24 h cells were collected, counted and about 2.5×10^3 cells were added to 3T3CD4CXCR4 or 3T3CD4CCR5 cell monolayers in 96-well plates in 100 μl of media in the presence or absence of the test compounds.

For the second assay we transfected 293T cells by the calcium phosphate precipitation method with the plasmid pIIIenv3-1 expressing the HIV-1 Env protein (HXB2 Env) with an LTR promoter, and cotransfected with a helper plasmid pCMVTAT at a ratio of 10:1; or with plasmids expressing SIV Env proteins using a CMV promoter as described above. After 48 h cells were collected and cocultured with uninfected cells as in the previous assay.

As a third system we used HUT78 cells persistently infected with HIV-1 IIIB or CEMx174 cells persistently infected with HIV-1 89.6. The infected cells were counted and cocultured with uninfected cells as in the previous assays.

For all fusion assays, after 5 or 20 h of cocultivation, the level of cell fusion induced by the untreated recombinant or virus-infected cells and the extent of fusion inhibition by the test compounds was evaluated by microscopic observation. Fusion activities were determined by counting the nuclei in syncytia compared with the total nuclei.

2.9. Influenza virus plaque assay

We used phthalocyanine stock solutions at concentrations of 5 mg/ml diluted 10-fold in medium DMEM without FCS; 5 μl of compounds were mixed with 45 ul influenza virus (A/PR/8/34[H1N1]) at a concentration of approximately 3×10^3 infectious particles, and left in the dark at room temperature for 1 h. For MDCK plaque assay, 50 μl of virus/compound mixture was mixed with 450 μl medium, and 200 μl of this mixture was added to wells with confluent MDCK cells (6-well plate). After 1 h incubation, the cells were washed and agar containing 2× Dulbecco's medium, 2.5 μg/ml trypsin, was added. After 2 days, agar with neutral red was added. After 3 days, the activity was measured by comparison of the number of plaques in wells infected with compound-treated virus to the number in wells infected with untreated virus.

2.10. Cytotoxicity test

Two approaches were used to determine cytotoxicity. For a trypan blue exclusion test (Strober, 1994), compounds at a concentration of 50 μg/ml in growth medium were added to 96-well plates with MAGI cells. After 72 h cells were detached by 0.25% trypsin–0.05% versene solution and diluted 1:10 in growth medium. To test cell viability we mixed 1 part of 0.4% trypan blue and 9 parts of diluted cells, incubated the mixture about 2 min at room temperature, and applied a drop of the trypan blue/cell mixture to a hemacytometer. Stained (nonviable) and unstained (viable) cells were counted using a binocular microscope. The fraction of viable cells was calculated as the number of unstained cells in the wells treated with compound as a percentage of the number in control wells.

The cytotoxicity of compounds was also measured by quantitative ^{3}H-thymidine incorporation assay (Tobin et al., 1996). For this assay, HUT78 or HeLaS3 cells were seeded in 96-well plates in growth media using about 3×10^{4} cells per well. After a 3 day incubation with test compounds of varying concentrations (500, 200, 100, 50, and 5 μg/ml), the cells were pulsed for 12 h with 1 μCi of ^{3}H-thymidine per well. The same test was also performed, with and without compounds, but without the 3 day incubation. The cells were harvested by a semi-automated multiwell harvester (SOP AVRP-009-01). Radioactive incorporation was quantitated by scintillation counting. The counts from wells containing compounds were compared to control wells to determine the concentration that inhibited the cell growth (DNA replication) by 50% (CC50).

3. Results

3.1. Inhibition of viral infection

Four classes of compounds were studied: phthalocyanines with positively charged substituents, phthalocyanines with carboxylate substituents, porphyrazines, and sulfonated phthalocyanines (Fig. 1). To evaluate the activity of these compounds we used an epithelial HeLa–CD4 cell line with an integrated LTR-β-galactosidase gene and a final compound concentration of 5 μg/ml. The positively charged phthalocyanines PcCat(1) and PcCat(2) exhibited very low anti-HIV activity; PcCat(1) did not block viral infection and PcCat(2) promoted growth of the virus (185 ± 5%). The phthalocyanines bearing carboxylate substituents had moderate to low activity. The Al and Co chelates blocked 40 ± 15 and 19 ± 2% of viral infection, respectively. The two porphyrazines tested had higher levels of activity; DX11 and DX18 blocked 72 ± 4 and 84 ± 4% of viral infection, respectively.

