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Broad spectrum anti-RNA virus activities of titanium and vanadium substituted polyoxotungstates

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Abstract

Seven polyoxotungstates substituted with vanadium or titanium atoms were examined for their activity against Flaviviridae (Dengue fever virus, DFV), Orthomyxoviridae (influenza virus type A, fluV-A), Paramyxoviridae (respiratory syncytial virus, RSV, parainfluenza virus type 2, PfluV-2 and canine distemper virus, CDV) and Lentiviridae (human immunodeficiency virus type 1, HIV-1) families. Among the seven polyoxotungstates examined, PM-43 $\{K_5[SiVW_{11}O_{40}]\}$, PM-47 $\{K_7[BVW_{11}O_{40}]\}$, and PM-1001 $[K_{10}Na(VO)_3(SbW_9O_{33})_2]26H_2O$ contained vanadium. PM-1002 had the same core structure of $(VO)_3(SbW_9O_{33})_2$ as PM-1001; however, three V atoms of PM-1001 consisted of two V^{IV} and one V^{V} and those of PM-1002 consisted of three V^{IV}. On the other hand, PM-518 $\{[Et_2NH_2]_7[PTi_2W_{10}O_{40}]\}$, PM-520 $[Pri_2NH_2]_5[PTiW_{11}O_{40}]$ and PM-523 $[PriNH_3]_6H[PTi_2W_{10}O_{38}(O_2)_2]H_2O$ all contained titanium. All compounds showed broad spectrum antiviral activity against all viruses examined except for PMs-43, -518 and -523 which did not exhibit inhibitory activity at $\geq$50 μM against PfluV-2, CDV and DFV, respectively. All compounds were inhibitory against HIV replication at an EC_{50} of less than 2.0 μM. Among them, PMs-1001 and -1002 showed the most potent inhibition. The compounds were not toxic for MDCK, HEp-2 and Vero cells at a concentration of 200 μM. For the exponentially growing MT-4 cells, the vanadium containing polyoxometalates (PMs-43, 47, 1001, 1002) showed toxicity at concentrations between 41 and 47 μM. On the other hand, titanium containing polyoxometalates (PMs-518, -520, -523) were not toxic at 100 μM. The mechanism of anti-HIV action of PM-1001 was analyzed: it affected the binding of HIV to the cell membrane and syncytium formation between HIV-infected and uninfected cells.

Keywords: Polyoxometalate; Antiviral; RNA virus; HIV; Dengue fever virus

1. Introduction

Polyoxometalates are negatively charged inorganic substances which contain early transitional metal ions such as tungsten (W), molybdenum (Mo) niobium (Nb), antimony (Sb), vanadium (V) and so on, and which make a cluster with the surrounding oxygen atoms. Several polyoxometalates have been reported to inhibit the replication of the human immunodeficiency virus (HIV), herpes simplex virus (HSV), influenza virus (fluV) and respiratory syncytial virus (RSV) (Yamamoto et al., 1992; Inouye et al., 1995; Ikeda et al., 1993; Huffman et al., 1997; Barnard et al., 1997; Rhule et al., 1998). We have previously reported that several Keggin (or Keggins sandwich) types of polyoxotungstates, i.e. $Na_{16}Fe_4(H_2O)_2$ $(P_2W_{15}O_{56})_2nH_2O$ (HS-054), $K_{10}Fe_4(H_2O)_2$ $(PW_9O_{34})_2H_2O$ (HS-058), K_9H_5 $(Ge_2Ti_6W_{18}O_{77})16H_2O$ (PM-504), and $(PriNH_3)_6H[PTi_2W_{10}O_{38}(O_2)_2]H_2O$ (PM-523) show potent activity against orthomyxo, praramyxoviruses and HIV (Shigeta et al., 1995, 1996, 1997).

Antibacterial activity was reported for the Keggin structure along with its lacunary species of polyoxotungstates against methicillin-resistant *Staphylococcus aureus* (MRSA) (Fukuda et al., 1999). Similarly, vanadium-containing polyoxometalates were reported to be inhibitory against the growth of *Streptococcus pneumoniae* (Fukuda and Yamase, 1997). Titanium seems also to be an important metal ion to afford antiviral action of polyoxotungstate in mice (Ikeda et al., 1993). Thus, we examined the antiviral activity of

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polyoxotungstates with a Keggin type structure and vanadium or titanium substituents.

