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Mechanism of Anti-Human Immunodeficiency Virus Action of Polyoxometalates, a Class of Broad-Spectrum Antiviral Agents

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SUMMARY

Various polyoxometalates proved inhibitory to the replication of a number of enveloped DNA and RNA viruses, i.e., herpesviruses (herpes simplex and cytomegalo), togaviruses (Sindbis), paramyxoviruses (respiratory syncytial), rhabdoviruses (vesicular stomatitis), arenaviruses (Junin and Tacaribe), and retroviruses [human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), simian immunodeficiency virus, and murine sarcoma virus]. The most potent compounds, i.e., JM1590 [$K_{13}[Ce(SiW_{11}O_{39})_2]\cdot 26H_2O$] and JM2766 [$K_6[BGa(H_2O)W_{11}O_{39}]\cdot 15H_2O$], inhibited HIV-1 and simian immunodeficiency virus at concentrations as low as 0.008–0.8 μM. The polyoxometalates also inhibited giant cell formation in co-cultures of HIV-infected HUT-78 cells and uninfected MOLT-4 cells. Studies designed to unravel the mechanism of action of these compounds revealed that they inhibit the reverse transcriptase activity associated with HIV. The polyoxometalates also proved inhibitory to the binding of HIV-1 virions to the cells. From "time of addition" experiments, whereby the polyoxometalates were added at different times after virus infection, their mechanism of anti-HIV action could be attributed to inhibition of virus-cell binding. There was a good correlation ($r = 0.84$) between the inhibitory effects of the compounds on HIV-1-induced cytopathicity and their inhibitory effects on syncytium formation and a close correlation ($r = 0.902$) between their inhibitory effects on syncytium formation and their interaction with gp120, whereas there was no correlation between their anti-HIV-1 activity and their inhibitory effects on HIV-1 reverse transcriptase. In flow cytometric studies, the compounds did not interfere with the binding of OKT4A/Leu-3a monoclonal antibody to the CD4 receptor of uninfected cells, but they inhibited binding of anti-gp120 monoclonal antibody to HIV-1-infected cells. Thus, the binding of the polyoxometalates to the viral envelope glycoprotein gp120 is responsible for their anti-HIV activity.

Sulfated polysaccharides, such as heparin, dextran sulfate, mannan sulfate, and pentosan polysulfate, and sulfated polymers, such as polyvinylalcohol sulfate, are well known inhibitors of a wide variety of DNA and RNA viruses, including HIV-1. Although these compounds are inhibitory to both HIV-1-associated RT activity and virus binding to the cells, their mechanism of action has been attributed to an inhibition of virus adsorption to the cell membrane (1–11).

The polyoxometalates represent another class of polyanionic compounds, which have been credited with antiviral activity *in vitro* and *in vivo* against a number of RNA and DNA viruses (12–16). Hill *et al.* (17, 18) reported on the anti-HIV-1 activity of a series of polyoxometalates and demonstrated that these compounds have anti-fusion/binding properties in cell culture. Also, during the course of our work, the heteropolyoxotungstate $K_7[PTi_2W_{10}O_{40}]\cdot 6H_2O$ (PM-19) was reported to inhibit HIV-1 replication and it was suggested, but not shown, that this compound might interfere with an early step of virus replication such as virus adsorption and/or virus penetration (19, 20).

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ABBREVIATIONS: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; HSV, herpes simplex virus; VSV, vesicular stomatitis virus; MSV, Moloney murine sarcoma virus; CMV, cytomegalovirus; RSV, respiratory syncytial virus; RT, reverse transcriptase; PBS, phosphate-buffered saline; RaM-IgG-F(ab′)$_2$-FITC, fluorescein isothiocyanate-conjugated F(ab′)$_2$ fragments of rabbit anti-mouse immunoglobulin antibody; $CCID_{50}$, 50% cell culture-infective dose; FCS, fetal calf serum; IC_{50}, 50% inhibitory concentration; AZT, 3′-azido-2′,3′-dideoxythymidine; DDI, 2′,3′-dideoxyinosine; TK$^-$, thymidine kinase-deficient; TIBO, tetrahydroimidazo-[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and -thione.

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None of these previous studies addressed the issue of whether polyoxometalates inhibit virus adsorption through interaction with the CD4 of the uninfected cells or through interaction with gp120 of the infected cells.

We now have evaluated a large variety of polyoxometalates for their activity against a wide variety of DNA and RNA viruses. Some of the polyoxometalates that were the subject of the present study were found to inhibit HIV replication at a concentration that is 10^4 to 10^5 times lower than their cytotoxic concentration. In our studies on the mechanism of action of these compounds, we found two targets for their anti-HIV action, (i) the virus-associated RT activity and (ii) virion binding to the cells. We then resolved the question of which of these two actions may actually account for the anti-HIV activity of the polyoxometalates and by which mechanism they achieve this anti-HIV activity.

Experimental Procedures

Compounds. The formulas of the polyoxometalates studied are presented below. Dextran sulfate (M_r 5000) was purchased from Sigma Chemical Co. (St. Louis, MO). AZT was synthesized by P. Herdewijn (Rega Institute, Leuven, Belgium), following the procedure described by Horwitz *et al.* (21); DDI was obtained from Pharmacia PL-Biochemicals (Piscataway, NJ). The TIBO derivative R82913 [(+)-(*S*)-4,5,6,7-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)-imidazol[4,5,1-jk][1,4]-benzodiazepin-2(1*H*)thione] was obtained from the Janssen Research Foundation (Beerse, Belgium). Ro31–8959 was obtained from Dr. N. Roberts (Roche, UK). The polyoxometalates (JM compounds) were stable in growth medium (RPMI 1640) during the 6 months of our experiments (based on activity testing in cell culture).