In general, the sulfonated phthalocyanines were the most active of the classes studied (Fig. 2). A strong correlation was observed between high activity and the absence of a ligand on the central metal. Thus, the most active compounds are the parent PcS (no metal, 94% blocking of HIV infection) and its chelates with copper [CuPcS(1), essentially no binding of axial ligands, 97% blocking of HIV infection] and nickel (NiPcS, weak binding of axial ligands, 93% blocking of HIV infection). The VO derivative, which has only a single tightly bound oxygen atom as an axial ligand, also displayed good activity (86% blocking of HIV infection).

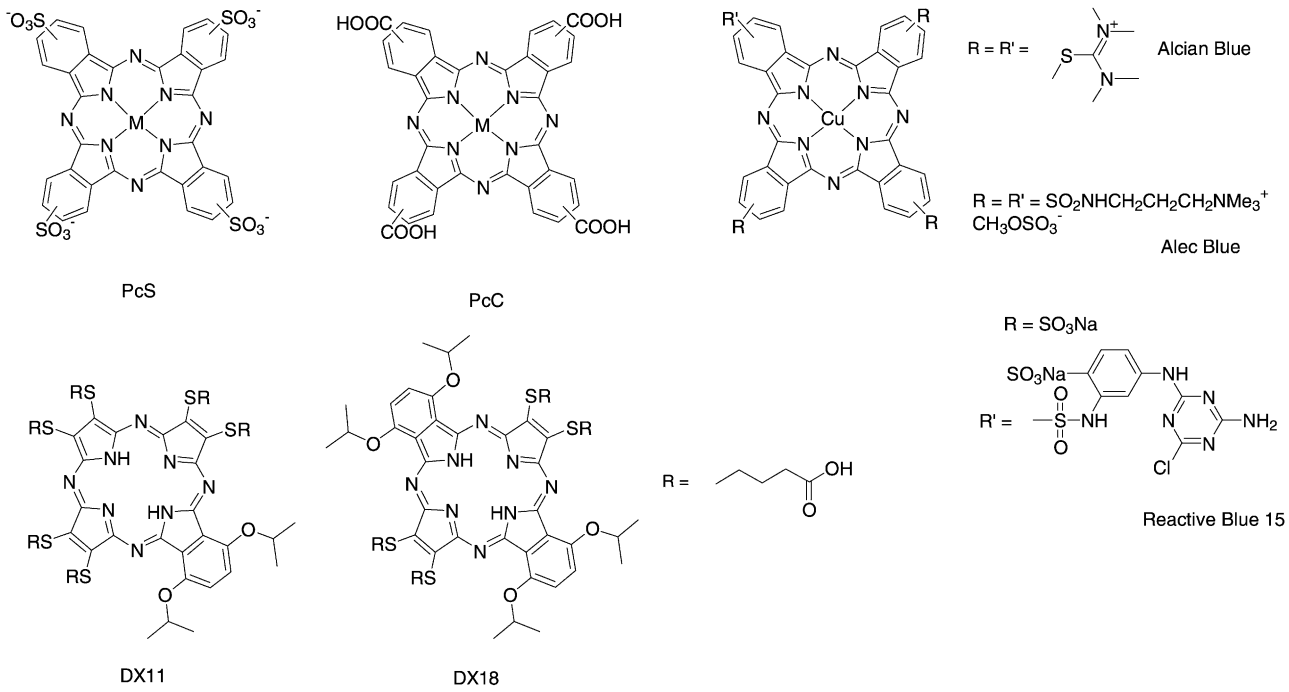

Fig. 1. Structures of phthalocyanines and porphyrazines.