2. Materials and methods

2.1. Compounds and antibodies

The polyoxometalates assayed for antiviral activity and cytotoxicity were synthesized and analyzed for chemical characteristics according to procedures published elsewhere. (Yamase et al., 1992, 1993, 2001, 2002; Ishikawa and Yamase, 1999). These compounds were fully characterized by chemical analysis, NMR, and infra-red spectroscopy. Dextran sulfate (DS, mol. wt. 5000) was purchased from Sigma Chemical Co. (St. Louis, MO). The bicyclam AMD3100 [1′,1′-(1,4-phenyl-bis(1,4,8,11 tetracyclostetradecane) octahydrochloride dihydrate) which is known as an inhibitor of the binding of gp120 to CXCR4 was provided by Dr. De Clercq, Catholic University of Leuven, Belgium. Ribavirin was provided by Yamasa Co. (Choshi, Chiba, Japan). 3′-azido-3′-deoxythymine (AZT) was a gift from GlaxoSmithKline Co., Tsukuba Research Center (Tsukuba, Ibaragi, Japan). Stock solutions of compounds were freshly prepared in water. Anti-gp120 antibody (goat) was polyclonal and conjugated with fluorescein isothiocyanate (FITC). It was purchased from Virostat Inc. (Portland, ME). Anti-CD4 and anti-CXCR4 antibodies were murine monoclonal antibodies. The former was purchased from Immumodiagnosis, Inc, Woburn, MA (AG-020096) and the latter was a product of R&D System, Minneapolis, MN (12G5). FITC conjugated anti-mouse-IgG antibody (goat) was a product of Serotec Ltd. (Oxford, UK).

2.2. Viruses & cells

FluV-A Ishikawa/7/82 (H3N2) was passed more than five times in MDCK cells prior to being used for the virus growth inhibition experiments in MDCK cells. RSV, Long strain was passed in HEp-2 cells more than 20 times and PfluV-2, Greer strain was passed 3 times in HMV cells (a human melanoma cell line) before use. CDV, Onderstepoort strain was provided by Dr. Kai of Tokyo University and passed five times in Vero cells. The Dengue fever virus (DFV) type 2 (9701021 strain) was provided by Dr. Kurane, National Institute of Infectious Diseases of Japan and passed in Vero cells more than 5 times. All cell lines which had been used for the passage of each virus were also used as the host cell of the virus infection in the antiviral experiments. The HIV-1, IIIb strain was passed more than 10 times in MOLT-4 cells before use. MT-4 and HeLa CD4-LTR/β-gal cells were used for HIV infection. MOLT-4/IIIb cells (persistently HIV-1 infected MOLT-4 cells) were used for the binding assay of anti-gp120 antibody and gp120, and also for the synsytium formation between HIV-infected and uninfected MOLT-4 cells.

HEp-2 and Vero cells were cultured in Eagle's minimal essential medium (MEM) supplemented with a 10% heat-inactivated newborn calf serum, 100 U of penicillin G and 100 μg of streptomycin per ml (growth medium). HMV-2, MT-4, MOLT-4 and MOLT-4/IIIb cells were cultured in RPMI1640 supplemented with the same percent of heat-inactivated newborn calf serum and antibiotics. For virus infection, newborn calf serum in medium was substituted with 2% bovine albumin for fluV or 2% heat inactivated fetal calf serum (FCS) for the other viruses (maintenance medium). HeLa CD4-LTR/β-gal cells were kindly provided by Dr. Emerman through the AIDS research and Reference Reagent Program, Division of AIDS, National Institute of Allergy an Infectious Diseases. (Bethesda Md.). Prior to use, HeLa CD4-LTR/β-gal cells were propagated in MEM supplemented with 10% FCS, 0.1 ng of hygromycin B per ml and 200 μg of Geneticin per ml. For the anti-HIV assays, cells were cultured in MEM with the addition of 50 U of penicillin G and 50 μg of streptomycin per ml.

