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Inhibition of proliferation of human immunodeficiency virus type 1 by novel heteropolyoxotungstates in vitro

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Summary

Fifteen heteropolyoxotungstates were tested for their effects on the proliferation of human immunodeficiency virus type 1 (HIV-1) using an in vitro system consisting of MT-4 cells and HTLV-III$_b$. Eight heteropolyoxotungstates (HPOTs) with the Keggin structure or dimerized deficient Keggin structure proved to be potent inhibitors of HIV-1. In contrast, seven non-Keggin HPOTs including HPA 23 did not have significant effects on HIV-1 proliferation at non-toxic doses. $[PTi_2W_{10}O_{40}]^{7-}$ (PM-19) was the most potent inhibitor of HIV-1 among the 15 HPOTs tested. The inhibition of HIV-1 replication by PM-19 presumably results from impaired virus adsorption and/or penetration into target cells. Viral spread of HIV-1 and HIV-2 on cell-to-cell basis was also susceptible to PM-19. In combination, PM-19 and 3′-azido-3′-deoxythymidine were synergistic in inhibiting HIV-1 proliferation.

Heteropolyoxotungstate; PM-19; Keggin structure; Human immunodeficiency virus type 1

Introduction

The potent in vitro and in vivo inhibition of replication of some DNA and RNA viruses by heteropolyoxotungstates (HPOTs) has been reported (Ablashi et al., 1977; Fujita et al., in preparation; Jasmin et al., 1973; Jasmin et al., 1974; Larnicol et al., 1981; Schonfeld et al., 1975). A Keggin HPOT, $[PTi_2W_{10}O_{40}]^{7-}$ (PM-19),

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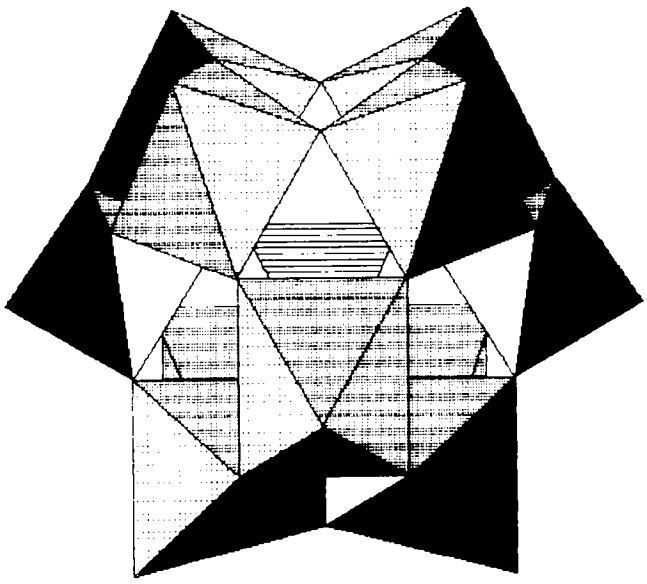

Fig. 1. Structure of Keggin compounds.

was reported to suppress the proliferation of human immunodeficiency virus type 1 (HIV-1) in MT-4 cells, the human HTLV-I-carrying T4 cell line (Inouye et al., 1990). The Keggin compounds are generally formulated as $[X^{n+}M_{12}O_{40}]^{(8-n)-}$ in which M = Mo (VI) or W(VI); X = P(V), As(V), Ti(IV), Zr(IV), B(III), Ge(IV), Si(IV) or As(V). The general structure is shown in Fig. 1. In PM-19, two tungsten atoms are replaced by titanium atoms and the central (hetero) atom is P(V). The tungsten and titanium atoms are roughly in the center of the octahedron of oxygen atoms which share corners and/or edges but not faces and the coordination of the central atom is tetrahedral. Another HPOT, $[NaSb_9W_{21}O_{86}]^{18}$ (HPA 23), which has no Keggin structure, inhibits the reverse transcriptases of some retroviruses such as avian myeloblastosis virus (AMV) and HIV-1. Treatment of AIDS patients with HPA 23 has no significant effect on the number of T4 lymphocytes or the ratio of the numbers of T4 and T8 lymphocytes (Moskovitz et al., 1988; Rozenbaum, 1985; Vittecoq et al., 1988), although there was a decrease in the reverse transcriptase activity in the cell-free supernatant of T cell lymphocyte culture. Balzarini et al. (1986) reported that HPA 23 did not prevent HIV-1 from inducing cytopathogenicity in ATH 8 cells or expressing p24 protein in H9 cells. Assuming that the three-dimensional structures of these compounds are closely related to their anti-HIV-1 potentials, we synthesized a series of HPOTs of both Keggin and non-Keggin structure and tested them for their effects on HIV-1 replication.

