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Prevention of HIV-1 infection by platinum triazines

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Abstract

To identify and explore the activity of compounds which may act as anti-HIV virucidal agents, we have investigated platinum compounds, especially those containing N-donor aromatic ligands. After screening over 70 related agents, including N-donor aromatic ligands and metal precursors, we have identified a novel class of platinum(II) complexes with 2-pyridyl-1,2,4-triazine derivatives and Pt(II) formulations with these derivatives (ptt compounds) as having the highest anti-HIV activity. The maximum activity was observed when the agents were added immediately post-infection. The ptt agents did not block cell fusion activity of HIV-1 Env proteins in cells bearing CD4X4 or CD4R5 receptors, indicating a lack of interaction with the Env protein. The ptt compounds exhibit low toxicity for human epithelial cells, and are thus promising candidates for use as microbicides or antiviral agents against HIV.

Keywords: HIV; Microbicides; Reverse transcriptase inhibitor

1. Introduction

Sexual transmission of infectious diseases continues to represent a major worldwide public health problem. Interference with the initial stages of infection by the application of effective topical microbicides provides an attractive approach for control of such diseases (Di Fabio et al., 2003; Foss et al., 2003; Harrison et al., 2003). Numerous agents have been proposed as topical anti-HIV microbicides, including surfactants, agents that enhance normal vaginal defense mechanisms, peptides, antibodies and polymers (Pauwels and De Clercq, 1996; Rosenthal et al., 1998; McCormack et al., 2001; De Clercq, 2002). The positive charge on the gp120 protein of X4 viruses, which use the CXCR4 chemokine as a receptor for entry, is significantly greater than that of the R5 viruses, which use CCR5 coreceptor for entry (Moulard et al., 2000), and the latter viruses may be able to escape the inhibitory action of negatively charged polymers, the most widely assessed group of candidate microbicides (Shattock and Doms, 2002). Antiviral agents that inhibit a key function of HIV replication prior to integration, such as reverse transcriptase (RT) inhibitors, may be useful in microbicide applications (McCormack et al., 2001; Pauwels and De Clercq, 1996; Borkow et al., 2002).

We have recently demonstrated potent anti-HIV activity for two classes of compounds: porphyrins and phthalocyanines (Vzorov et al., 2002, 2003). Both categories of compounds are small-molecule, negatively charged polyanions, and both were found to inhibit fusion activity of the HIV envelope (Env) protein and to block binding of the Env protein (gp120) to CD4. The presence of sulfonated aromatic groups and metals in the most active members of both groups motivated us to devise novel polyanionic sulfonated aromatic synthetic metal chelates for preliminary evaluation of their HIV-1 virucidal activity.

The name “inorganic medicinal agents” has recently been applied to metal-containing drugs (Orvig and Abrams, 1999). Assessments of antiviral activity of such agents usually employ metal chelates containing Cu(I) and Cu(II), which undergo redox reactions and rapid ligand exchange (Lebon et al., 2002; De Clercq, 1997). These properties

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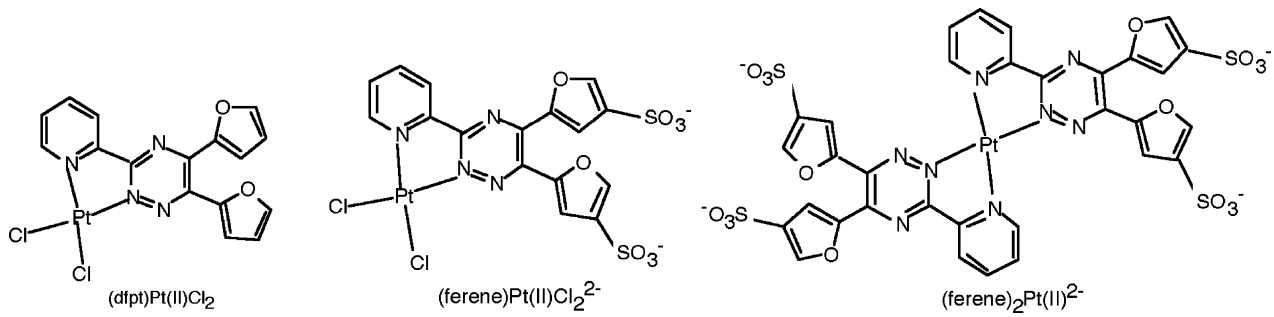

Fig. 1. Proposed structures of three related and representative ptt compounds tested in this study.

may be involved in the process leading to antiviral activity, but redox and rapid exchange reactions make defining the mechanism of action difficult. Antiviral testing of inorganic medicinal agents bearing aromatic sulfonic acid groups has been very limited. Levine and colleagues (Davis et al., 1995) clearly demonstrated that a pair of related tetrahedral d_{10} Cu(I) cationic and anionic compounds are toxic/inactive and non-toxic/active, respectively. These inorganic medicinal agents had two 2,9-dimethyl-1,10-phenanthroline (neocuproine) chelating moieties. Only the bis anionic bathocuproine disulfonate (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonate, or bcds) complex, with four peripheral phenylsulfonate groups, was active as an antiviral agent. This trianionic tetrahedral compound was reported to inhibit the HIV-1 protease. The Cu^{2+} ion itself inhibits the protease, but the work of Levine elegantly showed that this involves oxidation of cysteine by Cu^{2+} ion (Karlström and Levine, 1991). Also, the simple Cu^{2+} cation is inactive as an antiviral agent because it cannot penetrate cells or the viral envelope to reach the protease. It was logically concluded that ligands, such as bcds (dinegative at pH 4.5 or greater) facilitated the delivery of the complex to the viral target.