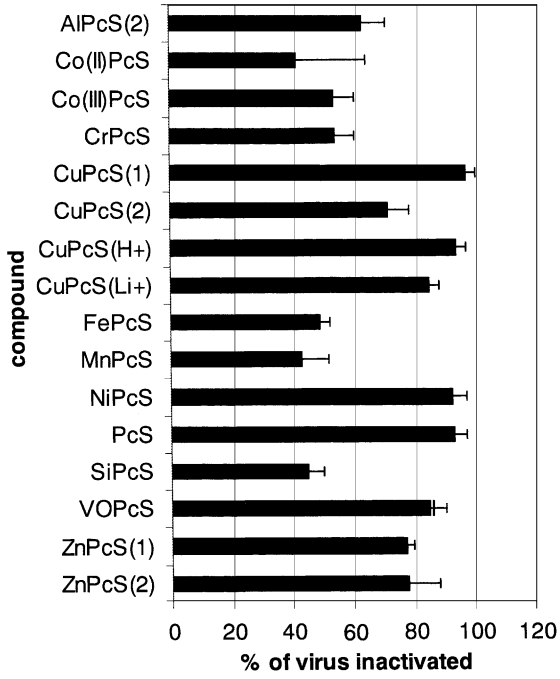

Fig. 2. Activity of sulfonated phthalocyanines against HIV-1 IIIB. Compounds at a concentration of 50 μg/ml were incubated with HIV-1 IIIB in the dark for 1 h, diluted 10-fold and the virus used to inoculate MAGI cells. HIV infectivity was measured after 3 days by removal of the media, fixation and staining with X-gal. The activity against HIV was measured by dividing the number of blue cells in wells infected with compound-treated virus by the number in wells infected with untreated virus. Data are plotted as the mean of three experiments, each replicated twice. Error bars represent standard deviations.

The Al, Co, Cr, Fe, Mn, Si, and Zn chelates, all of which have axial ligands, had lower activity, blocking 40–80% of HIV infection.

The copper chelate was studied further because it had high activity. In addition, because the copper is paramagnetic, copper phthalocyanines are not sensitive to light (essentially eliminating any possible photodynamic side effects). Four commercially available samples were studied. CuPcS(1), CuPcS(2), CuPcS(H+) and CuPcS(Li+) had activities of 97, 72, 94 and 85% virus inhibition, respectively. It appears that some components of these mixtures of sulfonated copper phthalocyanines are more active than others.

3.2. Kinetics of inactivation

To determine the kinetics of inactivation of viral infectivity, we incubated mixtures of HIV-1 IIIB with the test compounds and assayed residual infectivity at various time intervals. Two very active [NiPcS and CuPcS(1)], one moderately active [ZnPcS(1)] and two relatively inactive chelates [Co(II)PcS and AlPcC] were studied. For all except ZnPcS(1), the inactivation level observed at 2 min was constant over the time period studied (60 min) (Fig. 3). With ZnPcS(1), a somewhat longer time course was observed, and less inactivation was found at the last time point taken. In general, our results demonstrated that the interaction of phthalocyanines with HIV-1 IIIB is both rapid and independent of time after 2 min.

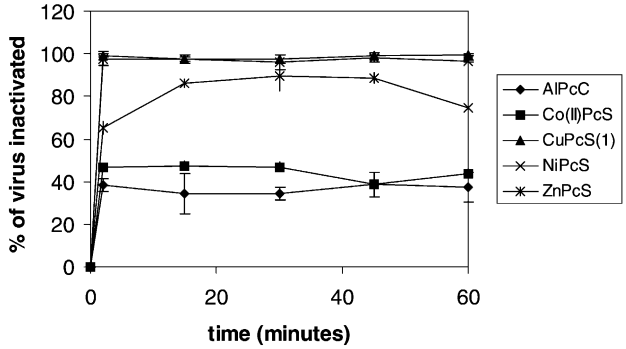

Fig. 3. Kinetics of inactivation of HIV-1 IIIB. Compounds at a final concentration of 50 μg/ml were mixed with virus and incubated at various time intervals (5, 15, 30, 45, 60 min), diluted 1:10 with complete medium, and infectivity titers determined. Data are plotted as the mean of three experiments, each replicated twice. Error bars represent standard deviations.