2.3. Antiviral assay

The evaluation of antiviral activity was based on the MTT assay as previously described (Pauwels et al., 1988; Watanabe et al., 1994; Mori et al., 1995; Shigeta et al., 1995), except for CDV which was examined by the plaque reduction method. Briefly, a four-fold dilution of compound (100 μl) was prepared in a 96-well tissue culture tray (Nunclon, 96-wells, Nunc A/S Roskilde, Denmark), using a maintenance medium with 4-wells for each dilution. To each well, 5×10^3 cells (in 50 μl maintenance medium) and 100 $CCID_{50}$ of virus (in 50 μl maintenance medium) was added. The plate inoculated with fluV, PfluV, RSV and DFV (except of HIV) was centrifuged at $700 \times g$ for 5 min and all trays were incubated at 35 °C for 4–5 days. During the incubation, the culture medium with or without compound was changed after 3 days with fresh medium containing the same concentration of compounds as before. To determine the median effective concentration (EC_{50}), 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a concentration of 7 mg/ml in phosphate-buffered saline (PBS, pH 7.2) was added to each well. The mixture was incubated at 37 °C for 2 h and the reduced MTT (formazan) was extracted by adding 100 μl of acidic isopropanol containing 4% Triton-X. The absorbance of blue formazan color was measured using a computer-controlled microplate reader (Bio Rad, Model 3550, Hercules, CA) at two different wavelengths (540 and 690 nm). Thus, the viability of mock- and virus-infected cells was evaluated by the absorbance of the color of formazan. The EC_{50} value was expressed as the concentration that achieved a 50% reduction of virus-infected cells from virus-induced destruction. The percentage protection was calculated using the following formula: $[(ODT)V-(ODC)V]/[(ODC)M-(ODC)V] \times 100$ where (ODT)V, (ODC)V and (ODC)M indicate the absorbance of the test sample, the virus-infected control (no

compound), and the mock-infected control (no virus and no compound), respectively.

Anti-HIV activity was also determined in the multinuclear activation of the galactosidase indicator (MAGI) assay (Kimpton and Emerman, 1992) with slight modification. Briefly, HeLa CD4-LTR/β-gal cells were plated in 96-well flat microtiter culture plates (10^4 cell per well). On the following day, the medium was aspirated and the cells were inoculated with HIV III_B strain (70 MAGI units which gave 70 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of drugs in fresh medium. At 48 h after viral exposure, all blue cells in each well were counted. EC_{50} and EC_{90} were determined as the concentrations of the compound which inhibited the appearance of blue cells to 50% or 10% of the control, respectively.

2.4. Binding inhibition assay

The inhibitory effects of the compounds on the binding of anti-CD4, anti-CXCR4 and anti-gp120 antibodies were analyzed using FACScan (Becton Dickinson). One million cells each of MT-4 (in the cases of anti-CD4 and anti-CXCR4) or MOLT-4/IIIb (in the case of anti-gp120) were treated with a determined concentration of the compounds for 30 min at 37 °C and exposed to the antibodies for 60 min at 4 °C. Then the cells were washed twice with PBS and MT-4 cells were again incubated with a FITC-conjugated anti-mouse IgG antibody for 30 min at 4 °C. The cells were washed twice again with PBS, fixed with 1% formamide in PBS, and subjected to FACScan analysis.

2.5. Syncytium formation inhibition assay

MOLT-4 cells (5×10^5 cells ml^{-1}) were cultured with an equal volume of MOLT-4/IIIb cells in a 96-well tray containing various concentrations of the test compounds. After a 24-h cocultivation period, the number of giant cells (syncytia) was recorded microscopically, as previously described (Baba et al., 1990).

2.6. Cytotoxicity

Cytotoxicity of the compounds was examined by the determination of cell viability by the MTT method 5 days after the treatment with the compounds. MDCK, HEp-2, Vero cells and HeLa CD4-LTR/β-gal cells were examined at stationary conditions (cultured at 35 °C with maintenance medium, number of viable cells increased 1.3–1.7 times between the beginning and the end of study). On the other hand, MT-4 cells were examined under exponentially growing conditions (cultured at 37 °C with growth medium, cell number increased 5.8 times). The median cytotoxic concentration was determined from the dose–response curve.

3. Results

3.1. Activities of PM-compounds against several RNA viruses

All PM-compounds examined were inhibitory against Flaviviridae (DFV), Orthomyxoviridae (fluV-A), Paramyxoviridae (RSV, PfluV-2, CDV) and Lentiviridae (HIV-1), except for PM-43 which was not inhibitory against PfluV-2; PM-518, which was not inhibitory against CDV; and PM-523, which was not inhibitory against DFV. The compounds which showed antiviral activity at a concentration of <1 μM were PMs-43, 47 versus HIV-1, PM-520 versus RSV, PM-523 versus HIV-1, PM-1001 versus DFV, RSV, HIV-1 and PM-1002 versus RSV, PfluV-2, HIV-1. All compounds showed anti-HIV activity at 2.0 μM or lower concentrations. Among the compounds, PMs-1001 and -1002 showed the most broad spectrum and potent antiviral activities against RNA viruses compared with the other PM-compounds (Tables 1 and 2).

