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Potent inhibition of respiratory syncytial virus by polyoxometalates of several structural classes

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Abstract

A series of polyoxometalates (POM) were synthesized and evaluated for anti-respiratory syncytial virus (RSV) activity. POM containing zirconium, tungsten, silicon, platinum, niobium or germanium of a variety of structural types have been evaluated. Sixteen of the compounds had very striking anti-RSV activity against a clinical isolate, Utah 89, with median effective concentration (EC_{50}) values $\leq 3\ \mu M$ and selective indices > 80 as determined by viral cytopathic inhibition effect, neutral red uptake and virus yield reduction assays. The EC_{50} values for all three assays correlated very well (Pearson correlation coefficients > 0.90). POM containing sodium cations were totally inactive. Germanium-, niobium-, tin- and zirconium-containing compounds were found to be highly potent and selective. The antiviral activity was not cell line-dependent. The median cytotoxic concentration (IC_{50}) values were generally greater than 100 μM. The compounds were also comparably active against a known laboratory RSV strain, A2, as well as other RSV strains. To detect any virus strain-specific inhibitory activity, seven POM were tested against other RSV strains; all were nearly equally inhibitory to the human virus strains, suggesting no strain specificity. Timing studies suggested that these compounds were most inhibitory during virus adsorption and penetration, although RSV was still significantly inhibited when the compound was added 3 h post-infection; which is considered well into the eclipse period. These data suggest that these potent, non-toxic compounds should be further studied as potential chemotherapeutic agents for treating RSV infections. © 1997 Elsevier Science B.V.

Keywords: Respiratory syncytial virus; Polyoxometalate; Antiviral; Cytopathic inhibition assay; Neutral red uptake assay

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1. Introduction

Polyoxometalates (POM) are heteropolyanions belonging to a class of polynuclear coordination complexes formed by transition metals of the chromium and vanadium series Fukuma et al., 1991. Many of them possess a cryptate structure with an alkaline or alkaline-earth cation in the central cage and charges on all oxygen atoms (Fisher et al., 1976). Ammonium 21-tungsto-9-antimoniate [HPA-23, $(NH_4)_{17}Na(NaSb_9W_{21}O_{86})$] has shown a wide spectrum of activity against both DNA and RNA viruses (Jasmin et al., 1973, 1974; Fisher et al., 1976). Recently, other POM have been reported to have RNA and DNA virus inhibitory activity (Shigeta et al., 1995a; Ikeda et al., 1993; Fukuma et al., 1991; Yamamoto et al., 1992). POM with Keggin-type structures have been shown to be inhibitors of arenaviruses (Yamamoto et al., 1992), human immuno-deficiency virus types 1 and 2 (Ikeda et al., 1993; Yamamoto et al., 1992; Inouye et al., 1991, 1992; Take et al., 1991, Hill et al., 1990a) and other retroviruses (Yamamoto et al., 1992), HCMV (Ikeda et al., 1993), herpes simplex viruses (Fukuma et al., 1991), thymidine kinase deficient herpes simplex virus (Ikeda et al., 1993), influenza viruses (Shigeta et al., 1995a,b), measles virus (Shigeta et al., 1995b), parainfluenza virus (Shigeta et al., 1995b), rhabdovirus (Yamamoto et al., 1992), RSV (Ikeda et al., 1993; Shigeta et al., 1995a,b) and togaviruses (Yamamoto et al., 1992). In addition, Hill et al. (1990b) have reported that silicon-containing POM had greater antiviral activity than the original model compound, HPA-23, that did not contain silicon.

The mechanism of anti-HIV inhibition of these types of compounds has been attributed to inhibition of virus-cell binding and syncytium formation between HIV-infected cells (Yamamoto et al., 1992; Take et al., 1991; Hill et al., 1990b). Similar compounds inhibited binding of HIV-specific gp120 monoclonal antibody to the gp120 protein (Yamamoto et al., 1992) and the anti-HIV activity was attributed to POM binding of gp120, thus blocking virus-mediated fusion and cell to cell spread of virus. Although some compounds inhibited reverse transcriptase (RT), it was found that the RT-inhibitory activity did not correlate with antiviral activity. Ikeda et al. (1993) found that polyoxosilico-tungstates displaced human cytomegalovirus (HCMV) virions bound to a heparin–sepharose matrix as has been demonstrated with sulfonated polymers (Hosoya et al., 1991), suggesting that the POM were able to compete with heparin sulfate for binding to those sites on the virion envelope required for interaction with cell surface heparin sulfate. Because sulfate polymers may interfere with influenza A virus fusion to cells and RSV binding/fusion to cells (Hosoya et al., 1991), the POM may also act in a similar manner to inhibit these two viruses (Shigeta et al., 1995a). Shigeta et al. (1995b) recently found that the POM evaluated for anti-influenza A activity inhibited the penetration of influenza virus, but not virus adsorption. Fukuma et al. (1991) has suggested that PM-19, a heteropolyanion with a Keggin-type structure, not only inhibited herpes simplex virus adsorption, but also a replication step after virus adsorption that influences virus DNA replication, since viral DNA synthesis was inhibited after addition of the compound 1 h post adsorption.