Materials and Methods

Chemicals

The preparation of the following compounds is well known: $K_5[BW_{12}O_{40}]\cdot 15H_2O$ (PM-1) (Rocchiccioli-Deltcheff et al., 1983; Yamase et al., 1986), $K_{27}[KAs_4W_{40}O_{140}]$ (PM-3) (Leyrie et al., 1978), $K_7[PTi_2W_{10}O_{40}]\cdot 6H_2O$ (PM-19) (Domaille et al., 1983), $K_8[P_2Co(H_2O)W_{17}O_{61}]\cdot 18H_2O$ (PM-29) (Malik et al., 1968), A-β-$Na_9[SiW_9O_{34}H]\cdot 23H_2O$ (PM-30) (Herve et al., 1977), $K_6[SiNi(H_2O)W_{11}O_{39}]\cdot 15H_2O$ (PM-40) (Weakley et al., 1967), $K_5[SiVW_{11}O_{40}]\cdot nH_2O$ (PM-43) (Herve et al., 1979), $K_5[PVW_{11}O_{40}]\cdot 6H_2O$ (PM-44) (Smith et al., 1973), $K_{13}[Eu(SiW_{11}O_{39})_2]\cdot 30H_2O$ (PM-48) (Ballardini et al., 1984), $(NH_4)_{17}Na[NaSb_9W_{21}O_{86}]\cdot 14H_2O$ (PM-71) (Fischer et al., 1976; Michelon et al., 1980). $K_{18}[KSb_9W_{21}O_{86}]$ (PM-2) and $(NH_4)_{18}[(NH_4)Sb_9W_{21}O_{86}]$ (PM-72), $K_6[BVW_{11}O_{40}]$ (PM-46) and $Na_4[PCr(H_2O)Mo_{11}O_{39}]$ (PM-98), and $K_7[BVW_{11}O_{40}]$ (PM-47) were prepared by the modified procedures for PM-71, -43 and -44, respectively. PM compounds prepared were purified as previously described. Analytical grade $Na_3[PMo_{12}O_{40}]$ (PM-102) was commercially obtained and used without further purification. IR or ^{183}W NMR spectra and polarographic analyses of PM compounds were used for the characterization of the anionic structure. In addition, structures of PM-19 and PM-48 were confirmed by single-crystal X-ray diffraction (Ozeki et al., 1990) and photoemission measurement (Ballardini et al., 1984), respectively. HPA 23 was generously provided by Prof. A. Teze, Université Pierre et Marie Curie. Their code numbers and molecular formulas are given in Table 1. PM-1, -19, -40, -43, -44, -46, -47, -98 and -102 have the Keggin structure, and PM-48 is a dimer of the deficient Keggin structure, whereas PM-2, -3, -29, -30, -71 and -72, and HPA 23 do not have the Keggin structure. Dextran sulfate (DS, average mol. wt. ca. 8000) was purchased from Sigma Chemical Co. and 3′-azido-3′-deoxythymidine (AZT) was kindly donated by Burroughs Wellcome Co. All other materials used in this study are commercial products of analytical grade.

Cells

MT-4 cells (Miyoshi et al., 1982) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum; penicillin, 100 units/ml; and streptomycin, 100 μg/ml. Uninfected Molt-4 cells (Minowada et al., 1972), HIV-1-infected Molt-4 cells (Molt-4/HTLV-III_b and Molt-4/ARV) (Koyanagi et al., 1985; Levy et al., 1984) and HIV-2(GH-1)-infected Molt-4 cells (Molt-4/0650) (Ishikawa et al., 1988) which were kindly provided by Prof. M. Hayami, Kyoto University, were subcultured in the same medium.

Virus and inhibition assay for viral infection

HTLV-III_b was obtained from the culture supernatant of Molt-4/HTLV-III_b as described by Harada et al. (1985b). The titer of the viral preparation determined

in MT-4 cells 6 days after the viral infection was 1×10^6 $TCID_{50}$/ml. MT-4 cells were infected with HIV-1 at a multiplicity of infection of 0.02. Portions (0.3 ml) of MT-4 cell suspension (6.6×10^5 cells/ml) were mixed with 0.2 ml of viral preparation (2×10^4 $TCID_{50}$/ml) in the wells of multiwell plates (Costar, 3424). After the plates were incubated for 1 h at 37°C, 0.5 ml portions of drug dilutions in the same medium were added to the cultures.