In order to examine the anti-HIV activities of PMs-1001 and -1002, EC_{50} values were determined by the MTT method using MT-4 cells and the MAGI method using HeLa CD4/LTR-β-Gal cells. EC_{90} values were determined only by the latter method. As shown in Table 3, antiviral activities of PMs-1001 and -1002 were almost comparable with or slightly greater than DS5000 and AZT. However, EC_{50} and EC_{90} values of both PM-compounds were higher than those of AMD3100. Selectivity indices (ratios of CC_{50} versus EC_{50}) were 304 and 1530 for PMs-1001 and -1002, respectively, by the MTT method and >10417 and >5556, respectively, by the MAGI assay.

3.2. Cytotoxicity of PM-compounds

All PM-compounds were examined for cytotoxicity towards MDCK, Vero, HEp-2 and MT-4 cells using the MTT method. All compounds did not show toxicity at concentrations up to 200 μM MDCK, Vero and HEp-2 cells. These cells were in a stationary culture condition of cell growth

Table 1
Chemical structure of polyoxotungstates

Compound[a]	Chemical formula	Species of structure
PM-43	$K_5[SiVW_{11}O_{40}]$	Keggin
PM-47	$K_7[BVW_{11}O_{40}]$	Keggin
PM-518	$[Et_2NH_2]_7[PTi_2W_{10}O_{40}]$	Keggin
PM-520	$[Pri_2NH_2]_5[PTiW_{11}O_{40}]$	Keggin
PM-523	$[PriNH_3]_6H[PTi_2W_{10}O_{38}(O_2)_2]H_2O$	Keggin
PM-1001[b]	$K_{10}Na[(VO)_3(SbW_9O_{33})_2]26H_2O$	Keggin sandwich
PM-1002[b]	$K_{11}H[(VO)_3(SbW_9O_{33})_2]27H_2O$	Keggin sandwich

[a] PM-518, PM-520 and PM-523 possess a Ti atom and PM-43, PM-47 and PM-1001 possess a V atom.

[b] PM-1001 and PM-1002 have the same core structure of $(VO)_3(SiW_9O_{33})_2$, however, V_3 of PM-1001 consists of two V^{IV} and one V^{V} and those of PM-1002 consists of three V^{IV}.

Table 2
EC_{50} (μM) of PM-compounds for several RNA viruses in vitro

Compound	EC_{50} (μM)[a]					
	DFV[b] Vero cells	FluV-A[b] MDCK cells	RSV[b] HEp-2 cells	PfluV-2[b] HMV-2 cells	CDV[c] Vero cells	HIV-1[b] MT-4 cells
PM-43	10.7 ± 6.7	8.4 ± 6.5	1.6[d]	>100	7.5 ± 0.95	0.3 ± 0.12
PM-47	10.5 ± 6.9	11.5 ± 0.6	29.0[d]	67.1[d]	6.0 ± 0.6	0.03 ± 0.01
PM-518	36.8[a]	62.3 ± 26.5	26.5[d]	53.2 ± 39.2	>50	2.0 ± 0.8
PM-520	11.7 ± 7.1	45.2 ± 25.8	0.74 ± 0.58	23.2 ± 2.4	7.4 ± 0.4	2.0 ± 0.5
PM-523	>61.5	5.6 ± 2.0	1.3 ± 0.46	2.5 ± 1.0	7.3 ± 1.1	0.3 ± 0.07
PM-1001	0.45[a]	1.75 ± 1.6	<0.16	1.1 ± 0.9	5.7 ± 0.5	0.14 ± 0.17
PM-1002	1.95 ± 1.4	4.6 ± 1.7	0.75 ± 0.05	0.75 ± 0.05	2.8 ± 1.0[d]	0.03 ± 0.01
Ribavirin	>100	5.0 ± 2.75	3.9 ± 3.1	14.0 ± 4.8	73.6 ± 34.5	Nd[e]

[a] Average values for 3–7 independent experiments.
[b] C_{50} was determined by the MTT method.
[c] EC_{50} was determined by the plaque reduction method.
[d] Average of two experiments.
[e] Not determined.