Polyoxometalates. The origin of the polyoxometalates is as follows:JM1583, $K_5[BW_{12}O_{40}]$ (22); JM1589, $Na_7[PrW_{10}O_{35}]\cdot 18H_2O$ (23); JM1590, $K_{13}[Ce(SiW_{11}O_{39})_2]\cdot 26H_2O$ (23); JM1591, $K_{12}H_2[P_2W_{12}O_{48}]\cdot 24H_2O$ (24); JM1596, $K_{10}[P_2Zn_4(H_2O)_2W_{18}O_{68}]\cdot 20H_2O$ (25); JM1809, $K_8H[P_2V_3W_{15}O_{62}]\cdot 34H_2O$ (26); JM2766, $K_6[BGa(H_2O)W_{11}O_{39}]\cdot 15H_2O$ (27); JM2800, $[Na/K]_6[Nb_4W_2O_{19}]\cdot 12H_2O$ (28); JM2815, $K_5[Si(TiCp)W_{11}O_{39}]\cdot 12H_2O$ (where Cp = cyclopentadienyl) (29); JM2820, $[Me_3NH]_8[Si_2Nb_6W_{18}O_{77}]$ (30); and HPA-23, $[NH_4]_{17}Na[NaSb_9W_{21}O_{86}]\cdot 14H_2O$ (31). These JM compounds belong to a variety of structural types, 1) The "Keggin" class, exemplified by JM1583, JM2766, JM2815, and JM2820, 2) The "Dawson" class, JM1591 and JM1809, 3) The "Keggin Sandwich" class, JM1589, JM1590, and JM1596, and 4) a niobate, JM2800.

Viruses. The following virus stocks were prepared as described previously: HSV-1 (KOS strain), HSV-2 (G strain) (5), and TK$^-$ HSV-1 (strains B2006 and VMW 1837) (5); vaccinia virus, VSV, poliovirus-1, and Sindbis virus (32); MSV (33); HIV-1 [HTLV-III$_B$ (LAI strain)] (34); HIV-2 (LAV-2$_{ROD}$ strain) (35); SIV (SIV$_{MAC251}$ strain) (C. Bruck, Smith Kline-RIT, Rixensart, Belgium); and parainfluenza virus type 3, reovirus type 1, CMV (AD169 strain, Davis strain), and Semliki Forest virus (American Type Culture Collection, Rockville, MD). Measles virus (Sugiyama strain) and RSV (Long strain) were provided by Dr. R. Matsumoto, (Tohoku College of Pharmacy, Miyagi, Japan) and Dr. Numazaki (National Senobi Hospital, Sendai, Japan), respectively. Junin virus and Tacaribe virus were provided by Dr. C. E. Coto (Faculty of Sciences, Buenos Aires, Argentina). HIV-1 and HIV-2 were prepared from the supernatant of HUT-78/HTLV-III$_B$ and HUT-78/LAV-2$_{ROD}$ cells, i.e., HUT-78 cells persistently infected with HTLV-III$_B$ or LAV-2$_{ROD}$, respectively.

Antiviral activity assays. The activity of the compounds against HIV-1, HIV-2, and SIV was monitored by the inhibition of virus-induced cytopathicity in MT-4 cells. Briefly, MT-4 cells were suspended at 3×10^5 cells/ml and infected with HIV-1, HIV-2, or SIV at 100 CCID$_{50}$/ml. Immediately after infection, 100-μl portions of the cell suspension were brought into different wells of a flat-bottomed microtiter plate containing varying concentrations of the test compounds. For the anti-HIV assays, the number of viable cells was determined after 5 days of incubation at 37° by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide method, as described previously (36). For the anti-SIV assays, trypan blue staining was used, as described previously (37).

For the anti-MSV assays, murine C3H/3T3 embryo fibroblast cells were infected with 80 focus-forming units of MSV and transformation of the cell cultures was examined microscopically at 5 days after infection, as described previously (38).

For the anti-CMV assays, human embryonic lung fibroblasts were grown in minimum essential medium supplemented with 10% inactivated FCS, 1% L-glutamine, and 0.3% sodium bicarbonate. The cells were infected with 100 plaque-forming units of CMV. Compounds and virus were added at the same time and the cells were further incubated at 37° in minimum essential medium supplemented with 2% FCS. Virus-induced cytopathic effect was monitored at 7 days after infection, as described previously (39).

The other antiviral assays were based on the inhibition of virus-induced cytopathicity in HeLa cells (for RSV, VSV, and poliovirus type 1), Vero cells (Sindbis virus, Semliki Forest virus, measles virus, parainfluenza virus type 3, Junin virus, Tacaribe virus, and reovirus type 1), or E$_6$SM cells (human embryonic skin-muscle fibroblast) (for HSV-1, HSV-2, and vaccinia virus), following previously established procedures (32, 40–42). Virus-induced cytopathicity was recorded at 1–2 days after infection for VSV, Semliki Forest virus, and poliovirus; at 2–3 days for HSV-1, HSV-2, TK$^-$ HSV-1, vaccinia virus, and Sindbis virus; and at 5 days for RSV, measles virus, parainfluenza virus, Junin virus, Tacaribe virus, and reovirus.

Giant cell formation assay. MOLT-4 cells (5×10^5) were co-cultured with an equal number of HUT-78/HTLV-III$_B$ cells or HUT-78/LAV-2$_{ROD}$ cells in microtiter plate wells containing various concentrations of the test compounds. After 24 hr of co-cultivation, the number of giant cells was recorded microscopically (43).

Time of addition experiment. As described previously (44), MT-4 cells (in microtiter plates) were infected at a multiplicity of infection of >1 with HIV-1(HLTV-III$_B$). After 60 min of incubation at 37°, unadsorbed virus was removed by three subsequent washing steps. At different times (1–24 hr) after infection the compounds were added at a standardized concentration, namely 100 times their IC$_{50}$ to inhibit by 50% the cytopathicity of HIV-1 in MT-4 cells infected with HIV-1 at 100 CCID$_{50}$/300,000 cells (see above). HIV-1 core protein (p24) production was evaluated at 29 hr after infection by a sandwich enzyme-linked immunosorbent assay (DuPont de Nemours, Brussels, Belgium).

Virus adsorption assay. The inhibitory effects of the test compounds on virus adsorption were measured by an indirect immunofluorescence-laser flow cytofluorographic method that had been specifically designed for this purpose (8). Briefly, MT-4 cells were exposed to HIV-1 in the presence or absence of the test compounds. The compounds were added 10–20 sec before virus addition. The cells were incubated for 30 min at 37° and washed twice in PBS to remove unbound virus. Then, high-titered polyclonal antibody derived from a patient with acquired immunodeficiency syndrome-related complex (diluted 1/500 in PBS) was added. After another 30-min incubation at room temperature, the cells were washed twice with PBS. The cells were then incubated with FITC-conjugated F(ab')$_2$ fragments of rabbit anti-human immunoglobulin antibody (Prosan, Ghent, Belgium) (diluted 1/30 in PBS) for 30 min at room temperature, washed once in PBS, resuspended in 0.5 ml of 0.5% paraformaldehyde in PBS, and analysed by laser flow cytofluorography.