We report on a series of POM that were not cytotoxic and showed striking in vitro anti-respiratory syncytial virus (RSV) activity against a variety of RSV strains.

2. Materials and methods

2.1. Compounds and synthesis

POM were evaluated for antiviral activity upon being submitted to the NIH Antiviral Substance Screening Program for respiratory viruses by R. Schinazi and M. Otto. The structural formulae of the POM and their code numbers are shown in Table 1.

The synthesis of HS-021, -027, -030, -033, -036 has been described by Hill et al. (1990a), HS-052 and HS-053 by Gómez-García et al. (1994), HS-057 by Gómez-García et al. (1993), HS-077, HS-081, and HS-086 by Weeks et al. (1992); HS-106, and JM-2922 by Finke and Droege (1984), HS-112 by Papaconstantinou et al. (1980), JM-1591

Table 1
Structural formulae of POM

POM code	Structural formula	Mol. wt.(kD)	Structural class
HS-021	α-$(NH_4)_nH_{(5-n)}BW_{12}O_{40}$	3110	α-Keggin
HS-027	α-$(NH_4)_5BW_{12}O_{40}$	2947	α-Keggin
HS-030	$(Me_4N)_4W_{10}O_{32}$	2648	W10[a]
HS-033	$(Arg\bullet H)_nH_{(5-n)}BW_{12}O_{40}$	3727	α-Keggin
HS-034	$(His\bullet H)_nH_{(4-n)}SiW_{12}O_{40}$	3494	α-Keggin
HS-036	$(Arg\bullet H)_nH_{(4-n)}SiW_{12}O_{40}$	3226	α-Keggin
HS-052	$Na_{16}Ni_4(H_2O)_2(P_2W_{15}O_{56})_2\bullet nH_2O$	8070	W.D.S.[b]
HS-053	$Na_{16}Mn_4(H_2O)_2(P_2W_{15}O_{56})_2\bullet nH_2O$	8055	W.D.S.[b]
HS-054	$Na_{16}Fe_4(H_2O)_2(P_2W_{15}O_{56})_2\bullet nH_2O$	8059	W.D.S.[b]
HS-057	$K_{10}Mn_4(H_2O)_2(PW_9O_{34})_2\bullet nH_2O$	5106	K.S.[c]
HS-058	$K_{10}Fe_4(H_2O)_2(PW_9O_{34})_2\bullet nH2O$	5110	K.S.[c]
HS-071	$(Arg\bullet H)_nNa_{7-n}PTi_2W_{10}O_{40}$	3827	Keggin
HS-077	$[(CH_3)_4N]_4[(NC(CH_2)_3Si)_2O]SiW_{11}O_{39}$	3070	O.S.K[d]
HS-081	$Cs_4[(Cl(CH_2)_3Si)_2O]SiW_{11}O_{39}$	3433	O.S.K[d]
HS-086	$[(CH_3)_4N]_4[(CH_2=CHSi)_2O]SiW_{11}O_{39}$	3097	O.S.K[d]
HS-093	$K_5[(CH_3)_2Si]PW_{11}O_{39}$	2931	O.S.K.[d]
HS-098	$K_6[(CH_3)_2Si]SiW_{11}O_{39}$	3045	O.S.K.[d]
HS-106	$(Me_3NH)_8Si_2W_{18}Nb_6O_{77}\bullet nH_2O$	5636	D.K.[e]
HS-112	$K_6P_2Mo_{18}O_{62}\bullet nH_2O$	3016	W.D.S.[b]
HS-116	$(Lysine\bullet H)_7KSi_2W_{18}Nb_6O_{72}\bullet 18H_2O$	6549	D.K.[e]
HS-158	$K_{12}Nb_6P_2W_{12}O_{62}$	4287	H.S.D.W.[f]
JM-1591	$K_{12}[H_2P_2W_{12}O_{48}]\bullet 24H_2O$	3940	W.D.H.D.[g]
JM-2768	$K_7H[Nb_6O_{19}]\bullet 13H_2O$	1370	H.M.[h]
JM-2775	$[Me_4N/Na/K]_4[Nb_2W_4O_{19}]$	1571	H.M.[h]
JM-2879	$Na_5[CH_3Sn(Nb_6O_{19})]\bullet 10H_2O$	1290	O.S.K.[d]
JM-2919	$(Me_3NH)_{10}(H)[Si_2(ZrOH)_3W_{18}O_{68}]\bullet 10H_2O$	5597	Barrel
JM-2921	$K_7[A\text{-}\alpha\text{-}GeNb_3W_9O_{40}]\bullet 18H_2O$	3244	α-Keggin
JM-2922	$K_7[A\text{-}\beta\text{-}SiNb_3W_9O_{40}]\bullet 20H_2O$	3235	β-Keggin
JM-2926	$K_7(H)[A\text{-}\alpha\text{-}Ge_2Nb_6W_{18}O_{77}]\bullet 18H_2O$	5843	D.K.
JM-2927	$K_8[A\text{-}\beta\text{-}Si_2Nb_6W_{18}O_{77}]$	5468	D.K.