In this study, we evaluated the potential as microbicides for HIV prevention of agents containing the 3-(2-pyridyl)-1,2,4-triazine group (Fig. 1), usually with aromatic sulfonic acid groups at the 5 and/or 6 positions, and the exchange-inert, redox-stable Pt(II) metal center. For simplicity, this class of compounds, many of them novel, will be designated as ptt. We evaluated the virucidal and antiviral activity of these ptt agents against HIV-1 and determined their effect on fusion activity of the viral Env protein as well as RT activity.

2. Materials and methods

2.1. Starting materials

3-(2-Pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine, disodium salt (ferene) (GFS Chemicals), 3-(2-(pyridyl)-5,6-diphenyl)-1,2,4-triazine (dppt) (Fluka, Milwaukee, WI), 5,6-di-2-furyl-3-(2-pyridyl)-1,2,4-triazine (dfpt) (Sigma–Aldrich, Milwaukee, WI), disodium 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine disulfonate (abb. Ferz, Ferrozine) (GFS Chemicals, Powell, OH), and tetra sodium 2,4-bis-(5,6-diphenyl-1,2,4-triazin-3-yl) pyridine tetrasulfonate (dppyts) (GFS Chemicals) were obtained from commercial sources. *cis*-$PtCl_2(DMSO)_2$ was prepared by the literature procedure (Price et al., 1972).

2.2. Physical measurements

^{1}H NMR spectra were recorded for DMSO-d_6 solutions on a Varian Unity 600 or a Bruker 500 spectrometer. Peak positions are relative to TMS.

2.3. Synthesis of dichloro(5,6-di-2-furyl-3-(2-pyridyl)-1,2,4-triazine)Pt(II) ((dfpt)$PtCl_2$)

A suspension of *cis*-$PtCl_2(DMSO)_2$ (0.051 g, 0.12 mmol) in methanol (75 ml) was treated with the dfpt ligand (0.035 g, 0.12 mmol), and the reaction mixture was stirred at 40 °C for 24 h. The yellowish-brown solid that precipitated was collected on a filter, washed with diethyl ether and chloroform, and dried in vacuo; yield, 0.044 g (65%). ^{1}H NMR (ppm): 9.60 (d, H6), 8.53 (d, H3), 8.29 (t, H4), 8.11 (t, H5), 7.27 (d, H3 (5-furyl)), 6.88 (t, H4 (5-furyl)), 8.29 (d, H5 (5-furyl)), 7.59 (d, H3 (6-furyl)), 6.93 (t, H4 (6-furyl)), 8.11 (d, H5 (6-furyl)). Anal. Calcd. for $C_{16}H_{10}Cl_2N_4O_2Pt{\cdot}H_2O$: C, 33.46; H, 2.11; N, 9.76. Found: C, 33.77; H, 2.00; N, 9.22.

2.4. Synthesis of disodium bis(3-(2-pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine)platinate(II) (Na_2[(ferene)$_2$Pt(II)])

A suspension of *cis*-$PtCl_2(DMSO)_2$ (0.3 g, 0.71 mmol) in methanol (50 ml) was treated with the disodium ferene salt (1.404 g, 2.84 mmol), and the reaction mixture was stirred at 40 °C for 24 h. The resulting red-brown solid was collected by filtration, washed with methanol and diethyl ether, and dried in vacuo; yield, 0.29 g, (36%). ^{1}H NMR (ppm): 10.7 (d, H6), 8.99 (d, H3), 8.81 (t, H4), 8.97 (t, H5), 7.04 (d, H3 (5-furyl)), 8.43 (d, H4 (5-furyl)), 6.82 (d, H3 (6-furyl)), 7.94 (d, H4 (6-furyl)). Anal. Calcd. for $C_{32}H_{16}N_8O_{16}PtNa_2S_4{\cdot}6H_2O$: C, 30.85; H, 2.27; N, 9.00. Found: C, 30.86; H, 2.16; N, 9.03.

2.5. Cell lines

The mouse NIH/3T3, canine MDCK, monkey CV-1, and human Hep2, HeLaS3, HEC-1-B, and CaCo-2 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The recombinant cell lines human MAGI, MAGI-CCR5, monkey sMAGI, mouse 3T3.T4, 3T3.T4.CXCR4, 3T3.T4.CCR5, and human T cell lines CEMx174 and HUT78 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS (NIAID, NIH). The recombinant human cell line JC53-BL, which is a derivative of HeLa cells that express high levels of CD4 and the HIV-1 coreceptors CCR5 and CXCR4 (Derdeyn et al., 2001) 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. The human 293T cell line was kindly provided by Dr. S.L. Lydy (Emory University, Atlanta, GA). NIH/3T3, MDCK, CV-1, Hep2, MAGI, MAGI-CCR5, sMAGI, 3T3.T4, 3T3.T4.CXCR4, 3T3.T4.CCR5, 293T HEC-1-B, and CaCo-2 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). HeLaS3, 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 containing 10% heat-inactivated fetal calf serum, 5 μg of concanavalin A (ConA) per milliliter, 5% interleukin 2 (IL-2), and antibiotics.

2.6. Viruses and plasmids

HIV-1 IIIB, HIV-1 89.6, influenza virus (A/PR/8/34 [H1N1]) and vaccinia virus (WR strain) were grown as previously described (Chen-Collins et al., 2003; Vzorov et al., 2002, 2003). Plasmids expressing HIV-1 Env proteins were described previously (Vzorov et al., 2002). The SIV/17E-Fr plasmid was obtained from Dr. J. Clements (Johns Hopkins University). To prepare SIVmac17E-Fr virus, 293T cells were transfected with pSIV/17E-Fr plasmids and at 72 h post-transfection medium containing virus was collected, precleared, aliquoted and frozen at −80 °C.