CD4 immunofluorescence assay. CD4 antigen expression was determined by FACSTAR (Becton-Dickinson) analysis, as described previously (45). Briefly, MT-4 cells were incubated for various times at room temperature in PBS in the absence or presence of serum, with or without test compound. The cells were then stained with optimal concentrations of the mAb OKT4A-FITC (Ortho Diagnostics) or anti-

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Leu-3a-phycoerythrin and Simultest immune monitoring kit control (FITC-labeled IgG1 and phycoerythrin-labeled IgG_2) (Becton-Dickinson) for 20 min at 4°, washed once in PBS, and fixed in 0.5 ml of 0.5% paraformaldehyde in PBS.

Glycoprotein gp120 immunofluorescence assay. Uninfected HUT-78 and HIV-1-infected HUT-78 (HUT-78/HIV-1) cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin G, and 20 μg/ml gentamicin. HUT-78/HIV-1 cells (200,000 cells) in 100 μl of RPMI supplemented with 10% FCS were washed twice in RPMI with 10% FCS, incubated at 20° for 15–20 min with the compounds at various concentrations in RPMI (10% FCS), washed twice with RPMI to remove residual compound, stained with anti-gp120 mAb (46) (9284; DuPont de Nemours, Brussels, Belgium) for 45 min at 37°, washed twice in PBS, incubated with RaM-IgG-F(ab′)$_2$-FITC (Prosan, Ghent, Belgium) for 40–50 min at 37°, washed twice in PBS, resuspended in 0.5 ml of 0.5% paraformaldehyde in PBS, and analyzed by flow cytometry, as described previously (47). The threshold of positivity for green fluorescence intensity was arbitrarily established; it was based on uninfected HUT-78 cells incubated with anti-gp120 mAb and RaM-IgG-F(ab′)$_2$-FITC or on HIV-1-infected HUT-78 cells incubated with only RaM-IgG-F(ab′)$_2$-FITC.

RT assay. Recombinant HIV-1 RT p66 was a kind gift from P. J. Barr (Chiron Corporation). The enzyme was expressed in yeast, purified, and used at a final concentration of 72 ng/ml (1.1 nM). The final total protein concentration in the RT reaction mixture when recombinant RT was used was 72 ng/ml. Virion particle-derived RT was obtained from the culture fluid of T cells persistently infected with HIV-1 (HUT-78/HTLV-III_B), as described previously (48). The final total protein concentration in the RT reaction mixture when virion-derived RT was used was 13 μg/ml, as determined by the Bradford method (Bio-Rad). The RT reaction mixture contained 50 mM Tris·HCl, pH 8.4, 10 mM $MgCl_2$, 100 mM KCl, 2.2 mM dithiothreitol, and 0.05% Triton X-100. The template/primer [poly(C)·oligo(dG)$_{12-18}$] (Pharmacia) and the substrate [^{3}H]dGTP (Amersham) were used at a final concentration of 65 μg/ml and 2.5 μM, respectively. The specific activity of [^{3}H]dGTP was 11 Ci/mmol. After addition of the enzyme and varying concentrations of the inhibitor, the reaction mixture was incubated for 1 hr at 37°. The incorporation rate was determined by a standard trichloroacetic acid precipitation procedure using Whatman GF/C glass fiber filters and liquid scintillation counting.

Results

Antiretroviral activity. The polyoxometalate derivatives were evaluated for their inhibitory effects on HIV-1-, HIV-2-, or SIV-induced cytopathicity in MT-4 cells and MSV-induced transformation of murine C3H/3T3 cells (Table 1). All the compounds, except for JM2800, showed an IC_{50} in the range of 0.1–1.3 μM against HIV-1(III_B); JM1590 ($K_{13}[Ce(SiW_{11}O_{39})_2]\cdot 26H_2O$) was the most active compound (IC_{50}, 0.03 μM). JM1591, JM1596, and JM1809 showed a comparable antiviral activity against the three HIV-1 and the two HIV-2 strains tested (IC_{50}, 0.03–0.2 μM). JM2815 and JM2820 were very potent inhibitors of the HIV-1(HE) strain (IC_{50}, 0.002 μM). JM2800 was the least active against HIV-1(III_B), HIV-1(RF), and HIV-2(ROD) (IC_{50}, 7.8–18.3 μM); it was inhibitory to HIV-1(HE) and HIV-2(EHO) replication with an IC_{50} of 1.5 and 1.0 μM, respectively. JM1583, JM2815, and JM2820 inhibited SIV replication with an IC_{50} of 0.002–0.003 μM. All test compounds, except for JM2800, were active against MSV, although less so than against HIV or SIV.

The polyoxometalates blocked giant cell formation and thus prevented destruction of the uninfected $CD4^+$ target cells by the HIV-infected HUT-78 cells (43). In the HIV-1(III_B)-induced syncytium formation assay, JM2766 and JM1809 were more active (IC_{50}, 1.9 and 2.0 μM, respectively) than dextran sulfate (IC_{50}, 4.4 μM), with JM1583, JM1589, and JM2815 being less active (IC_{50}, 13.2, 30.4, and 22.2 μM, respectively). JM1583, JM1590, JM1591, JM1809, JM2766, JM2815, and JM2820 proved active in the HIV-2(ROD)-induced syncytium formation assay at an IC_{50} of 0.2–0.8 μM; JM1589 and JM2800 were inactive (IC_{50}, > 30 μM) in both the HIV-1- and HIV-2-induced giant cell formation assays (Table 1).

Activity against other RNA viruses (other than retroviruses). When evaluated for their inhibitory effects on the replication of RNA viruses other than retroviruses (Table 2), the polyoxometalates were found to be particularly active against RSV, VSV, and Sindbis. The IC_{50} of JM1809 for RSV, VSV, and Sindbis was 0.04, 7, and 0.2 μM, respectively. Some of the compounds (JM1583, JM1809, JM2766, and JM2815) were also active against Junin and Tacaribe virus (IC_{50}, 2–18.6 μM). In addition, JM1809 showed activity against Semliki Forest and measles virus. The polyoxometalates had no activity against parainfluenza and the nonenveloped viruses poliovirus and reovirus (Table 2).