[a] W10, decatungstate.
[b] W.D.S., Wells-Dawson sandwich.
[c] K.S., Keggin sandwich.
[d] O.S.K., organosilyl Keggin.
[e] D.K, double Keggin.
[f] H.S.D.W.K., hexasubstituted Wells-Dawson Keggin.
[g] W.D.H.D., Wells-Dawson hexadefect.
[h] H.M., hexametalate.

by Contant and Tézé (1985), JM-2768 by Filowitz et al. (1979), JM-2919 by Finke et al. (1989) and JM-1591, JM-2775 by Dabbabi and Boyer (1976). HS-071 was made by a modified procedure of Knoth et al. (1983) and JM-2927 by slightly modifying the procedure of Finke and Droege (1984). HS-054, -058, -093, -094, -116, and HS-158, JM-2879, JM-2919, JM-2921 and JM-2926 were synthesized by C L. Hill (unpubl.). Ribavirin was obtained from ICN Pharmaceuticals (Costa Mesa, CA).

2.2. Cells and viruses

Bovine embryonic trachea cells (EBTr) and human larynx epidermoid carcinoma cells (HEp-2 cells) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Embryonic African green monkey kidney cells (MA-104) were obtained from BioWhittaker (Walkersville, MD). All cells were grown in minimal essential medium (MEM, Gibco, Gaithersburg, MD) supplemented

with 0.1% $NaHCO_3$ and 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT). When performing antiviral assays, serum was reduced to 2% and 50 μg/ml gentamicin (Sigma, St. Louis, MO) was added to the medium.

Respiratory syncytial virus (RSV), strains A2, Long and 9320, and bovine respiratory syncytial virus (BRSV, strain 375) were acquired from ATCC. RSV 18537 was obtained from Lee Biomolecular Research Laboratories (San Diego, CA). The Utah 89 strain was provided by the Utah State Diagnostic Laboratory (Salt Lake City, UT).

2.3. Experimental design for evaluating antiviral activity and cytotoxicity of POM

For all assays, stock solutions of compounds were prepared in sterile water. Antiviral activities of the POM were first evaluated by cytopathic effect (CPE) inhibition assay and verified by neutral red uptake assay. The Utah 89 clinical isolate was used as the initial strain for antiviral testing. Virus yield reduction assays were then done to confirm the antiviral activity. Cell yield assays, using actively growing cells, were done to verify the cytotoxicity. Virucidal assays were conducted to determine if the activity resulted from inactivation of infectious virions. Follow-up antiviral studies were done with active POM against other RSV strains. To determine if virus concentration affected POM inhibitory activity, multiplicity of infection studies were performed. Time of addition studies were done to determine the effects of POM on the stages of the virus replication cycle.

2.4. Virucidal assay

Various concentrations of compound were mixed with an equal volume of virus (multiplicity of infection = 0.001) and incubated at 37°C for 1.5 h. Surviving virus was assayed by CPE assay. Each concentration of compound was assayed in quadruplicate. That dosage of compound that reduced virus titer by 99% (EC_{99}) compared to untreated controls was then determined.