Table 3
Anti-HIV activity of PM-1001 and PM-1002

Cell[a]	Compound	EC_{50} (μM)[b]	EC_{90} (μM)[b]	CC_{50}[b](μM)	Si[c]
MT-4	PM-1001	0.14 ± 0.07	ND[d]	41.9 ± 2.9	304
	PM1002	0.03 ± 0.01	ND	45.9 ± 0.3	1530
	DS5000	0.65 ± 0.29	ND	>20	>30.8
	AMD3100	ND	ND	ND	ND
	AZT	0.032	ND	29.4 ± 8.4	919
HeLa CD4/LTR-β-Gal cells	PM-1001	0.0096 ± 0.0056	0.11 ± 0.06	>100	>10417
	PM-1002	0.018 ± 0.007	0.11 ± 0.05	>100	>5556
	DS5000	0.006 ± 0.0018	0.13 ± 0.055	>20	>3333
	AMD3100	0.0003 ± 0.0001	0.0036 ± 0.0012	>100	>333000
	AZT	0.037 ± 0.023	0.42 ± 0.33	>100	2703

[a] The results were determined using the MTT colorimetric method for MT-4 cells and MAGI assay for HeLa CD4/LTR-β-Gal cells, respectively.
[b] Each value is from three independent experiments.
[c] Selectivity index (CC_{50}/EC_{50}).
[d] Not determined.

during the examination. On the other hand, when cytotoxicity was examined for MT-4 cells which were in growing state of culture during the examination, PMs-43, -47, -1001 and -1002 all of which contain vanadium atoms, showed toxicity at concentrations of 41.9–47.4 μM (Table 4).

3.3. Time of drug addition experiment of PMs-1001 and -1002

The influence of the time-of-addition of compounds on the inhibitory effects against HIV infection in HeLa

Table 4
Cytotoxicity of polyoxotungstates for cell cultures

Compound	CC_{50} (μM)[a]			
	MDCK	Vero	HEp-2	MT-4
PM-43	>200	>200	>200	45.6 ± 0.43
PM-47	>200	>200	>200	47.4 ± 0.48
PM-518	>200	>200	>200	>100
PM-520	>200	>200	>200	>100
PM-523	>400	>400	>400	>100
PM-1001	>229.2 ± 36	362.7 ± 38.1	233.6 ± 21.5	41.9 ± 2.9
PM-1002	>200	>200	>200	45.9 ± 0.3

[a] Average values for four independent experiments. CC_{50}s were determined by the MTT method.

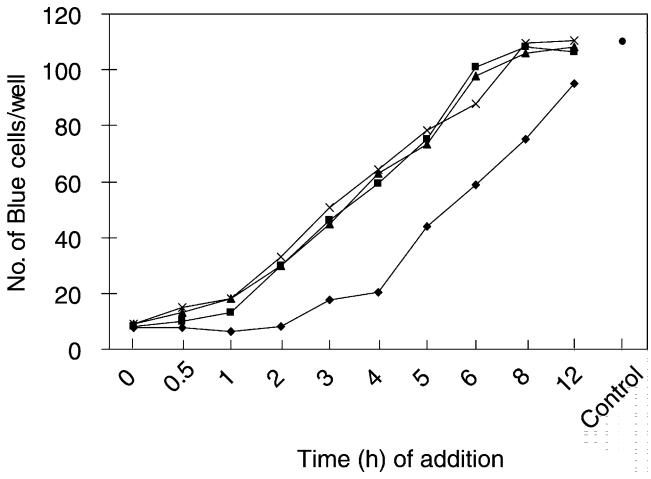

Fig. 1. Time of drug addition assay for HIV infection in HeLa CD4/LTR-β-Gal cells. Ten thousand HeLa CD4/LTR-β-Gal cells were inoculated with 100 MAGI units of HIV-1. Immediately after the infection and at the time indicated, 10 EC_{50} of each of the compounds was added to the culture. At 48 h after infection, the number of blue colored cells was counted. (■), DS5000; (▲), PM-1001; (×), PM-1002; (◆), AZT were added. (●) indicates an untreated control.