Activity against DNA viruses. When the polyoxometalates were examined for their inhibitory effects on the replication of DNA viruses, all polyoxometalates, except for JM1589 and JM2800, were found to inhibit HSV-1 (including TK^- HSV-1) and HSV-2 (Table 3). As a rule, the compounds were more active against CMV than against HSV. In fact, several polyoxometalates were found to inhibit CMV replication at a concentration of <1 μM. No activity was noted with any of the polyoxometalates tested against vaccinia virus.

Cytotoxicity. Except for HPA-23, which inhibited MT-4 cell proliferation at a $CCID_{50}$ of 42 μg/ml, the compounds did not prove inhibitory to MT-4 cell growth at concentrations up to 200 μg/ml. No microscopically detectable alteration of normal cell morphology was noted with any of the compounds when they were added to the cells (i.e., C3H/3T3, HeLa, Vero, or E_6SM) at concentrations up to 80 μM.

Inhibition of HIV-1 RT activity. When evaluated for their inhibitory effect on the RT activity of recombinant RT (p66/p66) with poly(C)·oligo(dG) as the template/primer, the polyoxometalates were found to inhibit RT activity in a concentration-dependent manner, with IC_{50} varying from 0.01 to >1.2 μM (Table 4). HIV-1(III_B) virion-derived RT was inhibited at a consistently higher IC_{50} than recombinant RT (Table 4). This is most likely caused by the nonspecific binding of polyoxometalates to proteins present in the virion-derived RT preparation. Indeed, the total protein concentration of the RT reaction mixture containing virion-derived RT was 180-fold higher than when recombinant RT was used. When the IC_{50} values for either recombinant RT or native RT inhibition were plotted against the IC_{50} values for inhibition of HIV-1(III_B) replication, there was a poor correlation between the anti-HIV activity of the compounds in cell culture and their inhibitory effects on RT activity ($r = 0.424$ for recombinant RT inhibition; $r = 0.045$ for native RT inhibition) (data not shown).

Time of addition experiment. To delineate the stage of the HIV-1 replication cycle at which the polyoxometalates interfered, the compounds were added at different times after exposure of the MT-4 cells to HIV-1 (Fig. 1). Dextran sulfate, AZT, DDI, and TIBO R82913 were run in parallel. The compounds were added at a concentration that was 100-fold higher than their IC_{50}. Dextran sulfate was not able to inhibit HIV-1 replication if added 2 hr after infection. AZT, DDI, and R82913

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TABLE 1

Inhibitory effects of polyoxometalates on retrovirus-induced cytopathicity, cell transformation, and syncytium formation

Except for HPA-23, which inhibited MT-4 cell proliferation at a $CCID_{50}$ of 5.9 μM, all polyoxometalates were nontoxic to the uninfected cells at concentrations up to 80 μM. All data represent mean values for at least two separate experiments.

Compound	IC_{50}[a]								
	Cytopathicity						Cell transformation, MSV	Giant cell formation	
	HIV-1			HIV-2		SIV(MAC_{251})		HIV-1(III_B)	HIV-2(ROD)
	III_B	RF	HE	ROD	EHO				
	μM								
JM1583	0.2 ± 0.1	2.6 ± 0.9	0.2 ± 0.1	0.2 ± 0.06	0.1 ± 0.07	0.003 ± 0.0003	4.6 ± 0.1	13.2 ± 2.9	0.7 ± 0.2
JM1589	1.3 ± 0.4	2.4 ± 1.1	0.3 ± 0.2	1.1 ± 0.3	0.2 ± 0.1	0.1 ± 0.003	4.6 ± 0.4	30.4 ± 4.6	>30
JM1590	0.03 ± 0.01	0.3 ± 0.1	0.08 ± 0.04	0.06 ± 0.03	1.3 ± 0.6	0.008 ± 0.002	0.9 ± 0.6	3.0 ± 1.6	0.3 ± 0.1
JM1591	0.1 ± 0.03	0.1 ± 0.05	0.03 ± 0.01	0.1 ± 0.04	0.2 ± 0.06	1.3 ± 0.5	4.3 ± 0.6	7.8 ± 2.2	0.8 ± 0.2
JM1596	0.2 ± 0.1	0.2 ± 0.07	0.05 ± 0.02	0.2 ± 0.1	0.1 ± 0.04	0.9 ± 0.4	2.5 ± 1.0	7.2 ± 4.6	3.6 ± 1.1
JM1809	0.2 ± 0.08	0.1 ± 0.03	0.08 ± 0.04	0.08 ± 0.03	0.2 ± 0.06	1.1 ± 0.8	1.8 ± 0.01	2.0 ± 0.9	0.8 ± 2.3
JM2766	0.1 ± 0.06	0.8 ± 0.2	0.2 ± 0.1	0.2 ± 0.07	1.1 ± 0.5	0.03 ± 0.02	20.2 ± 7.6	1.9 ± 0.6	0.6 ± 0.2
JM2800	18.3 ± 3.1	14.0 ± 3.2	1.5 ± 0.9	7.8 ± 2.4	1.0 ± 0.8	2.8 ± 2.6	>300	>60	>60
JM2815	0.6 ± 0.2	0.4 ± 0.3	0.002 ± 0.001	0.2 ± 0.09	0.5 ± 0.3	0.003 ± 0.0002	1.5 ± 1.4	22.2 ± 9.2	0.3 ± 0.09
JM2820	0.6 ± 0.4	0.3 ± 0.2	0.002 ± 0.002	0.5 ± 0.2	0.3 ± 0.2	0.002 ± 0.0003	0.5 ± 0.1	5.4 ± 3.1	0.2 ± 0.06
HPA-23	0.2 ± 0.06	0.1 ± 0.04	0.04 ± 0.02	0.06 ± 0.03	0.6 ± 0.3	0.008 ± 0.0004		2.9 ± 0.8	2.8 ± 0.6
Dextran sulfate	0.1 ± 0.02	0.06 ± 0.03	0.02 ± 0.01	0.02 ± 0.01	0.8 ± 0.4		4 ± 2.4	4.4 ± 2.7	0.8 ± 0.4

[a] Based on the inhibition of HIV-1- or SIV-induced cytopathicity in MT-4 cells, C3H/3T3 cell transformation by MSV, or syncytium (giant cell) formation upon co-culturing of MOLT-4 cells with persistently HIV-1 (strain IIIB)- or HIV-2 (strain ROD)-infected HUT-78 cells.