2.5. Cytopathic effect (CPE) inhibition assay

CPE inhibition assays for all viruses used in this study were performed as described by Sidwell and Huffman (1971) with slight modifications. Two test compounds per plate at varying concentrations and virus at a multiplicity of infection (MOI) = 0.001 were added to near confluent cell monolayers (1×10^5 cells/well for MA-104 and HEp-2 cells and 5×10^4 cells/well for EBTr cells) and incubated at 37°C until the cells in the control wells showed complete viral CPE as observed by light microscopy (usually 5–6 days). All compounds were assayed for virus inhibition in quadruplicate and for cytotoxicity in duplicate. For each compound, two wells were set aside as uninfected, untreated cell controls per test and four wells per test received virus only and represented controls for virus replication. When testing the effects of MOI on the antiviral activity of the POM, the MOI used were as shown in Fig. 1. The assay ended when the positive controls without drug at the lowest MOI tested showed +4 CPE (8 days). Changes due to viral cytopathic effect were graded on a scale of 1–4, grade 4 representing a scenario in which the entire (100%) monolayer in a well showed viral cytopathic effect. For all CPE-based assays, the 50% effective concentration (EC_{50}) was calculated by regression analysis using the means of the CPE ratings at each concentration of compound.

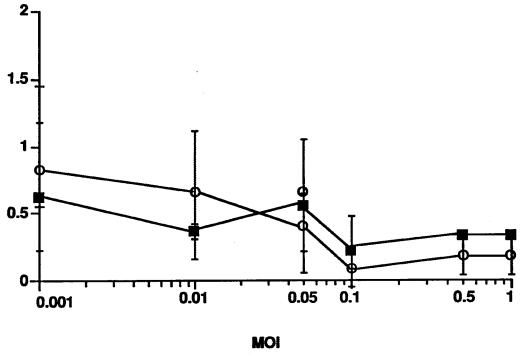

Fig. 1. Effects of multiplicity of infection on the inhibition of replication of RSV by selected POM. HS-116 (*B*) and HS-106 (*E*) were evaluated for activity against RSV (Utah 89) in a standard CPE inhibition assay using virus inocula at MOI = 0.01, 0.05, 0.1, 0.5 and 1. Values represent the average of two separate assays done for each MOI.

Morphological changes due to compound cytotoxicity were graded on a scale of 0–5; grade 5 was defined as 100% cytotoxicity. The 50% cytotoxic dose (IC_{50}) was calculated by regression analysis. A selective index (S.I.) was calculated for each compound ($S.I. = (IC_{50})/(EC_{50})$).

2.6. Neutral red assay of CPE inhibition and cytotoxicity

This assay was performed by a modified method as described by Cavenaugh et al. (1990). Briefly, medium was removed from each well of a plate scored for CPE from a CPE inhibition assay, 0.2 ml of neutral red (0.034% in PSS) was added to each of the wells of that plate and the plate incubated for 2 h at 37°C in the dark. The neutral red solution was removed from the wells and the wells rinsed twice with PBS (pH 7.4). Equal volumes (0.1 ml) of absolute ethanol and Sörenson citrate buffer (0.1 M sodium citrate, 0.1 M HCl, pH 4.2) were mixed together and added to each well. Plates were incubated in the dark for 30 min at room temperature to solubilize the dye. The plates were then gently mixed on a 96-well plate adapted vortexer for 1 min. Absorbances at 540 and 450 nm were read with a microplate reader (Bio-Tek EL 1309; Bio-Tek Instruments, Winooski, VT). All concentrations were assayed at least in triplicate. Absorbance values were expressed as percentages of untreated controls and EC_{50} and IC_{50} values were calculated by regression analysis.

2.7. Virus yield assay

Plates for this assay were set up as in the CPE inhibition assays for the amount of cells and virus. Virus was grown in the presence of various concentrations of compound in 96-well plates as described above. After 5–6 days, the plates were scored for CPE and then frozen and thawed. The crude lysates were then assayed for virus by plating 10-fold serial dilutions in triplicate in 96-well plates by CPE assay. The plates were incubated at 37°C until the positive control wells showed complete CPE. The remaining wells were scored for virus cytopathic effect and endpoints were expressed as $CCID_{50}$/0.1 ml, calculated using the 50% endpoint method (Reed and Muench, 1938).