The viable cells were counted by the trypan blue dye exclusion method after 4 days of culture. The calculation of HIV-1-induced cytopathic effect (CPE) was based on the numbers of viable cells in mock-infected and HIV-1-infected cultures (Harada et al., 1985a). The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of compound that reduced the cell number of the mock-infected MT-4 cells by 50%. The percent protection was calculated according to the method of Pauwels et al. (1988), except for the fact that the cell number was used instead of the optical density of the formazan product of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). The 50% effective concentration (EC_{50}) was defined as the concentration at which 50% protection was obtained.

Indirect immunofluorescence method

HIV-1-infected cells were smeared, dried and fixed with cold methanol. The fixed cells were treated with 1:100 diluted serum from a patient with hemophilia and fluorescein-isothiocyanate (FITC)-conjugated goat anti-human IgG anti-serum in that order at 37°C, each anti-serum for 40 min. The IF-positive cells were counted under the fluorescence microscope (Harada et al., 1985a).

Inhibition assay for syncytium formation

Syncytium formation was assayed by the method of Tochikura et al. (1988) with some modifications. Briefly, 0.35 ml portions of Molt-4 cell suspension (5×10^5 cells/ml), 0.15 ml portions of Molt-4/HTLV-III_b, Molt-4/ARV or Molt-4/0650 cell suspension (5×10^5 cells/ml) and 0.5 ml portions of drug dilutions in the medium (PM-19, 100 μg/ml; DS, 100 μg/ml; AZT, 1 μg/ml) were mixed in wells of multiwell plates. After 72 h of incubation, the cells were diluted with phosphate-buffered saline to 5×10^3 cells/ml and 2 ml portions were transferred to tissue culture dishes (Nunc 166508) to determine the number of giant cells.

Calculation of drug combination index

The multiple drug effect analysis of Chou et al. (1984) was used to calculate the effects of combined drugs. This method involves plotting dose-effect curves for each agent and for multiply diluted, fixed-ratio combinations of the agents.

A combination index (CI) is determined by the equation (non-exclusive formula):

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2 + (D)_1\ (D)_2/(Dx)_1\ (Dx)_2$$

where $(Dx)_1$ is the dose of agent 1 required to produce x percent effect alone,

and $(D)_1$ is the dose of agent 1 required to produce the same x percent effect in combination with $(D)_2$. Similarly $(Dx)_2$ is the dose of agent 2 required to produce x percent effect alone, and $(D)_2$ is the dose required to produce the same effect in combination with $(D)_1$. The fraction affected (fa) = $x/100$, *e.g.*, fa is 0.9 if CPE is inhibited by 90%. The CI values of <1, 1 and >1 indicate potentiation, addition and antagonism, respectively.

Results

Inhibition of HIV-1 replication by Keggin and non-Keggin heteropolyoxotungstates (HPOTs)

The inhibition of HIV-1 replication by 15 HPOTs including HPA 23 and 2 heteropolyoxomolybdates (HPOMs) is presented in Table 1. EC_{50} and CC_{50} values

TABLE 1

Chemical and biological properties of heteropolyoxometalates, dextran sulfate and AZT

Compound	Molecular formula	EC_{50} (μg/ml)	CC_{50} (μg/ml)	Anti-HIV index[a]
Keggin heteropolyoxotungstates				
PM-19	$K_7[PTi_2W_{10}O_{40}]\cdot 6H_2O$	4.1	600	16
PM 1	$K_5[BW_{12}O_{40}]\cdot 15H_2O$	2.9	380	4
PM-46[b]	$K_6[BVW_{11}O_{40}]$	6.6	390	4
PM-47[c]	$K_7[BVW_{11}O_{40}]$	2.0	320	4
PM-40	$K_6[SiNiW_{11}O_{42}H_2]\cdot 15H_2O$	3.0	140	1
PM-43	$K_5[SiVW_{11}O_{40}]\cdot nH_2O$	3.0	360	1
PM-44	$K_5[PVW_{11}O_{40}]\cdot 6H_2O$	4.8	360	1
Keggin heteropolyoxomolybdates				
PM-98	$K_4[PCr(H_2O)Mo_{11}O_{39}]$	not available[d]	420	not available[e]
PM-102	$K_3[PMo_{12}O_{40}]$	not available[d]	620	not available[e]
Other heteropolyoxotungstates				
PM-30	$Na_9[SiW_9O_{34}H]\cdot 23H_2O$	5.9	190	0.063
PM 29	$K_8[P_2Co(H_2O)W_{17}O_{61}]\cdot 18H_2O$	13	70	0.016
PM-48[f]	$K_{13}[Eu(SiW_{11}O_{39})_2]\cdot 30H_2O$	2.0	330	4
PM-2	$K_{18}[KSb_9W_{21}O_{86}]$	not available[d]	17	0.063
PM-72	$(NH_4)_{18}[NH_4Sb_9W_{21}O_{86}]$	not available[d]	34	0.016
PM-71	$(NH_4)_{17}Na[NaSb_9W_{21}O_{86}]\cdot 14H_2O$	not available[d]	66	0.032
HPA 23	$(NH_4)_{17}Na[NaSb_9W_{21}O_{86}]\cdot 14H_2O$	not available[d]	40	0.016
PM-3	$K_{27}[KAs_4W_{40}O_{140}]$	15	210	0.25
AZT		0.00045	4.5	128
Dextran sulfate		0.85	>800	16