TABLE 2

Activity of polyoxometalates against RNA viruses other than retroviruses

All polyoxometalates were not toxic to the uninfected cells at concentrations up to 80 μM.

Compound	IC_{50}[a]									
	Togaviruses		Paramyxoviruses			Arenaviruses		Rhabdoviruses, VSV	Picornaviruses, polio-1	Reoviruses, reo-1
	Sindbis	Semliki Forest	Measles	RSV	Parainfluenza	Junin	Tacaribe			
	μM									
JM1583	6.6 ± 2.1	99 ± 12	19.8 ± 3.6	1.3 ± 0.3	>132	5.3	7.9	28.1 ± 3.9	>132	>132
JM1589	>132	>132	>66	>66	>132	>66	>66	99 ± 19	>132	>132
JM1590	2.1 ± 1.0	22.5 ± 8	>30	1.8 ± 0.6	>60	15	15	5.9 ± 3.2	>60	>60
JM1591	5 ± 2.1	37.5 ± 13	>50	12.5 ± 2.4	>100	>50	>50	11.3 ± 3.9	>100	>100
JM1596	17.1 ± 9.9	12.6 ± 5.4	>18	>18	>72	>36	>36	12.6 ± 4.2	>36	>72
JM1809	0.2 ± 0.1	4 ± 2.1	4 ± 2.3	0.04 ± 0.01	>80	2	2	1 ± 0.2	>40	>80
JM2766	6.2 ± 1.2	93 ± 19	>62	21.7 ± 4.1	>124	17.1	18.6	14 ± 4.6	>124	>124
JM2800	>244	>244	>122	>122	>244	>122	>122	>244	>244	>244
JM2815	4.3 ± 2.1	46.5 ± 13	31 ± 12	4.3 ± 1.8	>124	13.3	15.5	6.2 ± 2.5	>124	>124
HPA-23	2.8 ± 0.9	9.8 ± 2.6	>28	7.2 ± 3.0	>56	1.4	1.7	2.8 ± 0.9	>5.6	>56
Dextran sulfate	2 ± 0.4	>80	>40	8 ± 1.6	>80	0.4	0.4	1 ± 0.3	>80	>80

[a] Based on the inhibition of the cytopathicity of polio virus, RSV, or VSV for Hela cells or of Sindbis, Semliki Forest, measles, parainfluenza, Junin, Tacaribe, or reovirus for Vero cells.

began to lose their inhibitory activity only if treatment was delayed until 6 hr after infection. The protease inhibitor Ro31-8959, which interacts with a late event in the replication cycle (assembly of mature virions), was still effective if added as late as 21 hr after infection. JM1590, one of the most potent HIV-1 inhibitors among the polyoxometalates (Table 1), was found to interact at an early stage in the replicative cycle, virtually coinciding with that at which dextran sulfate interacts (Fig. 1). When added at 3 hr after infection, JM1590 was no longer active. This contrasts with the behavior of the RT inhibitors AZT, DDI, and TIBO, which started to lose their activity only if their addition was delayed until 6 hr after infection.

Inhibition of HIV-1 adsorption to the cells. Using an indirect immunofluorescence technique with polyclonal anti-HIV-1 antibodies and flow cytometric analysis (Fig. 2), MT-4 cells to which HIV-1 particles had been adsorbed could be readily distinguished from MT-4 cells that had not been exposed to HIV-1. If MT-4 cells had been exposed to HIV-1 (in the absence of any compound), the fluorescence histogram of the HIV-1-loaded cells was clearly shifted to the right (greater fluorescence intensity) of the fluorescence histogram representing control cells, which had not been exposed to HIV-1 (Fig. 2a). When the same experiment was carried out in the presence of dextran sulfate (at 5 μM), the histogram representing the HIV-1-loaded cells was shifted back to the left and coincided almost completely with the histogram representing the control uninfected cells (exposed to the compound) (Fig. 2c). Likewise, JM1590 (at a concentration of 3.75 μM) caused an almost complete shift in the histogram representing HIV-1-loaded cells from right to left (Fig. 2b). These results indicate that both dextran sulfate and JM1590, at the concentrations used, block HIV-1 adsorption to MT-4 cells. When evaluated under the same conditions, JM2800 (at 15.25 μM) did not cause an appreciable inhibition of virus adsorption (data not shown).

Inhibition of anti-gp120 mAb binding to gp120. Because the polyoxometalates appeared to interfere with HIV adsorption to the cells, they were further evaluated for their ability to inhibit the binding of anti-gp120 mAb to gp120.

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TABLE 3

Activity of polyoxometalates against DNA viruses

All polyoxometalates were not toxic to the uninfected cells at concentrations up to 90 μM.

	IC_{50}*						
	Herpesviruses						Poxviruses, vaccinia
	HSV-1			HSV-2(G)	CMV		
	KOS	B2006 (TK⁻)	VMW 1837 (TK⁻)		AD-169	Davis	
	μM						
JM1583	49.5 ± 13.1	23.1 ± 4.6	36.3 ± 3.9	23.1 ± 4.6	4.6	1.7	>66
JM1589	>66	33 ± 11	>66	44.6 ± 9.2	35	13.5	>66
JM1590	6.8 ± 2.6	0.6 ± 0.2	2.1 ± 0.6	6.8 ± 1.9	0.6	0.2	>30
JM1591	5 ± 1.9	0.8 ± 0.4	1.3 ± 0.3	5 ± 2.1	1	1.3	>50
JM1596	7.2 ± 2.3	5.4 ± 1.6	4.3 ± 1.9	8.1 ± 3.6	0.4	0.5	>36
JM2766	21.7 ± 3.6	4.7 ± 2.1	17.1 ± 3.8	17.1 ± 4.7	0.3	0.6	>62
JM2800	>122	>122	>122	>122	12.2	9.2	>122
JM2815	21.7 ± 4.5	6.2 ± 2.9	12.4 ± 4.6	6.2 ± 2.3	1.2	0.9	>62
JM2820	3.6 ± 1.9	0.5 ± 0.2	2.2 ± 1.0	2.5 ± 0.7	0.2	0.2	>36
HPA-23	2 ± 0.3	0.4 ± 0.2	1 ± 0.3	2.8 ± 0.9	0.4	0.4	>28
Dextran sulfate	0.4 ± 0.2	0.4 ± 0.3	0.4 ± 0.2	0.8 ± 0.2	0.2	0.8	>40

* Based on the inhibition of the cytopathicity of HSV-1, HSV-2, or vaccinia for E_6SM cells or of CMV for human embryonic lung cells.