A 90% reduction in virus yield relative to untreated control wells was calculated by regression analysis. This represented a 1 $\log_{10}$ inhibition in titer when compared to virus controls not treated with compound. A virus yield S.I. was calculated by the formula $S.I. = IC_{50}/EC_{90}$. The IC_{50} was derived from a cell yield assay of log phase cells treated with compound.

2.8. Cell yield assay

Cytotoxicity was evaluated by determining the total number of cells after a 3 day exposure to several concentrations of compound using a Coulter counter (Model Zb-1; Coulter, Miami, FL). Twelve-well tissue culture plates were seeded with 5×10^4 cells suspended in growth medium and incubated for 4 h at 37°C; the cells were approximately 20% confluent. The medium was then replaced with growth medium containing the same concentrations of test compound as in the antiviral experiments plated in duplicate. The cells and drug were incubated at 37°C for 72 h (represented approximately 4–5 cell doublings, depending on the cell line used), at which time the medium was removed and the cells washed twice. Trypsin (0.25%) was added to each well and the cells incubated until they were rounded. The medium containing trypsinized cells from each well was then subjected to vigorous trituration by pipette to provide a uniform cell suspension. 0.2 ml Of each suspension was added to 9.8 ml of Isoton III (Coulter), an isotonic saline solution. The cell suspensions from each well were then counted three times. An IC_{50} was determined by regression analysis using the average cell number at each dilution.

2.9. Time of addition assays

To determine the part of the of the virus cycle inhibited by POM, varying concentrations of compound were added either simultaneously with virus (time = 0) or at various times after exposure of cells to virus and allowed to remain on the cells for the entire assay period (0–5 days). Virus was

Table 2
POM with strong anti-RSV inhibitory activity (S.I. > 75) in MA-104 cells

Compound	CPE inhibition assay			Neutral red assay		
	EC50 (μM)	IC50 (μM)	S.I. (IC_{50}/EC_{50})	EC50 (μM)	IC50 (μM)	S.I. (IC_{50}/EC_{50})
HS-027	1.0 ± 0.0	56 ± 62	56	0.7 ± 0.4	63 ± 53	90
HS-030[a]	3	50	17	1	83	83
HS-034	1.5 ± 0.7	>100[b]	>67	0.6 ± 0.6	75 ± 35	125
HS-086	1.8 ± 1.7	>100	>56	1.1 ± 1.3	93 ± 8	85
HS-106	0.8 ± 0.4	>100	>125	0.3 ± 0.1	75 ± 35	>250
HS-116	0.6 ± 0.6	>100	>167	0.3 ± 0.1	59 ± 59	197
JM-1591	0.1 ± 0.0	53 ± 4	530	0.3 ± 0.07	50 ± 13	166
JM-2768	1.9 ± 1.6	>100	>53	0.3 ± 0.0	>100	>333
JM-2879	0.7 ± 0.5	>100	>142	0.3 ± 0.2	95 ± 7	317
JM-2919	0.1 ± 0.0	80 ± 28	800	<0.1[c]	14 ± 2	>140
JM-2921	0.3 ± 0.05	>100	>333	0.1 ± 0.0	63 ± 11	630
JM-2922	0.5 ± 0.3	>100	>200	0.7 ± 0.4	80 ± 28	114
JM-2926	0.3 ± 0.3	>100	>333	0.1 ± 0.0	25 ± 2	250
JM-2927	0.3 ± 0.2	>100	>333	0.3 ± 0.2	62 ± 30	207

[a] Assay was done once, other values in the table are from two experiments and represent EC_{50} and $IC_{50} \pm$ S.D.
[b] Concentrations greater than 100 μM were not evaluated for cytotoxicity.
[c] Concentrations lower than those shown were not used in the assay.

diluted and absorbed as described above for the CPE inhibition assay. The maintenance medium which contained virus was removed and fresh maintenance medium containing drug was added to the cells and incubated until the virus controls without compound showed 100% CPE. Plates were then scored for viral CPE as described above. EC_{50} values were determined by regression analysis.

2.10. Statistics

Significant differences were determined by analysis of variance (ANOVA) or by two sample t-test. Correlations of values obtained by neutral red assay, virus yield assay or CPE inhibition assay were made by calculating product-moment coefficients according to the method of Pearson (Sokal and Rohlf, 1981). All statistical analyses were done on a personal computer using the Minitab Release 10xtra for Macintosh® (Minitab, State College, PA) statistical package.