3.3. Effective concentration

To determine the effective concentration of some of the more active compounds, virus samples were mixed with phthalocyanines at concentrations from 0.6 to 50 μg/ml (Fig. 4). The most effective concentration was generally the highest concentration tested of 50 μg/ml. However, four of the six compounds studied [CuPcS(1), CuPcS(H+), NiPcS, and VOPcS] also exhibited approximately 50% activity at concentrations of 6.25 μg/ml or less. Thus, the most active compounds had an EC50 of less than 6.25 μg/ml.

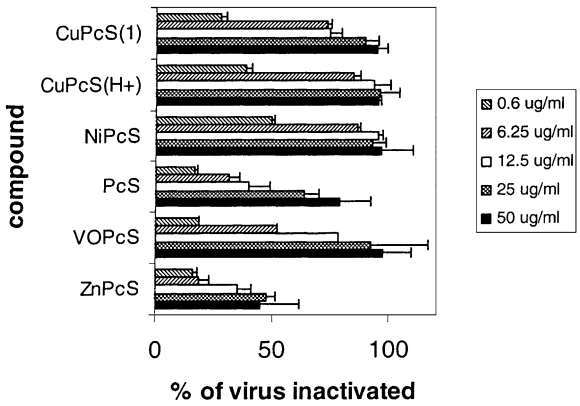

Fig. 4. Activity of selected phthalocyanines as a function of concentration. Virus samples were mixed with phthalocyanines at the indicated concentrations and infectivity titers determined. Data are plotted as the mean of three experiments, each replicated twice. Error bars represent standard deviations.

3.4. Activity after removal of phthalocyanine

It may be desirable for a potential microbicide to be virucidal, e.g. for inhibition of viral infectivity to continue even after the concentration of the compounds is substantially decreased. To show that the virus, once treated, was still rendered noninfectious once the unbound compound had been removed we used a filtration-dilution protocol. In this protocol, solutions of the virus and compound were filtered until only about 10% of the original volume remained. The solution that had not gone through the filter was diluted to the original volume and the process repeated four times. Control spectroscopic assays showed that the dilutions resulted in sulfonated tetrapyrrole concentrations being reduced by approximately 100-fold. Three compounds were evaluated, NiPcS, PcS and ZnPcS(1). After exposure to ZnPcS(1), filtration-dilution resulted in partial recovery of infectivity. In contrast, NiPcS and PcS were both very effective in blocking of HIV infection, and after filtration-dilution there was only minimal recovery of infectivity. These results indicated that PcS and NiPcS are truly virucidal; that is, the virus is inactivated irreversibly by the compound.

3.5. Activity against other viruses

To investigate the specificity of the compounds with high activity against HIV, we extended our studies to the HIV-1 primary isolate 89.6, SIVmac1A11 and influenza virus (A/PR/8/34[H1N1]). We selected the most active compounds against HIV-1 IIIB: CuPcS(1), NiPcS and PcS. These three compounds inactivated HIV-1 primary isolate 89.6 to the extents of 73, 83 and 83%, respectively. We also observed that SIVmac1A11 was also sensitive to the compounds: 82, 89, and 59% inactivation, respectively. To determine the specificity of these results we used influenza virus (A/PR/8/34[H1N1]) which is an unrelated enveloped virus. Influenza virus was less sensitive to these compounds: CuPcS(1), NiPcS, and PcS inactivated 44, 37 and 46% of the infectivity, respectively.

3.6. Toxicity

To confirm that the decreased numbers of HIV-infected cells observed in the MAGI assay were not a result of the toxicity of the phthalocyanines, we assessed the toxicity of the more active phthalocyanines. Compounds at a concentration of 50 μg/ml in growth medium were added to MAGI cells. This concentration is 10-fold higher than that used when the compounds are applied to MAGI cells for virus assay. After 72 h, a trypan blue assay was used to compare cell viability in cells treated with compounds to untreated cells. Cells treated with CuPcS(1), CuPcS(H+), NiPcS, and PcS, retained about 88, 65, 72 and 53% viability, respectively.