2.7. Screening of ptt compounds for activity against HIV-1

Ptt 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 virus stock. Samples were left in the dark at room temperature for 1 h. For MAGI, sMAGI or MAGI-CCR5 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). The samples were tested in duplicate. 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). For determining virus titers we used MAGI (Kimpton and Emerman, 1992), sMAGI (Chackerian et al., 1995) or MAGI-CCR5 (Chackerian et al., 1997) assays. 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 percent).

2.8. Cell fusion assays

For fusion assays, we used 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 as described previously (Vzorov et al., 2002).

As a positive control for fusion inhibition assays we used CuPcS(1), a compound which was able to inhibit HIV Env-induced cell fusion completely in 3T3CD4CXCR4 or 3T3CD4CCR5 cells as described previously (Vzorov et al., 2003). For all fusion assays, after 5 or 20 h of cocultivation, the level of cell fusion induced in 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 ptt compounds at concentrations of 500 μg/ml prepared in DMEM medium without FCS; 5 μl of compounds was mixed with 45 μl of influenza virus (A/PR/8/34[H1N1]) at a concentration of approximately 3×10^3 infectious particles, left in the dark at room temperature for 1 h, and used for plaque assays on MDCK cells as described (Vzorov et al., 2003).

2.10. Vaccinia virus plaque assay

We used ptt compounds at concentrations of 500 μg/ml prepared in DMEM medium without FCS; compounds were mixed with virus stocks (WR strain) at a final concentration of 50 μg/ml. After 1 h of incubation (in the dark) the virus–drug mixture was diluted 10-fold in DMEM (without FBS) and used for plaque assay as described (Chen-Collins et al., 2003).

2.11. Inhibition of HIV-1 infection in human PBL

To determine the ability of compounds to block infection of primary hPBL, serial dilutions of the compounds (0.5, 5,

and 50 μg/ml) were mixed with 5×10^2 cell-free infectious particles of HIV-1 IIIB or JR-FL. The mixture was incubated for 1 h in the dark, diluted 10-fold with medium, and added to 96-well plates with 3×10^4/well of cells. After 2 h incubation in medium with 15 μg/ml DEAE-dextran the unbound virus and residual compound was removed by three washes. After 3 days, the culture supernatant was harvested from each well and assayed for infectious particles by a highly sensitive β-galactosidase assay in JC53-BL cells (Derdeyn et al., 2001). Comparison of the number of infected cells in wells with samples of compound-treated virus to the number found in wells with samples with untreated virus was used to determine residual viral infectivity (expressed in percentage). The p24 content was also determined 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 with untreated virus was used to determine effects on viral production (expressed as a percentage).

To determine the effect of compounds on virus replication, the compounds with 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, the culture supernatant was harvested and tested for p24 content by ELISA Core Antigen assay (Coulter Corporation).

2.12. Reverse transcriptase assay

To determine the effect of ptt's on RT activity, serial dilutions of the compounds (5, 50, 500 μg/ml) were mixed with cell-free HIV-1 IIIB or JR-FL. The amount of virus used corresponded to 3.5 ng of RT (1×10^4 particles, which is 20-fold higher than that used for the MAGI-screening assay). The mixture was incubated for 1 h in the dark, diluted 10-fold with medium, and pelleted for 1 h at 13,000 × *g* at 4 °C in an Eppendorf centrifuge. The RT activity was determined by using the template/primer hybrid poly(A)x oligo $(dT)_{15}$. The detection and quantification of synthesized DNA as a parameter for RT activity followed a sandwich ELISA protocol (Roche, Mannheim).

2.13. RT assay with recombinant HIV-1 RT

To determine the inhibitory effect of ptt's on RT activity, serial dilutions of the compounds (2, 10, and 50 μg/ml) in lysis buffer were mixed with 5 ng of recombinant HIV-1 RT (Roche, Mannheim). The mixture was incubated for 30–60 min in the dark, diluted 10-fold with lysis buffer and the reverse transcriptase activity was determined by using the template/primer hybrid poly(A)x oligo $(dT)_{15}$. The detection and quantification of synthesized DNA as a parameter for RT activity followed a sandwich ELISA protocol (Roche, Mannheim).

2.14. Cytotoxicity test

Three approaches were used to determine cytotoxicity; two of them were described previously (Vzorov et al., 2003). For a trypan blue exclusion test (Strober, 1994) compounds at varying concentrations (1000, 200, 100, and 50 μg/ml) in growth medium were added to 96-well plates with MAGI cells. After 72 h of incubation, the percent of viable cells was determined by counting unstained cells as percent of total cells. The cytotoxicity of compounds was also measured by determining effects on cell proliferation using a quantitative ^{3}H-thymidine incorporation assay (Tobin et al., 1996). For this assay we used suspension HeLaS3 cells because this significantly reduces the assay time as compared with adherent cells, and permits the evaluation of large number of compounds at different dilutions. It also reduced variability between samples because we used a semi-automated multi-well harvester. The 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 counts from wells containing compounds were compared to control cells to determine the concentration that inhibited cell growth (DNA replication) by 50% (CC_{50}). In parallel with these two assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Pauwels et al., 1988) was performed using the human endometrial adenocarcinoma cell line HEC-1-B, and the human colon epithelial cell line CaCo-2. For MTT assay, compounds of varying concentrations (500, 100, 20, and 2 μg/ml) in growth medium were added to 96-well plates with HEC-1-B or CaCo-2 cells. Following 72-h incubation, cells were washed with Hank's balanced salt solution to remove colored compounds and 100 μl of growth medium with 10 μl of MTT (10 mg/ml) reagent was added to each well. After 4 to 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 spectrophotometer. The absorbance at 690 nm was automatically subtracted from the absorbance at 540 nm to eliminate the effects of non-specific absorption.