3. Results

3.1. Antiviral activity

Four compounds were found to be weakly active (S.I. = 5–20); HS-021, HS-071, HS-112 and HS-158. The EC_{50} values for these compounds ranged from 0.2–10 μM. Five compounds and ribavirin were moderately inhibitory (S.I. = 20–75); HS-033, HS-036, HS-058, HS-081 and HS-098. EC_{50} values for these compounds ranged from 1–1.5 μM; for ribavirin the EC50 = 30 μM. Cytotoxicity values (IC_{50}) for all of the compounds mentioned above ranged from 2–100 μM. Fourteen compounds were found to have striking anti-RSV inhibitory activity (S.I. > 75, Table 2). Materials classified as weakly or moderately inhibitory had significantly higher EC_{50} values by both CPE inhibition and neutral red uptake assays than those compounds classified as strongly inhibitory ($P < 0.001$, $P < 0.01$, ANOVA). Therefore, the high S.I. values calculated for the strongly inhibitory compounds were not necessarily due to less cytotoxicity of these compounds.

A significant majority of EC_{50} values obtained by CPE inhibition assay were no different from

Table 3
Inhibition of virus yields by POM.

Compound	$EC_{50}(\mu M)$	IC50 (μM)[a]	S.I. (IC_{50}/EC_{90})	Compound	EC_{50} (μM)	IC50 (μM)[a]	S.I. (IC_{50}/EC_{90})
HS-027	1	>100[b]	>100	HS-112	10	54	5
HS-030	2	56	28	HS-116	0.1	>100	>1000
HS-033	0.7	>100	>143	HS-158	0.6	10	17
HS-034	1	79	79	JM-1591	2	66	33
HS-036	0.8	>100	>125	JM-2768	1	>100	>100
HS-058	7	>100	>14	JM-2789	0.4	>100	>250
HS-071	7	>100	>14	JM-2919	<0.1[c]	>100	>1000
HS-081	1.0	>100	>100	JM-2921	0.1	44	440
HS-086	2	>100	>50	JM-2922	0.8	>100	>125
HS-098	1	>100	>100	JM-2926	0.1	>100	>1000
HS-106	0.4	>100	>250	JM-2927	0.1	>100	>1000
				Ribavirin	123	1350	11

[a] Determined using actively growing cells by cell yield assay.
[b] > Signifies that concentrations greater than 100 μM were not evaluated for cytotoxicity.
[c] < Indicates that concentrations lower than those shown were not evaluated.

the same values obtained by the neutral red assay when those values in Table 2 were compared (Pearson correlation coefficient (PCC) = 0.989 (a value of 1 being 100% correlation). The IC_{50} values for both assays also compared favorably with one another with few exceptions and were also not significantly different (PCC = 0.936).

To confirm the antiviral activity detected by the CPE inhibition assays and neutral red assays, a number of compounds were evaluated for anti-RSV activity by virus yield reduction assay (Table 3). When compared to those values obtained by the CPE inhibition assays and neutral red assays, the values from the virus yield assays were not much different and correlated well with the other two assays (PCC = 0.949, 0.950 for CPE inhibition assays and neutral red assays, respectively). Surprisingly, the compounds were not very toxic to log phase cells exposed to the compounds for 3 days after seeding. These IC_{50} values correlated very well with the IC_{50} values obtained for the same compounds by visual observation and neutral red assay (PCC = 0.983, 0.922 for CPE inhibition assays and neutral red assays, respectively), even though the cells used in the latter two assays were stationary cells.

Four compounds (HS-081, -086, -106 and -116) were assayed for virucidal effects and none were found to be virucidal. The following compounds were found to be inactive in the primary assay for antiviral activity: HS-052, -053, -054, -057, -077, and -093 and JM-2775.

3.2. Structure-activity relationships

The POM containing sodium cations (i.e., HS-052, -053, -054 and -057, JM-2775; see Table 1), with the exception of JM-2879, were found to have SI values equal to 0 and were considered inactive. However, the addition of potassium cations resulted in inhibitory activity, including a number of strikingly potent agents (JM-1591, -2768, -2921, -2922, and -2926). Simple changes, such as replacement of the manganese cations of the inactive POM HS-057 with iron cations, yielded an active compound (HS-058). Germanium- (JM-2921, JM-2926), niobium- (HS-106, HS-116, JM-2768, JM-2879, JM-2921, JM-2922, JM-2926, JM-2927) tin- (JM-2879) and zirconium- (JM-2919) containing POM were found to be highly active. Compounds with molybdenum cations were weakly active (HS-112) or inactive (JM-2775). Addition of the amino acid histidine