To determine the possible effects of cell division on the assay, we also compared the cytotoxic effects of active compounds at different time points by using a quantitative ^{3}H-thymidine incorporation cell growth assay. Two suspension cell lines, HUT78 and HeLaS3, were used; the latter are closely related to MAGI cells, which are derived from HeLa cells. We observed that either without incubation or after a 3 day incubation, none of the compounds tested [CuPcS(1), CuPcS(H+), NiPcS, PcS, VOPcS, ZnPcS(1)] were toxic in either cell line, with CC50 > 500 μg/ml for all except CuPcS(Li+) which had CC50 > 200 μg/ml. Based on these assays, therapeutic indices were calculated to be: CuPcS(1), >165; CuPcS(H+), >80; CuPcS(Li+), >13; NiPcS, >70; PcS, >70; VOPcS, 35; and ZnPcS(1), >20, respectively.

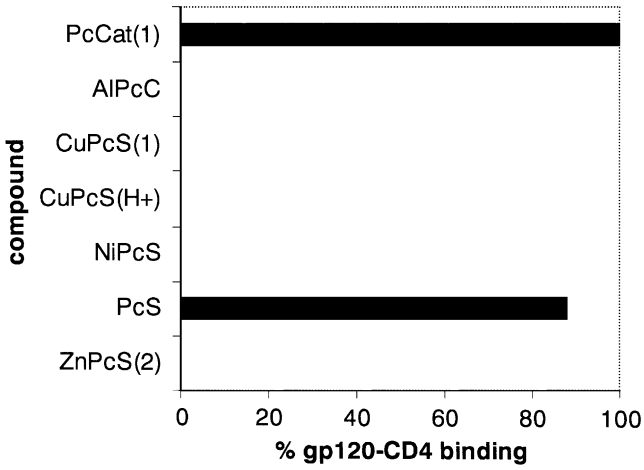

Fig. 5. Inhibition of gp120–CD4 binding. A 96-well plate coated with soluble CD4 was incubated with HIV-1 IIIB gp120 in the presence or absence of compounds for 1 h at room temperature. After extensive washes the bound gp120 was detected by anti-gp120 peroxidase conjugated antibodies. Results are given as percentage of gp120 binding compared to untreated gp120 sample (100%).

3.7. Effect of phthalocyanines on interaction of gp120 with CD4

To investigate the site of action of the compounds, we studied the effect of phthalocyanines on binding of gp120 to its primary receptor, CD4 (Dalgleish et al., 1984). CD4 binding results in a conformational change in gp120 (Sattentau and Moore, 1991) that enables it to interact with a coreceptor, generally either CCR5 or CXCR4 (Lapham et al., 1996). A gp120–CD4 binding assay was used as described in Section 2. CuPcS(1), CuPcS(H+), NiPcS, and ZnPcS(2), which all were effective to very effective in blocking HIV infection, also showed substantial (or complete) inhibition of gp120–CD4 binding (Fig. 5). However, PcS, which also is effective in blocking HIV infection (94%), did not block binding of gp120 to CD4 effectively. AlPcC had moderate activity against HIV (40% blocking of HIV infection) but completely inhibited binding of gp120 to CD4. Thus, there seems to be no correlation of anti-HIV activity and inhibition of gp120–CD4 binding in these experiments, suggesting that more than one mechanism of blocking of HIV infection is operative.

Table 1
Inhibition of virus-induced cell fusion by phthalocyanines

Compounds/constructs	pIIIenv X4 8 h/21 h	VVenv1 X4 8 h/21 h	VV89.6 envt X4 8 h/21 h	VV89.6 envt R5 8 h/21 h
Control	4+/4+	4+/4+	4+/4+	4+/4+
AlPcC	2+/4+	4+/4+	4+/4+	4+/4+
AlPcS(1)	3+/4+	3+/4+	4+/4+	4+/4+
Co(II)PcS	−/3+	−/3+	4+/4+	3+/4+
Co(III)PcS	−/−	−/−	−/4+	3+/4+
CrPcS	−/−	−/3+	−/4+	2+/4+
CuPcS(1)	−/−	−/−	−/−	−/−
CuPcS(2)	−/−	−/−	−/−	−/−
CuPcS(H+)	−/−	−/−	−/−	−/−
CuPcS(Li+)	−/−	−/−	−/−	−/−
MnPcS	−/−	−/−	−/−	−/−
NiPcS	−/−	−/−	−/−	−/−
PcS	−/1+	−/−	−/−	−/−
SiPcS	2+/4+	4+/4+	4+/4+	4+/4+
VOPcS	−/−	−/−	−/−	−/−
ZnPcS(1)	−/1+	−/−	−/2+	1+/4+
ZnPcS(2)	2+/4+	2+/4+	3+/4+	3+/4+