CD4/LTR-β-Gal cells was examined for PMs-1001 and -1002. One hundred MAGI units of HIV were inoculated onto HeLa CD4/LTR-β-Gal cells, and both PM-compounds, DS5000 and AZT were added to the culture immediately after and at the hours indicated in Fig. 1. At 48 h after the virus inoculation, the number of blue cells were counted by the MAGI method. When the compounds were added immediately after the virus inoculation, the appearance of blue cells was almost completely inhibited. When they were added at 4 h after virus inoculation, the number of blue cells recovered from 54 to 58% of the untreated control in the case of PMs-1001, -1002 and DS5000, whereas, it was suppressed to 19% in the case of AZT. When treatment by PM-compounds or DS5000 started 8 h after virus inoculation, the number of blue cells was almost the same as the control, whereas it was 68% following AZT treatment (Fig. 1). Thus, the antiviral effects of PMs-1001, -1002, and DS5000 were expressed in the early stage of virus infection and assumed to inhibit virus adsorption or entry into the cells.

3.4. Inhibitory effect of PMs-1001 and -1002 on sycytium formation by HIV

When PMs-1001 and -1002 were examined for their inhibitory effects on the formation of syncytia (multinucleated giant cell formation induced by cocultivation of MOLT-4 cells and MOLT-4/IIIb cells) at concentrations of 10 and 2 μM, they completely inhibited syncytium formation at 10 μM. At 2 μM, 40 and 60% inhibition was observed by PMs-1001 and -1002, respectively.

Table 5
Inhibitory effect of DS5000, PM-1001 and AMD-3100 on binding of antibodies to CD4, CXCR4 and gp120

Compound (10 μM)	Antibody	% inhibition[c]
DS5000	Anti-CD4[a]	<5
	Anti-CXCR4[a]	<5
	Anti-gp120[b]	27.0
PM-1001	Anti-CD4	<5
	Anti-CXCR4	<5
	Anti-gp120	97.0
AMD3100	Anti-CXCR4	98.0

A representative result is shown; (a) for the binding assay of anti-CD4 and anti-CXCR4 antibodies, MT-4 cells were used; (b) for the binding assay of anti-gp120 antibody, Molt-4/IIIb cells were used; (c) Percent of the antibody-bound cells were determined by FACScan analysis.

3.5. Inhibitory effect of PM-1001 on the binding of antibodies to CD4, CXCR4 and gp120

In order to analyze the target molecule to which PM-1001 interacts with during the adsorption/penetration process, we examined the inhibitory effect of PM-1001 against the binding of the antibodies to CD4, CXCR4 and gp120. We used mouse anti-CD4 and anti-CXCR4 monoclonal antibodies along with mouse polyclonal anti-gp120 antibody, and we examined its adsorption to MT-4 cells or MOLT-4/IIIb cells by FACScan analysis. As a result, PM-1001 and DS5000 inhibited interaction between gp120 and its antibody, whereas, they did not inhibit interactions between HIV receptors (CD4 and CXCR4) and their antibodies. PM-1001 as well as DS5000 may be inhibitory for the interaction of gp120 with its receptor(s), but the binding site was shown to be gp120. On the other hand, AMD3100 exhibited anti-HIV activity by binding to CXCR4 (Table 5).

4. Discussion

Polyoxometalates have been proven to inhibit the replication of several enveloped DNA and RNA viruses. The antiviral effect of PM-523 was shown to cover broad spectrum for orthomyxoviruses and paramyxoviruses (Shigeta et al., 1996). In this study, we examined seven polyoxometalates including four vanadium-containing compounds and three titanium containing compounds. All compounds were inhibitory against the replication of DFV, fluV-A, RSV, PfluV-2, CDV and HIV-1 with a few exceptions. The exceptions were PMs-43, -518 and -523 which were not inhibitory towards PfluV-2, CDV and DFV, respectively, at ≥50 μM.

All compounds were also inhibitory to the replication of HIV-1(IIIb) at concentrations between 0.03 and 2.0 μM. Among them, two Tris (vanadyl)-substituted tungstoantimonates (PMs-1001 and -1002) were potent inhibitors of HIV-1. The EC_{50} values of these compounds were 0.03–0.14 μM for infected MT-4 cells (by MTT method) and 0.0096–0.018 μM for HeLa CD4/LTR-β-Gal cells (by

MAGI assay). Both compounds were also potent inhibitors of several enveloped RNA viruses in addition to HIV-1. They inhibited DFV replication at 0.45–1.95 μM and RSV replication at 0.16–0.75 μM. Both DFV and RSV have been known as clinically important pathogens. The former is a causative agent of Dengue fever, which has been reported recently to migrate from the tropical zone to the temperate zone. The latter is the agent of a respiratory infection that causes bronchiolitis and pneumonia among infants.