TABLE 4

Inhibition of HIV-1 RT activity by polyoxometalates

Compound	IC_{50}*	
	Recombinant RT	Native RT
	μM	
JM1583	0.58	1.85
JM1589	>6.6	>6.6
JM1590	0.04	1.25
JM1591	0.16	0.9
JM1596	0.23	3.6
JM1809	0.01	0.08
JM2766	0.02	0.19
JM2800	>12.2	>12.2
JM2815	0.28	1.74
JM2820	0.23	1.06
HPA-23	0.16	1.48
TIBO R82150	0.14	0.24

* IC_{50} required to inhibit RT activity by 50%. The template/primer was poly(C)·oligo(dG)$_{12-18}$ and the enzyme preparations tested were either recombinant RT (p66) or HIV-1(III$_B$) virion-derived native RT.

Exposure of persistently HIV-1-infected HUT-78 cells to the polyoxometalates for only 15 min resulted in a concentration-dependent loss of staining of the cells by anti-gp120 mAb (Table 5). The IC_{50} of JM2800 was >61 μM and the other polyoxometalates had an IC_{50} in the range of 1.03–21.2 μM. When evaluated for their ability to inhibit the binding of OKT4A/Leu 3a mAb to the CD4 receptor of MT-4 cells, JM2820, JM2815, HPA-23, and dextran sulfate did not prove inhibitory at concentrations up to 10 μM (data not shown).

When the IC_{50} values for inhibition of viral cytopathicity (Table 1) were plotted against the IC_{50} values found for inhibition of syncytium (giant cell) formation (Table 1), a good correlation (r = 0.843) was obtained between the IC_{50} of the compounds in the two assays (Fig. 3A). Furthermore, the IC_{50} values found for inhibition of anti-gp120 mAb binding (Table 5) correlated closely (r = 0.902) with the inhibition of giant cell formation (Fig. 3B).

Discussion

The present study demonstrates that polyoxometalates of varied classes have a broad-spectrum antiviral activity against

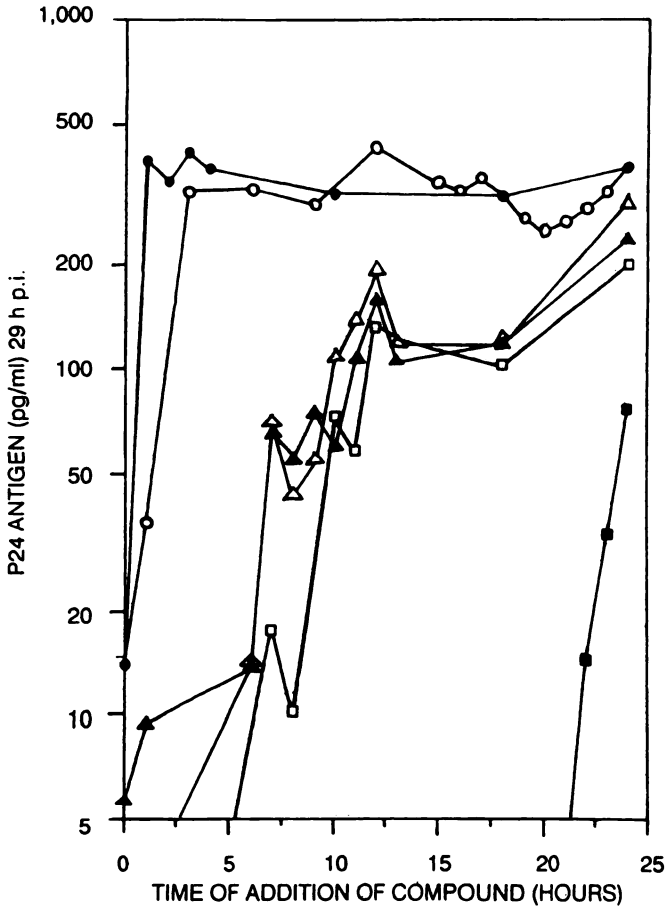

Fig. 1. Time of addition experiment. MT-4 cells were infected with HIV-1 at a multiplicity of infection of >1. The compounds were added at different times after infection. Viral p24 antigen production was determined at 29 hr after infection by a sandwich enzyme-linked immunosorbent assay. The compounds were used at 100 times their IC_{50}. ●, Dextran sulfate (10 μM); ○, JM1590 (3 μM); ▲, AZT (0.19 μM); △, DDI (423 μM); □, R82913 (0.155 μM); ■, Ro31–8959 (0.065 μM).

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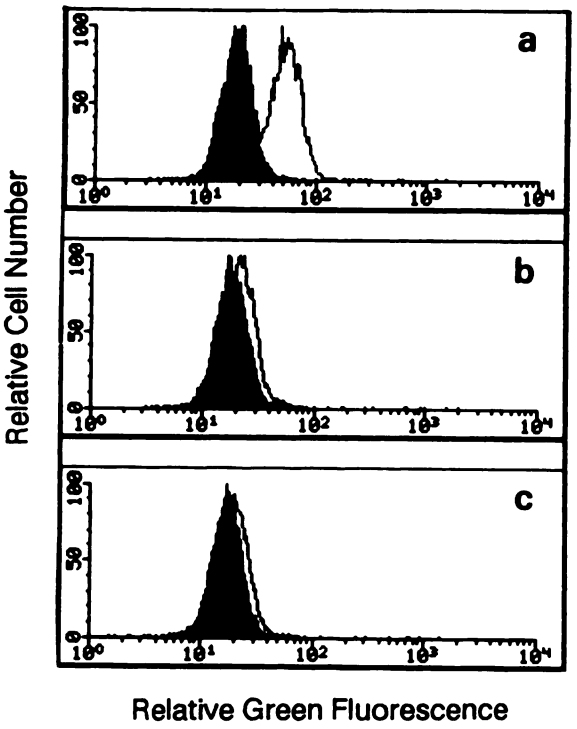