Fusion activities were determined by counting the nuclei in syncytia compared with the total nuclei: 4+, more than 50% of nuclei are present in syncytia; 3+, 30–50% of nuclei are present in syncytia; 2+, 30–10% of nuclei are present in syncytia; +, less than 10% of nuclei are present in syncytia; −, no syncytia were observed.

3.8. Inhibition of HIV Env-induced cell fusion by phthalocyanines

One possible mechanism by which phthalocyanines might block viral infection is inhibition of the fusion activity of the viral Env proteins, which is required for viral entry. This was investigated by assaying cell fusion activity (Table 1, Fig. 6). We compared three different expression systems for the Env proteins. These systems differ in their expression of other encoded proteins.

Our first studies were done with a recombinant vaccinia expression system, which is able to express high levels of Env. Two recombinants were used: one expressing the HIV-1 IIIB Env which has tropism for the X4 coreceptor (VVenv1), and the second expressing the Env of HIV-1 89.6, a primary viral isolate with dual tropism for both X4 and R5 coreceptors (VV89.6 envt). With these systems, we observed essentially complete inhibition of HIV Env-induced cell fusion with the parent, nickel, manganese and vanadyl sulfonated phthalocyanines (PcS, NiPcS, MnPcS and VOPcS), and all forms of the copper phthalocyanines [CuPcS(1), CuPcS(2), CuPcS(H+), and CuPcS(Li+)]. With the exception of the manganese chelate, these phthalocyanines bind axial ligands either not at all (parent, VO) or weakly, leading to planar structures. In contrast, phthalocyanines bearing metals expected to bind axial ligands were not very effective at inhibiting fusion. All of AlPcC, AlPcS, Co(II)PcS, Co(III)PcS, CrPcS, ZnPcS(1) and ZnPcS(2) had at least 30% of nuclei in syncytia at 8 h in one of the tests. Thus, inhibition of fusion is apparently most easily achieved with planar phthalocyanines [i.e., those with no, or small (VO), axial ligands]. MnPcS is an exception; perhaps other

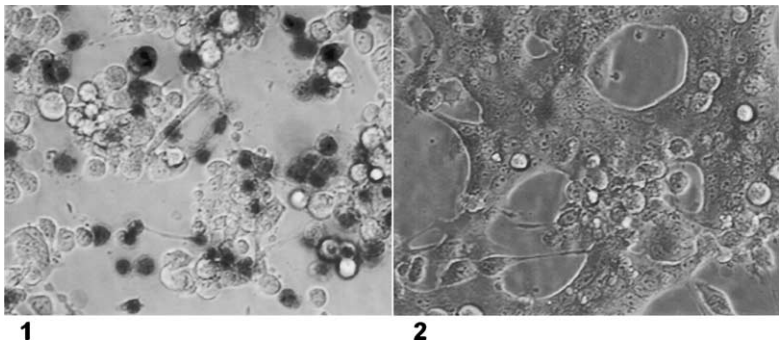

Fig. 6. Inhibition of Env-induced cell fusion by phthalocyanines. About 2.5×10^3 HEp2 cells infected with VVenv1, the vaccinia recombinant expressing the HIV-1 IIIB Env protein (1, 2), were added to 3T3CD4CXCR4 cells in a 96-well plate in the presence (1) or absence (2) of NiPcS and incubated for 5 h for syncytium analysis; samples were fixed, and photographed under a phase-contrast microscope.

mechanisms are important for this chelate, which is not considered further. We also observed a correlation between inhibition of fusion and blocking of viral infection. All the phthalocyanines that showed essentially complete inhibition of HIV-induced cell fusion, with the exception of CuPcS(2), blocked more than 80% of viral infection. In contrast, the chelates that did not inhibit fusion activity significantly blocked less than 80% of viral infection and most blocked less than 60%.