3. Results

As an approach to explore the utility of novel and unexplored small molecules to function as antiviral or virucidal agents, we have investigated platinum compounds containing N-donor aromatic ligands. From a screening of over 70 agents, including the ligands and the metal precursors, we have identified platinum(II) 2-pyridyl-1,2,4-triazine derivatives and formulations with these derivatives as having high activity in a range of assays.

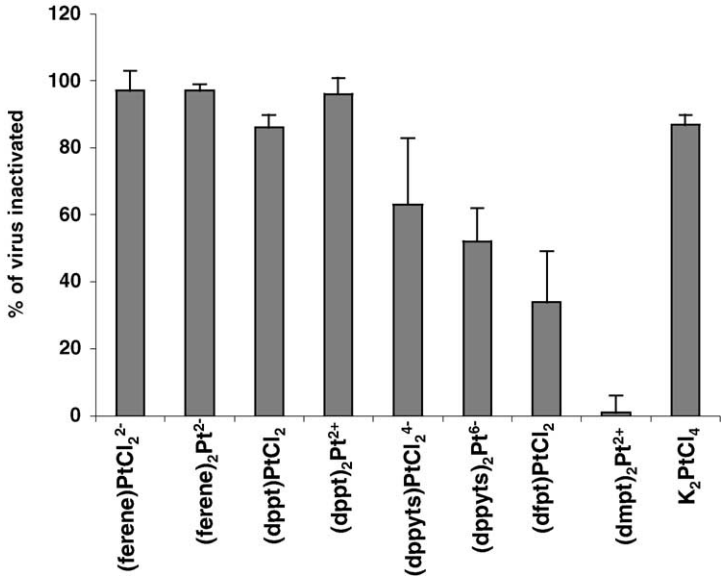

Fig. 2. Inhibitory activity of ptt's against HIV-1 infection. Compounds at a concentration of 50 μg/ml were incubated with HIV-1 IIIB in the dark for 1 h, diluted 10-fold and used to inoculate MAGI cells. Inhibition of HIV infection was measured by removal of the media after 3 days, fixation and staining of cells with X-gal (Vzorov et al., 2002). The percent 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.

3.1. Inhibition of viral infection

To evaluate the ability of ptt's to prevent HIV infection, we pretreated virus with test compounds at a final concentration of 5 μg/ml and assayed residual infectivity using an epithelial HeLa-CD4 cell line with an integrated LTR-β-galactosidase gene. The most active ptt compounds were found to result in about 96–99% inhibition of infection (Fig. 2).

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. Three active compounds (ferene)$PtCl_2^{2-}$, $(ferene)_2Pt^{2-}$ and $(dppt)PtCl_2$were compared. For $(ferene)_2Pt^{2-}$, about 90% inhibition of infectivity was observed at 2 min and about 97% of virus was inactivated after 15 min, with no further change over the 60 min time period studied (Fig. 3). Another compound, $(dppt)PtCl_2$ showed maximum inactivation only after 60 min of incubation; the other compounds tested showed maximum inhibition after 15–45 min. The results demonstrate that the interaction of (ferene)$PtCl_2^{2-}$ and $(ferene)_2Pt^{2-}$ with HIV-1 IIIB is rapid, but the kinetics of inactivation vary among the compounds tested.

3.3. Effective concentration

To determine the effective concentration of selected active compounds, virus samples were pretreated with ptt's at a series of concentrations (Fig. 4). The most effective concentration was generally found to be 50 μg/ml, the highest concentration tested. However, two of three compounds studied, (ferene)$PtCl_2^{2-}$ and $(ferene)_2Pt^{2-}$, also exhibited activity at concentrations of less than 20 μg/ml. Of the most active compounds, $(ferene)_2Pt^{2-}$ had an EC_{50} of 7 μg/ml (Table 1).

3.4. Activity against other viruses

To investigate whether these compounds would non-selectively inactivate other enveloped viruses, we extended our studies to the primary isolate HIV-1 89.6, SIVmac17E-Fr, influenza virus (A/PR/8/34[H1N1]) and vaccinia virus

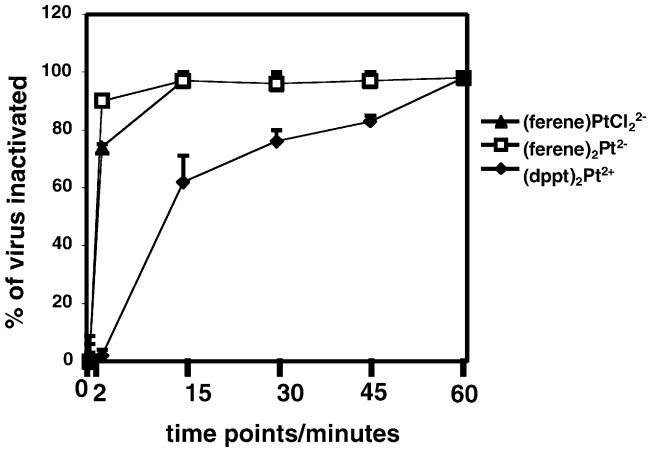

Fig. 3. Kinetics of inactivation of HIV-1 by ptt compounds. The compounds were mixed with virus at a final concentration of 50 μg/ml and incubated for various time intervals (2, 15, 30, 45, 60 min), diluted 1:10 with complete medium, and residual infectivity titers determined as in Fig. 2.