or lysine resulted in the highly active compounds HS-034 and HS-116, whereas those with arginine side chains (HS-033, HS-036, HS-071) were only weakly to moderately active. POM with phosphorus and tungsten centers (HS-052,-053, -054, -057, -058, and -158) were either inactive or weakly active to moderately active with the exception of JM-1591. This relationship is further strengthened by the comparison of HS-093 with HS-098, where the substitution of silicon in the place of the phosphorus group resulted in a very active compound (HS-098). In addition, compounds belonging to the structural class (see Table 1) designated as Wells-Dawson sandwich type compounds were either inactive or only weakly active. In contrast, compounds belonging to the double Keggin group of compounds all had strikingly potent inhibitory RSV activity, as did most of those belonging to the α-Keggin class of compounds.

3.3. Cell line dependency

To determine if the anti-RSV activity was cell line-dependent, four compounds (HS-81, -86, -106 and -116) were evaluated for inhibition of RSV (Utah 89) in MA-104 and HEp-2 cells by CPE inhibition assay and neutral red uptake assay. Inhibition of virus replication was comparable to that found in HEp-2 cells for all compounds but HS-81 (data not shown). Overall, there was no significant cell-dependent antiviral activity or drug cytotoxicity (ANOVA, $P > 0.5$). Both assays were again equally sensitive in measuring antiviral activity (PCC = 1) and cytotoxicity (PCC = 0.923).

3.4. Virus strain specificity

To determine if the antiviral effect observed was viral strain-specific, the inhibitory spectrum of the POM against other strains of RSV was determined (Table 4). The EC_{50} doses for the other RSV strains were comparable to those data shown in Table 2. For the A2 virus, PCC = 0.848, 0.813 for CPE inhibition assays and neutral red assays, respectively; for the Long strain 0.93, 0.932; for strain 9320 0.994 and 0.999; strain 18357 (type B RSV) 0.990 and 0.997, and for bovine RSV (BRSV) 0.992 and 1. However, BRSV was very resistant to ribavirin.

3.5. Effects of multiplicity of infection

Two experiments were done to determine if the antiviral effects of the POM were dependent on the multiplicity of infection (MOI). The effects of MOI, ranging from 0.01 to 1, were evaluated for two active compounds, HS-116 and HS-106 (Fig. 1) against the Utah 89 strain of RSV. The antiviral efficacy of these two compounds was not dependent on the virus concentrations used in the assays (ANOVA, $P > 0.06$)

3.6. Timing studies

In an effort to determine the part of the replication cycle inhibited by these compounds, an experiment was done in which compound was added at times from 1 h pre-infection to 12 h post-infection and left on for the duration of the experiment. It showed that HS-116 equally inhibited virus at all times assayed (average $EC_{50} = 0.26 \pm 0.05$ μM). This experiment suggested that an early event such as adsorption or penetration may have been inhibited and that the continued presence of compound may have prevented adsorption to or penetration of cells as long as it was present.

4. Discussion

These experiments demonstrate that the POM represent a class of antiviral compounds with potent and selective anti-RSV activity independent of virucidal effect and MOI. Of the active compounds evaluated against other virus strains, each was nearly equally inhibitory to all human RSV strains against which it was tested even though the clinically effective drug, ribavirin, was not. This suggests that these compounds probably inhibit all RSV strains in some analogous manner. This is consistent with their putative mode of inhibition, which is to inhibit some step between attachment and penetration (Hosoya et al., 1991; Shigeta et al., 1995a). Our data suggest that other steps in the replication cycle may also be inhibited which is consistent with the findings of Fukuma et al. (1991), although the inhibition of the post-penetration parts of the virus replication cycle may

Table 4
Inhibition of other RSV strains by POM.