Fig. 2. Inhibitory effects of JM1590 on HIV-1 binding to MT-4-cells. *Black histogram*, fluorescence of MT-4 cells that were not exposed to HIV-1; *white histogram*, fluorescence of MT-4 cells that were exposed to HIV. The MT-4 cells were exposed or not exposed to HIV-1 in the absence of any compound (a), in the presence of JM1590 at 3.75 μM (b), or in the presence of dextran sulfate at 5 μm (c).

enveloped RNA and DNA viruses, including retroviruses (i.e., HIV-1, HIV-2, SIV, and MSV), togaviruses (i.e., Sindbis and Semliki Forest), paramyxoviruses (i.e., RSV), arenaviruses (i.e., Junin and Tacaribe), rhabdoviruses (VSV), and herpesviruses (i.e., HSV-1, HSV-2, and CMV) (Tables 1–3). In terms of antiviral potency, marked differences were noted from one compound to another and from one virus to another; JM1809 was the most potent against Sindbis, RSV, VSV, and arenaviruses, whereas other compounds (i.e., JM2815 and JM2820) were equally active or even more active than JM1809 against HIV-1, HIV-2, and SIV.[4]

For HIV-1(III_B), JM1590 was the most potent inhibitor (IC_{50}, 0.03 μM). Inouye *et al.* (19) found that $K_7[PTi_2W_{10}O_{40}]\cdot 6H_2O$ inhibits the replication of HIV-1(III_B) in MT-4 cells by 50% at a concentration of 1.3 μM and with a selectivity index of 156. The selectivity index of JM1590 for inhibition of HIV-1(III_B) replication was 1150 and that of JM2766 was >1250. With JM2815 and JM2820, selectivity indexes of >5000 against HIV-1(HE) were obtained. JM1583, JM1590, JM2815, and JM2820 were particularly potent inhibitors of SIV replication (IC_{50}, 0.003, 0.01, 0.003, and 0.002 μM, respectively; selectivity index, >20,000, >4,000, >50,000, and >50,000, respectively).

The anti-HIV potency of the polyoxometalates in MT-4 cells varied from one strain to another. It has been reported previously that the antiviral effects of sulfated polysaccharides (49–51) and other polyanionic compounds (52) may vary considerably depending on the cell type used and the HIV strain tested.

[4] In confirmatory testing, Dr. Raymond Schinazi (Emory University) has concurrently shown that JM2820 has excellent antiviral activity in his HIV and HSV assays (manuscript in preparation).

TABLE 5

Inhibitory effect of polyoxometalates on the binding of anti-gp120 mAb to persistently HIV-1-infected HUT-78 cells, as detected by fluorescence-activated cell sorting analysis

HIV-1-infected HUT-78 cells (200,000 cells/100 μl of RPMI with 10% FCS) were washed twice, incubated with the compounds at the indicated concentrations at 20° for 15 min, stained, and analyzed as described in Experimental Procedures. The inhibitory index for inhibition of anti-gp120 mAb binding (II_{gp120}) was calculated according to the formula $II_{gp120} = 1 - (MF_{gp120X} - MF_C/MF_{gp120} - MF_C)$, whereby MF_{gp120} is the mean channel fluorescence (*MF*) for the cells incubated with only anti-gp120 mAb, MF_{gp120X} is the mean fluorescence for the cells incubated with test compound and anti-gp120 mAb, and MF_C is the mean fluorescence of the cells incubated with RaM-IgG-F(ab′)$_2$-FITC. Mean channel fluorescence was determined by the Consort 30 program (Becton-Dickinson).

Compound	Concentration	Mean channel fluorescence	II_{gp120}[a]
	μM		
JM1583	33	31.4	0.79
	6.6	38.4	0.37
	1.32	43.4	0.08
	0.26	45.0	0.00
JM1589	33	33.2	0.68
	6.6	37.9	0.40
	1.32	43.0	0.10
	0.26	44.0	0.04
JM1590	15	27.4	1.00
	3	32.6	0.72
	0.6	41.2	0.20
	0.12	44.4	0.01
JM1591	25	31.7	0.77
	5	35.1	0.57
	1	39.7	0.29
	0.2	41.3	0.20
JM1596	18	28.4	0.97
	3.6	36.1	0.51
	0.72	37.2	0.44
	0.14	43.2	0.08
JM1809	20	25.0	1.00
	4	30.5	0.84
	0.8	37.1	0.45
	0.16	40.0	0.27
JM2766	31	28.4	0.97
	6.2	29.1	0.93
	1.24	34.7	0.59
	0.25	41.7	0.17
JM2800	61	38.4	0.37
	12.2	43.1	0.09
	2.44	43.7	0.05
	0.49	45.0	0.00
JM2815	31	34.1	0.63
	6.2	39.6	0.30
	1.24	44.8	0.00
	0.25	45.1	0.00
JM2820	18	32.0	0.75
	3.6	33.4	0.67
	0.72	38.9	0.34
	0.14	43.2	0.08
HPA-23	14	31.0	0.81
	2.8	31.0	0.75
	0.56	40.4	0.25
	0.11	45.0	0.00
Dextran sulfate	20	25.0	1.00
	4	31.1	0.81
	0.8	35.4	0.55
	0.16	38.6	0.36
Ram-IgG-F(ab′)$_2$-FITC		27.9	
Anti-gp120 mAb + RaM-IgG-F(ab′)$_2$-FITC		44.6	

The differences in susceptibility among the various HIV strains probably reflect the differences in the interaction of the polyanions with the HIV envelope glycoproteins. These findings apparently extend to polyoxometalates. Because the polyoxo-