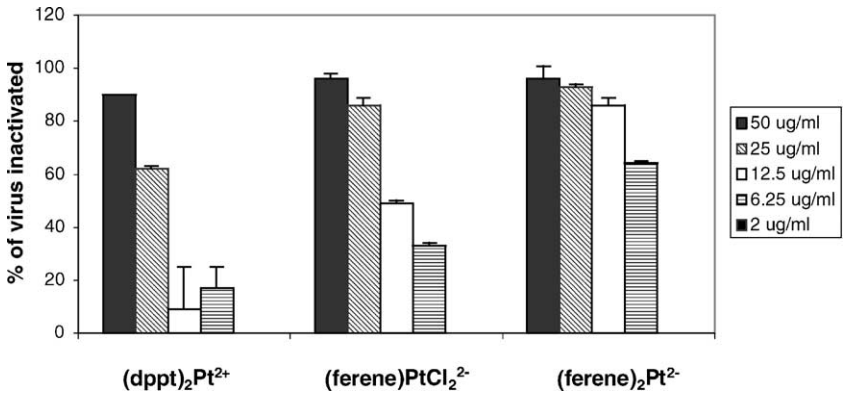

Fig. 4. Activity of selected ptt's as a function of concentration. Virus samples were mixed with ptt's at varying concentrations of 50, 25, 12.5, 6.25, and 2 μg/ml, incubated in the dark for 1 h, and residual infectivity titers determined as in Fig. 2. The 2 μg/ml results are indistinguishable from the baseline.

(WR). We selected the two most active compounds against HIV-1 IIIB: (ferene)$PtCl_2^{2-}$ and (ferene)$_2Pt^{2-}$, which effectively inactivated HIV-1 89.6 to the extents of 82% and 96%, respectively (Fig. 5A). (Ferene)$_2Pt^{2-}$ had intermediate activity with SIV and vaccinia virus, and did not display any activity against influenza virus. In contrast, (ferene)$PtCl_2^{2-}$ demonstrated low activity against all these viruses, blocking infection by about 40% or less. These results indicate that (ferene)$PtCl_2^{2-}$ and (ferene)$_2Pt^{2-}$ have selective activity against HIV as compared with the other viruses tested, suggesting a specific interaction with a viral component.

To investigate whether the compounds with high activity against HIV-1 IIIB and 89.6 would also inactivate R5 viruses, we extended our studies to the primary isolate HIV-1 JR-FL. (dppt)$PtCl_2$ effectively inactivated HIV-1 JR-FL (90% inactivation). (dppyts)$PtCl_2^{4-}$ and (dppt)$_2Pt^{2+}$ also showed activity to the extents of 67% and 58%, respectively. (Ferene)$PtCl_2^{2-}$, (ferene)$_2Pt^{2-}$, and (dmpt)$_2Pt^{2+}$ were not active. These results indicate that some ptt's also have activity against R5 viruses (Fig. 5B).

3.5. Toxicity

In order to confirm that the reduction of β-gal expressing cells observed after treatment with compounds was not a result of toxicity, we assessed the toxicity of the more active ptt compounds. Compounds at various concentrations (50, 100, 200, and 1000 μg/ml) in growth medium were added to MAGI cells. The minimum concentration (50 μg/ml) was 10-fold higher than that used for virus assay. After 72 h, a trypan blue assay was used to compare cell viability in cells treated with compounds to that of untreated cells. Both (ferene)$PtCl_2^{2-}$ and (ferene)$_2Pt^{2-}$ showed less toxicity than the other compounds tested, with CC_{50} = 80 μg/ml and 60 μg/ml, respectively.

To extend these results we used a colorimetric MTT assay, which provides an indication of the mitochondrial integrity and activity as a measure of cell viability and/or number. For this assay, we compared effects on two human cell lines: endometrial HEC-1-B and colon epithelial CaCo-2 cells. The CC_{50} values of compounds were found to be similar for both cell lines: (ferene)$PtCl_2^{2-}$ = 300 μg/ml; (ferene)$_2Pt^{2-}$ = >500 μg/ml; (dppt)$PtCl_2$ = 100 μg/ml; K_2PtCl_4 = 200 μg/ml; and (dppt)$_2Pt^{2+}$ = 11 μg/ml.

To determine the possible effects of the compounds on cell division, we also compared the cytotoxic effects of active compounds at different times by using a quantitative ^{3}H-thymidine incorporation cell growth assay. A suspension cell line, HeLaS3, was used; these cells are closely related to MAGI cells, which are derived from HeLa cells. We observed that after a 3-day incubation the most toxic compound was (dppt)$_2Pt^{2+}$ (CC_{50} > 5 μg/ml). However, (ferene)$_2Pt^{2-}$ showed little toxicity (CC_{50} > 500 μg/ml). From these assays, the therapeutic index for (ferene)$_2Pt^{2-}$ was calculated to be >71 (Table 1).

Table 1
Effective concentration and therapeutic indices of selected compounds

Compound	CC_{50} (μg/ml)[a] (cell viability)	EC_{50} (μg/ml)[b]	Therapeutic index[c]
(ferene)$PtCl_2^{2-}$	300	12	25
(ferene)$_2Pt^{2-}$	>500	7	>71
(dppt)$PtCl_2$	100	21	5

[a] Results obtained by MTT assay using the human endometrial adenocarcinoma cell line HEC-1-B, and the human colon epithelial cell line CaCo-2.
[b] Results obtained by counting infected cells using the MAGI assay.
[c] The therapeutic index value was defined as the CC_{50} (cell viability)/EC_{50} ratio.