[a] 10% cytotoxic concentration on MT-4/100% suppressive concentration against HIV antigen expression.
[b] Vanadium (V).
[c] Vanadium (IV).
[d] The maximum protection is lower than 50%.
[e] 100% suppressive concentration against HIV antigen expression could not be obtained.
[f] PM-48 has a deficient dimerized Keggin structure.

are shown. For four 21-tungsto-9-antimoniates (PM-2, PM-71, PM-72, HPA 23) we were unable to obtain EC_{50} values since protection with these compounds did not reach 50%.

The anti-HIV-1 index was defined as the ratio of 10% cytotoxic concentration on MT-4 cells and 100% suppressive concentration for HIV-1, as determined by the expression of viral-specific antigens.

The anti-HIV-1 indexes of all seven Keggin HPOTs and PM-48 are in a range of 1 to 16, while those of non-Keggin HPOTs are less than 1. In the cases of two HPOMs, 100% suppression of HIV-1 antigen expression was not observed even at the toxic concentrations. PM-19 was the most potent inhibitor of HIV-1 replication among the Keggin compounds tested. It has the same anti-HIV-1 index as DS. These results indicate the importance of the Keggin structure in anti-HIV-1 activities of HPOTs; Keggin HPOT at non-toxic concentrations completely protects MT-4 cells against HIV-1 expression of viral-specific antigens (anti-HIV-1 index ≥ 1).

Mechanism of inhibition of HIV-1 replication by PM-19

Under standard assay conditions, inhibitors are added to the cultures 1 h after viral infection. This makes it difficult to specify the primary sites of action of the inhibitors. To understand how HPOTs intervene in the life cycle of HIV-1, we modified the assay procedures as follows, using PM-19 as a model compound. The cells were washed with fresh medium after 1 h of viral infection. Different concentrations of PM-19 were added to cultures for 1 h either before, during or after viral infection. The highest suppression of the HIV-1 replication was attained when PM-19 at 12.5 μg/ml or higher was added to the cell culture during viral infection (Fig. 2). In contrast, there was no significant effect when PM-19 was present for one hour at any other time (data not shown). In Fig. 3, 50 μg of PM-19/ml was added simultaneously with the initiation of HIV-1 infection, or 1, 5, 10

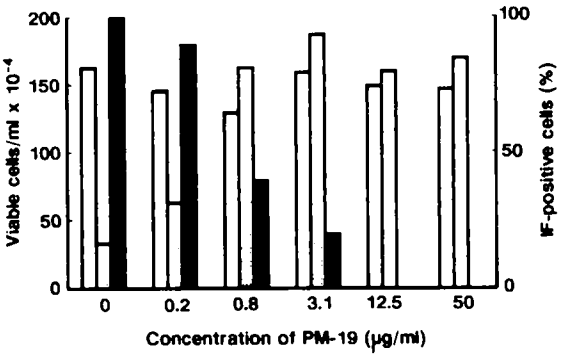

Fig. 2. Dose dependence of the suppression of virus adsorption. The mixture of 200 μl of HIV-1 (2×10^4 $TCID_{50}$/ml), 500 μl of varied concentrations (0, 0.4, 1.6, 6.2, 25 and 100 μg/ml) of PM-19 and 300 μl of MT-4 cell suspension (6.6×10^5 cells/ml) was incubated at 37°C for 60 min in a humidified atmosphere of 5% CO_2. The cells were washed with and resuspended in 1 ml of fresh medium. On day 4, the viable cells (□ uninfected, ▫ HIV-1-infected) and % IF-positive cells among HIV-1-infected population (■) were counted.