In a second set of experiments, we used pIIIenv, a plasmid expression system that is able to express Env proteins in the absence of other HIV proteins or vaccinia proteins. As above, we observed essentially complete inhibition of HIV Env-induced cell fusion with PcS, NiPcS, MnPcS, VOPcS, and all forms of the copper phthalocyanines [CuPcS(1), CuPcS(2), CuPcS(H+), and CuPcS(Li+)]. PcS did not completely block fusion in the pIIIenv recombinant (after 21 h incubation, 10% of the cells were fused), though it did inhibit fusion in the recombinant vaccinia expression system. PcS also blocked gp120–CD4 binding by only about 35%.

For comparison, we also used cells persistently infected with HIV-1 IIIB or HIV-1 89.6 for cocultivation with uninfected target cells in the presence or absence of test compounds. The fusion activity observed in this assay was comparable with that found using plasmids expressing the HIV Env protein, and lower than observed with Env expressed by vaccinia virus. The results of inhibition of HIV-induced fusion by the compounds tested correlated well with results observed using both other expression systems (data not shown).

These results demonstrate that the compounds with high or intermediate levels of anti-HIV activity are able to effectively inhibit the membrane fusion activity of the viral Env proteins, a biological function that is important for viral entry as well as the induction of viral cytopathic effects.

4. Discussion

The central goal of our study was to identify novel compounds with high activity in blocking HIV infection, which could be useful as topical microbicides to provide a defense against infection by sexually transmittted virus. The vaginal and gastrointestinal surfaces play a major role in the pathogenesis of infection by HIV-1 as potential routes for viral entry. Several reports have described HIV attachment and infection of epithelial cells (Fantini et al., 1993; Yahi et al., 1995; Trujillo et al., 2000). Langerhans cells (LCs) are CD4+ CD1a+ antigen-presenting cells in the dendritic cells (DC) family that are present within the epidermis and mucosal epithelium and that are believed to be the initial targets for HIV infection (Zambruno et al., 1995; Turville et al., 2001). Galactosylceramide (GalCer) has been implicated in HIV-1 entry into some CD4 negative epithelial cell lines (Harouse et al., 1991, 1995; Yahi et al., 1992; Fantini et al., 1993). HIV-1 entry into human intestinal cells is reported to involve both GalCer and CXCR4/fusin (Delezay et al., 1997). HIV-1 isolates such as 89.6 that are able to use CXC4/fusin as a coreceptor, but do not bind to GalCer, do not infect these cells. These data may indicate that CXCR4/fusin can function as a coreceptor for HIV-1 entry into CD4−/GalCer+ intestinal epithelial cells (Delezay et al., 1997). This also correlates with a recent report showing that for infection of colonic epithelial HT-29 cells by HIV-1, both CXCR4 and GalCer receptors were critical (Trujillo et al., 2000).

As an assay for anti-HIV activity of test compounds, we used a MAGI assay which is based on usage of an epithelial cell line. We did not evaluate the use of human PBMCs as a target cell, because it is unlikely that these cells would be relevant to prevention of initial infection at mucosal surfaces. HeLa cells stably transfected with human CD4 (HeLa–CD4 cells) are permissive for T cell line-adapted X4 or dual usage R5X4 viruses, because they express the coreceptor CXCR4. All the anionic phthalocyanines studied were active with various degrees of efficacy against HIV-1 IIIB. Overall, our results indicate that the most promising compounds were CuPcS(1), CuPcS(H+), and NiPcS. These compounds were also able to inhibit infection by the dual tropic HIV-1 primary isolate 89.6 as well as SIVmac1A11 viruses. Although we did not observe complete inactivation of infectivity, it is relevant to note that mucosal infection by HIV and SIV is a very inefficient process that requires multiple exposures to the virus. Thus, even partial prevention of infection is expected to reduce the likelihood of virus transmission. The phthalocyanine compounds may also be useful in combination with other potential microbicides. Since sexual transmission may involve either cell-free or cell-associated virus, it will also be important to evaluate the effect of the phthalocyanines to prevent the latter type of infection.