3.6. Additional antiviral properties of ptt compounds

We extended our results by testing the anti-HIV activity of the leading compounds using primary human T cells PBL (Table 2). We tested three active compounds, (ferene)$PtCl_2^{2-}$, (ferene)$_2Pt^{2-}$ and (dppt)$PtCl_2$ for inactivation of HIV-1 IIIB or JR-FL before addition to cells or for inhibition of replication by addition of compounds to cells post-infection. The p24 content in the supernatant by ELISA Core Antigen assay was determined at 3-day post-infection. We found that (ferene)$PtCl_2^{2-}$ at 50 μg/ml inactivated about 83% of HIV-1 IIIB, exhibited no activity against JR-FL

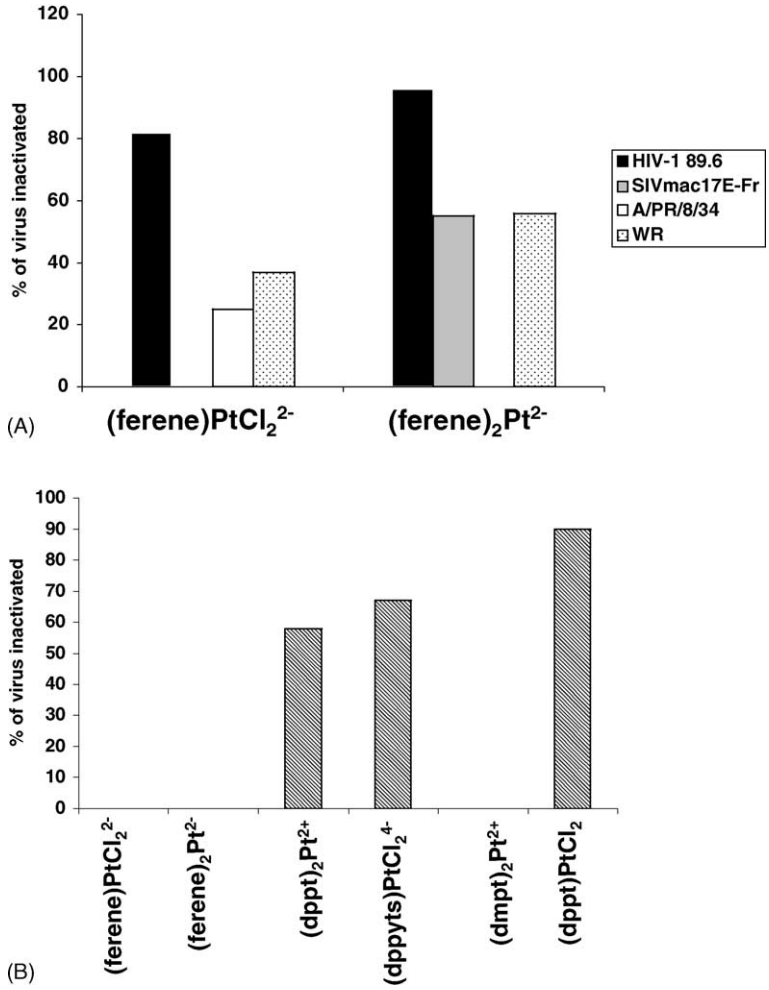

Fig. 5. Activity of ptt's against other viruses. Compounds at a concentration of 50 μg/ml were incubated with HIV-1 89.6, SIVmac17E-Fr, influenza virus (A/PR/8/34[H1N1]), vaccinia virus (WR) (A) or HIV-1 JR-FL (B) in the dark for 1 h, diluted 10-fold and used to inoculate appropriate cells as described in Section 2. Residual infectivity was measured after 2 or 3 days by assays for the virus used, as described in Section 2.

when preincubated with the virus before addition to cells, and blocked 84% of HIV-1 IIIB or 70% of JR-FL replication when present during infection. $(Ferene)_2Pt^{2-}$ showed similar results, but was even more effective in blocking JR-FL replication. We found that $(dppt)PtCl_2$ inactivated about 78% of HIV-1 IIIB, and 37% of JR-FL when preincubated with virus before addition to cells. We also tested two highly negatively charged platinum compounds: $(dppyts)PtCl_2^{4-}$ and $[(PPDTS)PtCl_2]^{3-}$. These compounds were less active against HIV-1 JR-FL but showed antiviral activity against HIV-1 IIIB. The culture supernatants were also assayed for infectivity by a β-galactosidase assay in JC53-BL cells, and these results were similar to those obtained by p24-ELISA assay. The results indicate that selected ptt compounds

Table 2
Inhibition of HIV-1 infection in primary human PBL

Compound	Virucidal activity (preincubation of virus with compounds before addition to cells)				Antiviral activity (incubation with test compounds during infection)			
	Percentage of inhibition HIV-1 IIIB		Percentage of inhibition HIV-1 JR-FL		Percentage of inhibition HIV-1 IIIB		Percentage of inhibition HIV-1 JR-FL	
	50 μg/ml	5 μg/ml	50 μg/ml	5 μg/ml	50 μg/ml	25 μg/ml	50 μg/ml	25 μg/ml
$(ferene)PtCl_2^{2-}$	83	56	4	0	84	82	70	2
$(ferene)_2Pt^{2-}$	85	62	16	5	90	89	93	73
$[(PPDTS)PtCl_2]^{3-}$	20	0	16	3	62	18	19	0
$(dppyts)PtCl_2^{4-}$	56	0	4	0	73	20	46	19
$(dppt)PtCl_2$	78	16	37	0	66	47	27	15