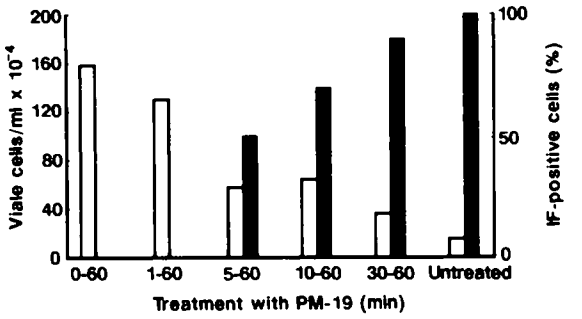

Fig. 3. Time dependence of the suppression of virus adsorption. The mixture of 200 μl of HIV-1 (2×10^4 $TCID_{50}$/ml), 300 μl of MT-4 cell suspension (6.6×10^5 cells/ml) was incubated at 37°C. Simultaneously or with a delay of 1, 5, 10 or 30 min, 500 μl of PM-19 (100 μg/ml) was added to this mixture. After 1 h of HIV-1 infection, the cells were washed with and resuspended in 1 ml of fresh medium. On day 4, the viable cells (□) and % IF-positive cells (■) were counted.

or 30 min after the initiation. When the infection was terminated (at 60 min), the cells were collected by centrifugation to remove unadsorbed HIV-1 and PM-19. Then the cells were resuspended in 1 ml of fresh medium and cultured for 4 days in a humidified atmosphere of less than 5% CO_2 in air. HIV-1 proliferation was completely suppressed when PM-19 was added to cell culture simultaneously with or 1 min after the initiation of HIV-1 infection. If HIV-1 infection was continued for 5 min or longer in the absence of PM-19, more than half of cell population expressed HIV-1 antigens. This shows that PM-19 probably interferes with the interaction of HIV-1 and target cells as in the cases of DS and glycyrrhizin (GL) (Ito et al., 1987).

Inhibition of syncytium formation by PM-19

The cell-to-cell transmission of HIV-1 is effected by a mechanism in which the cells expressing the HIV-1 envelope glycoprotein gp120 fuse with uninfected cells that bear the CD4 surface protein (Sodroski et al., 1986). Besides direct intervention in the interaction of HIV-1 and target cells, DS and GL have been reported to inhibit the syncytium formation effected by the cell-to-cell infection by HIV-1 in vitro (Tochikura et al., 1988). Nucleoside analogs such as AZT and 2′,3′-didehydro-3′-deoxythymidine do not affect HIV-1-related syncytium formation (Tochikura et al., 1988). The effect of PM-19 on the cell-to-cell transmission of HIV-1 and -2 was examined using an in vitro assay system consisting of Molt-4 and chronically virus-infected Molt-4 cells (Molt-4/HTLV-III_b, Molt-4/ARV or Molt-4/0650). As seen in Table 2, PM-19 completely inhibited syncytium formation at 50 μg/ml. The results with the reference compounds AZT and DS were in good agreement with the previous findings (Tochikura et al., 1988).

TABLE 2

Inhibition of syncytium formation by PM-19, dextran sulfate and AZT

Cell line	Syncytia formed/10^4 cells treated with			
	None	PM-19 (50 μg/ml)	DS (50 μg/ml)	AZT (0.5 μg/ml)
Molt-4	5			
Molt-4/HTLV-III_b	9			
Molt-4 + Molt-4/HTLV-III_b	58	6	12	74
Molt-4/ARV	9			
Molt-4 + Molt-4/ARV	85	8	9	69
Molt-4/0650	6			
Molt-4 + Molt-4/0650	56	15	7	64

HIV-infected and mock-infected Molt-4 cells were cultured individually or in combination at the ratio of 3 to 7 for 72 h in the presence of PM-19, 50 μg/ml; Dextran sulfate (DS), 50 μg/ml; or AZT, 0.5 μg/ml. Each culture was established with 2.5×10^5 cells in a total volume of 1 ml.