Compound	CPE Inhibition Assay[a]			Neutral red assay[a]		
	EC_{50} (μM)	IC_{50} (μM)	S.I. (IC_{50}/EC_{50})	EC_{50} (μM)	IC_{50} (μM)	S.I. (IC_{50}/EC_{50})
RSV A2/Mα-104 cells						
HS-058	<0.1[b]	34	>340	<0.1	64	>640[c]
HS-086	4	>100	>25	1	>100	>100
HS-106	1	>100	>100	0.7	>100	>143
HS-158	<0.1[b]	25	>250	0.1	26	260
JM-2919	0.1	>55	>550	0.1	>100	>1000
JM-2921	1	>100	>100	2	>100	>50
JM-2926	3	>100	>33	2	>100	>50
JM-2927	3	55	18	1	100	100
Ribavirin	20	1310	66	40	520	13
RSV (Long)/HEp-2 cells						
HS-081	4	>100[c]	>25	1.0	>100	>100
HS-086	4	>100	>25	5	>100	>20
HS-106	1	>100	>100	0.4	>100	>250
HS-116	1	>100	>100	0.3	>100	>333
JM-2921	1	>100	>100	1	>100	>100
JM-2926	2	>100	>50	1	>100	>100
Ribavirin	10	740	74	4	500	125
RSV (9320)/Mα-104 cells						
HS-081	10	>100	>10	1	>100	>100
HS-086	5	>100	>20	<0.3[b]	>100	>333
HS-106	3	>100	>33	0.3	>100	>333
HS-116	5	>100	>20	0.5	>100	>200
Ribavirin	120	2825	24	30	3000	100
RSV (18357)/Mα-104 cells						
HS-081	3	>100c	>33	3	>100	>33
HS-086	5	>100	>20	1	>100	>100
HS-116	1	>100	>100	0.3	>100	>333
JM-2921	1	>100	>100	1	>100	>100
JM-2926	2	>100	>50	1	>100	>100
Ribavirin	40	1400	35	30	1530	51
Bovine RSV (375)/EBtr cells						
HS-081	8	>100	>12	2	>100	>50
HS-086	10	>100	>10	4	>100	>25
HS-116	3	>100	>33	2	>100	>50
Ribavirin	410	4100	10	120	4100	34

[a] Each concentration of compound was tested in quadruplicate. The EC_{50} or IC_{50} values were derived from those concentrations representing the linear part of the dose-response curve.
[b] < Indicates that concentrations lower than those shown were not evaluated.
[c] > Signifies that concentrations greater than 100 μM were not evaluated for cytotoxicity.

not be as important for preventing virus production as blocking early steps such as adsorption and penetration. At high concentrations these compounds could simply cover up all the receptors to which the virus attaches. Alternatively, the compounds may very well interact with a glycoprotein of RSV or a cellular receptor to which the virus binds to prevent attachment or penetration as described by Ikeda et al. (1993). An interaction of the POM with a nascent glycoprotein (RSV G

or F) produced by virus replication may account for the inhibition seen at later times (i.e. compound addition at 3 h post-infection) during the eclipse period, preventing proper assembly of virions or infection of cells by newly released virus. However, the data presented here argue strongly for inhibition of virus attachment as the primary mode of inhibition, since inhibition of virus replication only during the virus adsorption period was at least four-fold greater than at any other time period evaluated. In addition, the data also suggest that the compounds may interact with cellular receptors to block virus adsorption, since pretreatment with compound resulted in the most potent inhibition of virus replication.

It would be expected that if the POM inhibited attachment of virus, they would interact with glycoprotein G or its cellular receptor, since attachment of RSV is mediated by glycoprotein G (Levine et al., 1987). The RSV G protein is usually thought to be very antigenically dimorphic among clinical isolates and the two major antigenic subgroups (Collins et al., 1996); but the POM anti-RSV activity shown in this study seemed to be strain-independent. There is an antigenically conserved region of 13 amino acids (164–176) midway in the extracellular domain of G that is thought to be a good candidate for being involved in receptor binding (Johnson et al., 1987). This sequence is highly conserved throughout both subgroups (A and B) of viruses, although only six of the amino acids and the four cysteine residues are conserved in BRSV (Lerch et al., 1990). It is perhaps this sequence with which the POM may interact with each of the viruses in the subgroups tested. The fact that this region was not completely conserved in the G protein of BRSV could relate to the host specificity of BRSV (Lerch et al., 1990) and could also explain why BRSV was relatively refractory to inhibition by the POM. Alternatively, the POM may not interact with cellular receptors for BRSV to inhibit virus adsorption as they may do with receptors for human RSV strains.

These data also show that the assays employed for these experiments were comparably sensitive and reliable. Cytopathic inhibition assays done by visual observation of cytopathic effects were quantitatively and statistically as accurate as neutral red uptake assays and virus yield reduction assays for determining antiviral activity. Similar results were seen with methods for quantitating cytotoxicity (visual observation, neutral red uptake assay and cell yield assay).

Thus, the very potent activity of the polyoxometalates and lack of cytotoxicity discovered in vitro suggests that these compounds warrant further evaluation as potential agents for use against RSV infections in combination with or as alternatives to ribavirin.

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