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

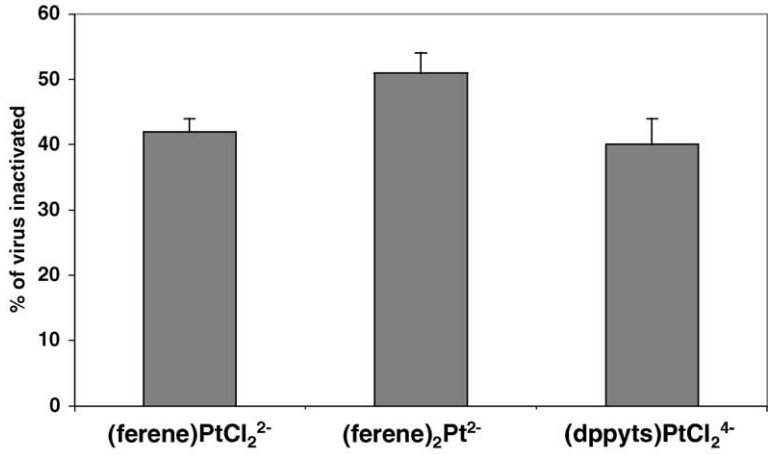

Fig. 6. Prevention of cell-associated virus transmission. Various amounts of HUT78 cells (3×10^2, 3×10^3, 3×10^4) infected with HIV-1 IIIB were exposed to test compounds at a concentration of 50 μg/ml in the dark for 10 min at 37 °C, diluted 10-fold and used to inoculate MAGI cells; residual infectivity titers were determined as in Fig. 2. The data shown were obtained with 3×10^3 HUT78 cells infected with HIV-1 IIIB.

have virucidal and antiviral activity against HIV-1 IIIB and antiviral activity against HIV-1 JR-FL in primary hPBL.

To determine if antiviral activity is retained when cells are pretreated with compounds, MAGI cells were exposed to various concentrations of the test compounds (0.05, 0.5, 5, and 50 μg/ml) 2 h prior to infection and the compounds kept present during infection. Pretreatment of cells with compounds showed some reduction in antiviral effects of ptt's. In particular, $(ferene)_2Pt^{2-}$ had an EC_{50} of 16 μg/ml (two-fold lower), and $(ferene)PtCl_2^{2-}$ had an EC_{50} of >50 μg/ml (>four-fold lower), respectively.

To investigate the activity of ptt's against cell-associated virus we used HUT78 cells infected with HIV-1 IIIB virus. $(Ferene)_2Pt^{2-}$ and $(ferene)PtCl_2^{2-}$ were found to block 30–50% of HIV infection (Fig. 6); these results indicate that these ptt compounds are more effective in preventing infection by cell-free virus.

3.7. HIV Env-induced cell fusion in the presence of ptt compounds

One possible mechanism by which ptt's might block viral infection is inhibition of the biological activity of the viral Env proteins, which is required for viral entry. We investigated effects of the compounds on cell fusion activity by using plasmid-based or vaccinia expression systems for the Env proteins as described previously (Vzorov et al., 2003). We tested seven compounds, $(ferene)PtCl_2^{2-}$, $(dmpt)PtCl_2$, $(dmpt)_2Pt^{2+}$, $(ferene)_2Pt^{2-}$, $(dppt)PtCl_2$, $(dppyts)PtCl_2^{4-}$, and K_2PtCl_4, none of which was found to block fusion activity of HIV-1 IIIB or HIV-1 89.6 Env proteins with cells bearing CD4X4 or CD4R5 receptors (not shown). Thus, neither the ptt compounds nor K_2PtCl_4 affect the fusion activity of the Env protein.

3.8. Inhibition of HIV-1 RT by ptt compounds

To determine if ptt's have an inhibitory effect on RT activity, various concentrations of the test compounds (5, 50, and 500 μg/ml) were mixed with cell-free HIV-1 IIIB, incubated for 1 h in the dark, diluted 10-fold with medium, and pelleted, followed by an assay of RT activity. We observed that the ptt compounds tested were able to block recombinanat RT activity at a concentration of 500 μg/ml (not shown). The compounds $(dppt)_2Pt^{2+}$, $(dppt)PtCl_2$, and $(dmpt)_2Pt^{2+}$ completely blocked RT activity; $(ferene)PtCl_2^{2-}$ and $(ferene)_2Pt^{2-}$ blocked 99% and 95% of RT activity, respectively. Low levels of inhibitory activity were exhibited by $(dppyts)PtCl_2^{4-}$.

To confirm our results we also determined the effects on activity of recombinant RT protein (Table 3). We observed that the ptt compounds tested were able to block recombinant RT activity at a concentration of 50 μg/ml. $(Ferene)PtCl_2^{2-}$ blocked 89% of RT activity, compared to 60% for $(dppyts)PtCl_2^{4-}$ and 33% for $(ferene)_2Pt^{2-}$. The compounds $(dppt)_2Pt^{2+}$, $(dmpt)_2Pt^{2+}$, and $(dppt)PtCl_2$ were not active (Fig. 7). These results show that some ptt's are inhibitors of HIV RT activity, and this effect is concentration dependent. Only one compound had substantial activity against recombinant RT. However, the active compounds including this RT-active compound may have a multi-target mechanisms of action.

4. Discussion

Various synthetic compounds have been evaluated for antiviral activity (inhibition of virus replication), but only a relatively small number have been evaluated for virucidal activity (Garg et al., 1999). The relevant literature on small sulfonated compounds describes antiviral activity or the in-

Table 3
Effect of ptt compounds on RT activity % inhibition of RT % HIV-1 inactivated

Chemical name	Percentage of inhibition of RT		Percentage of inactivated HIV-1 IIIB/X4 (50 μg/ml)
	RT assay with HIV-1 IIIB virus particles (500 μg/ml)	RT assay with recombinant RT (50 μg/ml)	
(ferene)$PtCl_2^{2-}$	99	89	85
$(ferene)_2Pt^{2-}$	95	33	91
(dppyts)$PtCl_2^{4-}$	43	60	63
(dppt)$PtCl_2$	100	0	86
$(dppt)_2Pt^{2+}$	100	0	70

hibition of viral enzymes, some of which, such as protease, occur later in the viral replication cycle (Lubkowski et al., 1998; Pommier et al., 2000; Pommier and Neamati, 1999). Sulfonates have generally proven to be non-toxic to cells (Mohan et al., 1991a,b; Rosenthal and Ben-Hur, 1989).