Effect of the combination of PM-19 and AZT on HIV-1 replication

In Fig. 4, PM-19 and AZT were employed at the concentrations of 0.625–20 μM (or 1.84–58.8 μg/ml) and 1.25–40 nM (or 0.00034–0.01 μg/ml), respectively. The ratio of PM-19 and AZT in combination experiments was fixed at 500:1 on a molar basis. At this ratio, both drugs protect MT-4 cells from HIV-1-infection to the same extent. The CI values calculated by a non-exclusive formula indicate that PM-19 potentiates AZT against HIV-1 in vitro.

Similarly the combined toxic effect on MT-4 cells was evaluated at the higher concentrations in the vicinity of their CC_{50} values; the ratio of PM-19 and AZT in combination experiments was also fixed at 500:1. The results indicate that both drugs are not synergistic in terms of cytotoxicity since the CI values are higher than 1 (data not shown).

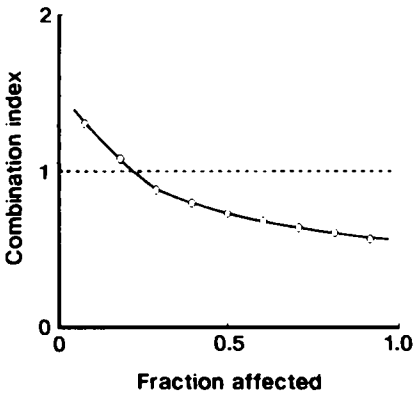

Fig. 4. Effect of the combination of PM-19 and AZT on the replication of HIV-1 in MT-4 cells. The drug combination index (CI) with respect to the fraction affected for the inhibition of the cytopathic effect of HIV-1 by a mixture of PM-19 and AZT (molar ratio, 500:1) is shown.

Discussion

We studied 17 heteropolyoxometalates consisting of 15 HPOTs and 2 HPOMs in the assay system for anti-HIV activity and found only HPOTs with either the Keggin structure or its deficient dimer structure to be potent inhibitors of HIV-1 replication. As shown in Fig. 1, the Keggin compounds are nearly spherical with a diameter of approximately 10 Å. These morphological properties are shared by all Keggin HPOTs and HPOMs listed in Table 1. In contrast, 21-tungsto-9-antimoniates which contain three lobes equivalent to each other through the threefold axis are much bulkier (ca. 18 Å in diameter) (Fischer et al., 1976). The faces of the bicyclic cavity surrounding the central ion (e.g., sodium in the case of HPA 23) are so large that it might be possible to exchange central ions through the faces (Fischer et al., 1976). In fact, the compounds in this group show quite similar biological properties; poor anti-HIV activity and marked cytotoxicity. The anti-HIV activity of 40-tungsto-4-arsenate PM-3 (20 Å in diameter) is moderate. One or two tungsten atoms in the Keggin structure [$XW_{12}O_{40}$] (X is a central atom) can be replaced with transition metal ions to form ternary heteropolyoxotungstates (PM-19, PM-43, PM-44, PM-46, PM-47 in Table 1), among which PM-19 is unique having two tungsten atoms replaced. It remains to be solved, however, whether these structural features of PM-19 are related to its anti-HIV index. These findings suggest that the anti-HIV activity of heteropolyoxometalate is determined by many factors such as shape, size, surface charge, species of polyanions, etc.

PM-19 inhibited HIV-1 replication by intervening in the early steps before virus penetration into target cells in vitro, as in the case of DS (Mitsuya et al., 1988; Baba et al., 1988). Like DS, PM-19 inhibited syncytium formation caused by viral spread from cell to cell.

Recent studies have explored drug combinations against HIV-1 in vitro (Hartshorn et al., 1986, 1987; Mitsuya et al., 1987b; Resnick et al., 1986; Vogt et al., 1987). Although many combinations show synergistic interactions, combinations of ribavirin and pyrimidine 2′,3′-dideoxynucleosides such as AZT, 2′,3′-dideoxythymidin-2′-ene, 2′,3′-dideoxycytidine and 2′,3′-dideoxycytidin-2′-ene (Vogt et al., 1987; Baba et al., 1987), and recombinant soluble CD4 and DS (Hayashi et al., 1990) are antagonistic. Some compounds, such as recombinant alpha A interferon and acyclovir, potentiate AZT against HIV-1 in vitro (Hartshorn et al., 1987; Mitsuya et al., 1987a), but DS is simply additive with AZT (Ueno et al., 1987). PM-19 potentiates AZT against HIV-1 in vitro, but does not potentiate cytotoxicity against MT-4 cells. The combined administration of PM-19 and AZT could be expected to enhance the anti-HIV-1 effect without increasing the toxic side effects.

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