Anti-HIV activity of PMs-1001 and -1002 was examined using MAGI assay, a more sensitive method for the detection of anti-HIV activity. The EC_{90}s of the compounds for HeLa CD4 /LTR-β-Gal in the MAGI assay were 0.11 μM. Selectivity indices (CC_{50}/EC_{50}) of PMs-1001 and -1002 for HIV-1 were >10,000 and 5500, respectively. In order to analyze the mechanism of anti-HIV-1 activity of PMs-1001 and -1002, a time-of-addition experiment was performed and it revealed that both compounds as well as DS5000 and AMD3100 acted at a very early stage of virus infection (<2 h post infection). When we examined the inhibitory effect of PM-1001 against the bindings of specific antibodies to gp120 and to the receptors, PM-1001 strongly inhibited the binding of anti-gp120 antibody to HIV-1 infected MT-4 cells, whereas it did not inhibit binding of anti-CD4 and anti-CXCR4 antibodies to Molt-4 cells. This result indicates that PM-1001 binds to viral gp120 and interferes with the interaction between gp120 with its receptors (CD4 and CXCR4), whereas it does not bind to either CD4 or CXCR4 directly. Several types of polyoxotungstates which have been known as "JM" series were reported to inhibit the binding of anti-gp120 antibody to the persistently HIV-1 infected HUT-78 cells. The inhibitory activity of the compounds for the binding of anti-gp120 antibody to the cells correlated closely with the inhibitory activity for the syncytium formation by HIV-1 (Yamamoto et al., 1992). Thus, polyoxotungstates may be assumed to bind to the HIV-1 gp120 molecule and to interfere with the binding of gp120 to its receptors, thereby inhibiting syncytium formation between HIV-1-infected cells and uninfected cells. PM-19, a Keggin type, titanium containing polyoxotungstate [$K_7(PTi_2W_{10}O_{40})6H_2O$] was also reported to inhibit the syncytium formation by HIV-1 (Inouye et al., 1995).

We have previously demonstrated that polyoxotungstates inhibited fusion between the viral envelope (or virus-infected cellular membrane) and uninfected cellular membrane. The mechanism of anti-fluV-A activity of PM-523 [$PriNH_3$]6H[$PTi_2W_{10}O38(0_2)_2$]H_2O, proved to be the inhibition of the fusion between the viral envelope and cellular membrane by the inhibition of the dequenching of fluorescence from the rhodamin labeled viral envelope (Shigeta et al., 1996). Another polyoxotangstate HS-058 [$K_{10}Fe_4(H_2O)_2(PW_9O_{34})_2$ nH_2O] was also shown to inhibit both adsorption of RSV to cells and syncytium formation by RSV (Shigeta et al., 1995). It is conceivable that PM-523, which was reported to show catalytic activity for the epoxidation of carbohydrate (Ishikawa and Yamase, 1999), may act as the inhibitor of membrane fusion.

In this study, titanium-containing polyoxometalates were less toxic for actively growing cells than their vanadium-containing counterparts. PMs-19 and -523 are Keggin type, titanium containing polyoxometalates. PM-19 was reported to be inhibitory for HSV-2 infection in immunosuppressed mice and PM-523 showed therapeutic effect for fluV-A respiratory infection in mice. The former enhanced peritoneal macrophage function in mice and the latter exhibited a synergistic antiviral activity in combination with ribavirin (Ikeda et al., 1993; Shigeta et al., 1997). Thus, titanium-containing polyoxometalates may be safe and potent for therapeutic use in vivo virus infections. In conclusion, titanium- and vanadium-containing polyoxotungstates proved to be potent inhibitors of a variety of enveloped RNA viruses such as DFV, FluV-A, PfluV-2, CDV, RSV and HIV-1. The mechanism of anti-HIV activity of a vanadium-containing tungstoantimonate (PM-1001) resides in the inhibition of the binding of gp120 to the cell membrane.

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