The potency of organic medicinal aromatic sulfonic acid compounds as viral inhibitors has a long history (Åkerfeldt et al., 1971; Mohan et al., 1991a,b, 1990), some of the first indications that such compounds can act to prevent viral entry into cells coming as early as 1971. Active research on organic sulfonates continues because such compounds interfere with many processes in the viral life cycle besides preventing cell entry. Examples exist in which a single compound was shown to have multiple modes of action (Brinkworth and Fairlie, 1992; Mohan et al., 1991a,b; Pommier et al., 2000).

The present study shows that platinum compounds containing N-donor aromatic ligands have a high potential as anti-HIV microbicides. The results show the importance of the ligand and especially its peripheral charge. As mentioned above for the Cu(I) compounds, the complex with the neutral ligand was inactive and relatively toxic, whereas the negative complex with the negative ligand, bcds, was active and relatively non-toxic. Likewise, the negative ptt complexes with either one or two negative aromatic ligands were active and relatively non-toxic. This trend for ptt compounds is best described with two such active species shown in Fig. 1 illustrating agents containing the ferene ligand, which is rendered dinegative by possessing two aromatic sulfonated furan rings and an analogous agent containing the "desulfonated" derivative of ferene. The latter complex, which has one neu-

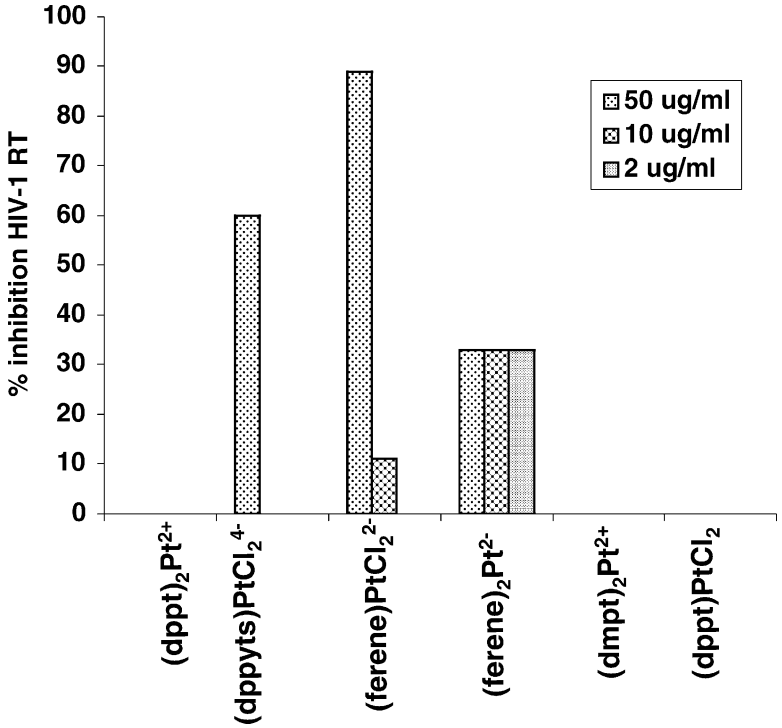

Fig. 7. Inhibition of HIV-1 RT activity by ptt's. Compounds at concentrations of 2, 10, and 50 μg/ml with recombinant HIV-1 RT was incubated in the dark for 30–60 min, diluted 10-fold with lysis buffer, and used for a Roche Mannheim colorimetric reverse transcriptase assay. Results are given as percentage of RT detected compared to untreated HIV-1 sample (100%).

tral ligand, also shown in Fig. 1, had low activity (Fig. 2) and was relatively toxic to cells (data not shown). In contrast, the negatively charged agents with ferene had high activity (Fig. 2) and low toxicity (Table 1).

The ptt compounds were able to inactivate cell-free virus rapidly. Some compounds were effective against both X4 and R5 viruses and showed low toxicity for human epithelial cells. Further studies to develop more active compounds of this class are thus warranted.

Several categories of agents have been studied as candidate microbicides for HIV prevention; these include surfactants that disrupt the viral membrane integrity, such as nonoxynol-9. Unfortunately, this compound has failed in human clinical trials, due to drug-induced vaginal lesions and inflammation that might actually promote virus transmission (Fichorova et al., 2001; Richardson et al., 2001; Van de Wijgert and Coggins, 2002). Other candidate microbicides include negatively charged large polymers, such as the polysaccharide sulfates, chondroitin sulfate and carrageenan (D'Souza et al., 2000; De Clercq, 2002), however, these may not be as active against R5 viruses, which are involved in sexual transmission (Moulard et al., 2000; Shattock and Doms, 2002). Reverse transcriptase inhibitors may be also useful as microbicides. One of these compounds, PMPA (Tenofovir) requires cellular activation to the diphosphate for antiviral activity, and is therefore unlikely to act directly to inactivate HIV virions (Barkow et al., 1997). Non-nucleoside RT inhibitors (NNRTIs) have also been suggested as potential microbicides (Pauwels and De Clercq, 1996), but it has been shown that not all NNRTIs have anti-HIV microbicidal activity (Barkow et al., 1997; Parniak and Sluis-Cremer, 2000). The ptt compounds offer advantages for possible use as topical microbicides in that they are highly stable and nearly colorless at effective concentrations. Their structural features are also quite amenable to modification in an effort to develop more active compounds. In future studies, it will also be of interest to test this class of compounds in combination with other potential microbicides, which differ in their mechanisms of action.

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