Polyanionic compounds have been shown to inhibit HIV replication by preventing virus attachment (adsorption) to the surface of the host cell (De Clercq, 2002b). These negatively charged species might be expected to interact with the positively charged amino acids in the V3 loop of the HIV glycoprotein, gp120, which is rich in arginine and lysine residues (Neurath et al., 1995). In doing so, the polyanions shield the V3 loop and therefore may interfere with binding of the HIV virions to cell surface components (Gallaher et al., 1995). The sulfonated phthalocyanines studied are small polyanionic molecules. They inhibit viral binding and fusion/entry into susceptible cells. In this respect, they are similar to other polyanionic species, including sulfated polymers.

To determine the specificity of the phthalocyanine–virus interaction, we compared the activity of the compounds against HIV-1 IIIB with that against influenza virus. The compounds with the highest activity against HIV were only moderately active against influenza virus. These results may indicate that the virucidal effect of these compounds is a result of their interaction with specific viral components, in

addition to a general disruptive effect on enveloped virus structure.

We also observed that compounds with anti-HIV activity were able to inhibit the cell fusion activity of the HIV Env protein. To exclude the possibility that such an inhibitory effect could be due to an indirect effect on surface expression of the Env protein, we demonstrated that cell fusion induced by recombinant vectors in the absence of any other HIV protein was also sensitive to inhibition by the compounds. These results provide strong evidence that the phthalocyanines are able to inhibit an important function of the Env protein that is needed for viral entry. Structure activity correlations indicate that planar or nearly-planar phthalocyanines are more effective than phthalocyanines with axial ligands both in inhibition of fusion and blocking of viral infection.

The interaction of the phthalocyanines with HIV appears to be very rapid. For PcS and NiPcS, removal of free compound did not result in significant recovery of infectivity, indicating that these are effective virucidal agents. Virucidal activity is desirable, but presumably not necessary, in that the compounds when used as microbicides will continue to be present at sites of transmission during exposure to virus in vivo. Sulfonated phthalocyanines therefore are a promising class of compounds for further development as microbicides to prevent HIV transmission. Specific structural features, particularly the central metal and perhaps the extent and placement of the sulfonic acid groups, also play a role in determining the activity of these compounds.

There is substantial current interest in the development of microbicides to prevent HIV transmission (Gabelnick and Harper, 1999; Upmalis, 1999; Darroch and Frost, 1999; Hammett et al., 2000; Harrison, 2000; van de Wijgert and Coggins, 2002). Nonoxynol-9 (N9), a surfactant that inactivates viruses, was one of the first candidates to be investigated. It was found that treatment with N9 and related molecules can lead to inflammation and ulceration of human tissues (Stafford et al., 1998). Clinical trials have shown that N9 increases the risk of acquiring HIV infection during sexual transmission (Fichorova et al., 2001; Richardson et al., 2001; van de Wijgert and Coggins, 2002). It is therefore no longer being considered as an agent to prevent HIV infection. A second group includes agents that enhance normal vaginal defense mechanisms; examples include lactobacilli, acid buffers, and peroxidases (Clarke et al., 2002). It is uncertain whether this approach will result in full inactivation of the virus particles under conditions of expected clinical use. A third group of compounds includes peptides and antibodies; these also enhance the normal vaginal defense mechanisms (Weber et al., 2001; Mascola, 2002). Although these appear promising in many respects, a limitation may be the difficulty of formulating these compounds into vaginal microbicides. A fourth group includes polymers such as the sulfonated polysaccharides chondroitin sulfate and carrageenan (D'Souza et al., 2000; De Clercq, 2002a). Carraguard, a vaginal microbicide gel containing carrageenan, has been shown to block HIV and other sexually transmitted agents in vitro and is entering phase II clinical trials (Spieler, 2002). It is not yet clear whether polymers or small molecules will be more effective in blocking infection by HIV and it will be of interest to test such agents in combination.

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