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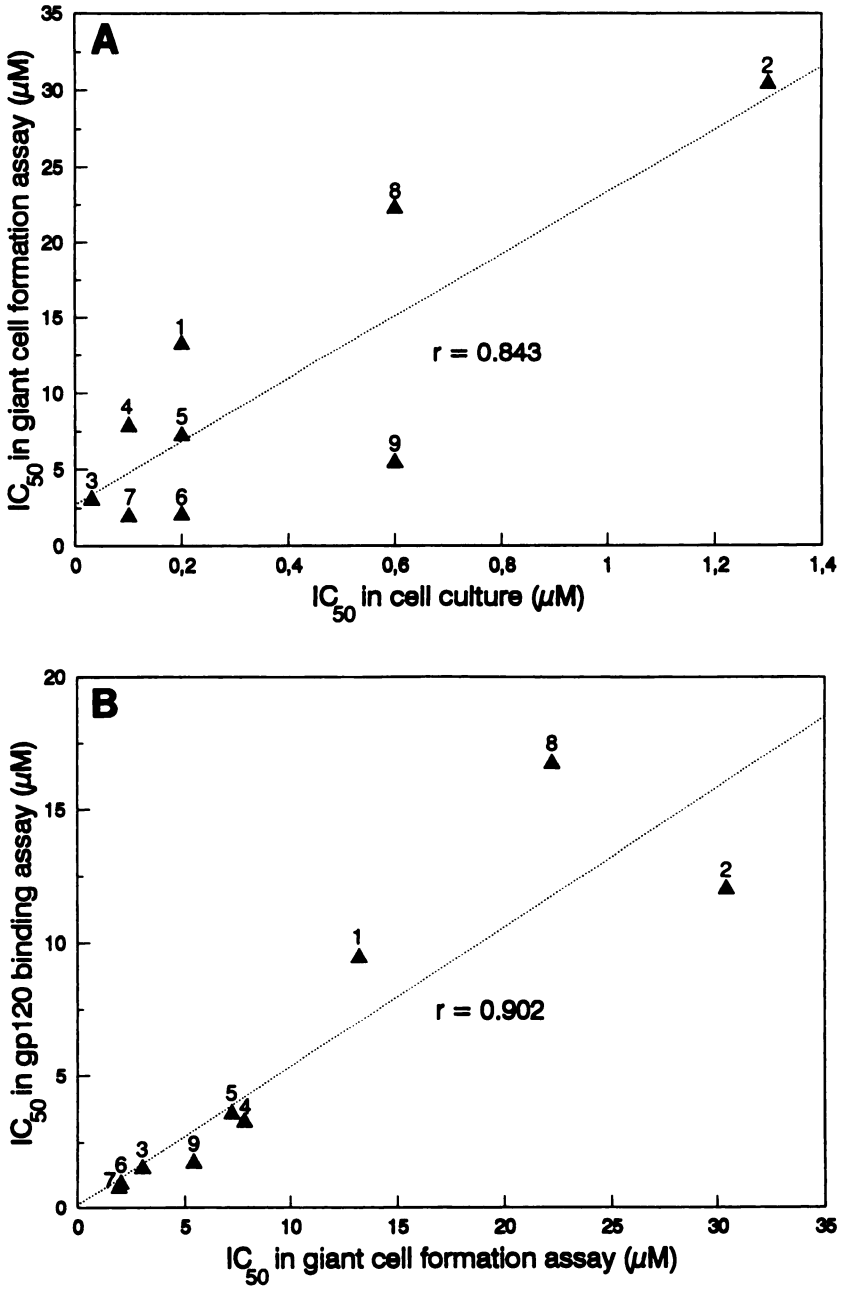

Fig. 3. A, IC_{50} of polyoxometalates for giant cell formation inhibition plotted against their IC_{50} for inhibition of HIV-1(III_B) replication in MT-4 cells. *1*, JM1583; *2*, JM1589; *3*, JM1590; *4*, JM1591; *5*, JM1596; *6*, JM1809; *7*, JM2766; *8*, JM2815; and *9*, JM2820. A correlation coefficient (*r*) of 0.843 was obtained. B, IC_{50} of polyoxometalates for the inhibition of anti-gp120 mAb binding to gp120 plotted against their IC_{50} for inhibition of HIV-1-induced giant cell formation. The numbering of the compounds is the same as in A. A correlation coefficient (*r*) of 0.902 was obtained.

metalates represent diverse classes and specific discrete structures, it is clear from the results described above that specific structural differences in the polyoxometalates are critical for their ability to block specific viral adsorption.

As demonstrated by flow cytometric analysis (8), the polyoxometalates, like dextran sulfate, block the binding of HIV-1 particles to the cells (Fig. 2). They do not interfere with the binding of OKT4A/Leu-3a mAb to the CD4 receptor of MT-4 cells, but they inhibit the binding of anti-gp120 mAb to persistently HIV-1-infected HUT-78 cells (Table 5).

The binding of the polyoxometalates to the viral envelope gp120 may be responsible for their anti-HIV activity, because there was a good correlation between their interaction with gp120 and their inhibitory effects on syncytium formation, which, in turn, correlated with their inhibitory effects on viral cytopathicity (Fig. 3).

Except for JM1589 and JM2800, all polyoxometalates tested were able to inhibit the activity of both the recombinant and native RT of HIV-1 (Table 4). However, their potency as RT inhibitors did not correlate with their inhibitory potency against HIV-1 replication in cell culture. From the time of addition experiments (Fig. 1) it was also evident that the primary site of interaction of the polyoxometalates had to be an earlier stage of the virus replicative cycle than the reverse transcription process. According to the time of addition experiments, the site of interaction of the polyoxometalates could be identified as the virus adsorption process. That the polyoxometalates interfere with virus-cell binding was then directly shown by flow cytometric analysis (Fig. 2).

Thus, polyoxometalates are able to interact with two processes in the HIV replicative cycle, (i) RT and (ii) virus adsorption. However, their mode of anti-HIV action can be ascribed

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primarily, if not solely, to inhibition of virus adsorption. The latter by itself suffices to account for the inhibitory effects of the polyoxometalates on virus replication, virus-induced cytopathicity, and syncytium formation. Any follow-up studies on the pharmacology, pharmacokinetics, and structure-activity relationships of these compounds should take into consideration the fact that their primary target of anti-HIV action is on the outside of the cells (virus adsorption) rather than inside (reverse transcription).

In conclusion, polyoxometalates show a broad-spectrum antiviral activity against a wide variety of enveloped DNA and RNA viruses, including HIV-1 and HIV-2. The primary target for their antiviral action appears to be the viral envelope, where they may occupy the sites required for interaction with the cell surface.

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