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Inhibition of HIV-1 infection by zinc group metal compounds

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Abstract

Thirty-seven metal compounds were examined for inhibitory activities against infection with human immunodeficiency virus type 1 (HIV-1). Zinc group metal compounds, namely, zinc acetate, zinc chloride, zinc nitrate, cadmium acetate and mercury chloride, showed anti-HIV-1 activities. Cadmium and mercury compounds at 1-10 μg/ml and zinc compounds at 100 μg/ml strongly inhibited HIV-1 infection, although the cadmium, mercury and zinc compounds had severe cytotoxities at 100, 100 and 1000 μg/ml, respectively. They inhibited transcription of HIV-1 RNA and HIV-1 production at concentrations at which they did not affect the growth of HIV-1-producing cells. They had little effect on syncytium formation resulting from cocultivation of uninfected with HIV-1-producing cells. Nor did they affect HIV-1 DNA synthesis following HIV-1 infection. The metal compounds may owe their anti-HIV-1 effects to inhibition of HIV-1 DNA to RNA transcription, rather than inhibition of the adsorption, penetration or reverse transcription step of HIV-1 infection. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Zinc; Cadmium; Mercury; Inhibition of HIV-1 production

1. Introduction

Several metal ions or metal compounds affect the replication of human immunodeficiency virus type 1 (HIV-1). For example, calcium ions are necessary for syncytium formation induced by HIV-1-producing cells (Dimitrov et al., 1993). Divalent cations are necessary for the activity of reverse transcriptase (Sommerfelt and Weiss, 1990). Zinc stimulates the activity of HIV-1 integrase in vitro (Lee and Han, 1996). Copper, zinc, silver and mercury inhibit the protease of HIV-1 (Karlstrom and Levine, 1991; Zang et al., 1991). Purified Tat protein requires zinc, cadmium or mercury for dimerization (Frankel et al., 1988).

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The nucleocapsid protein of HIV-1 p15 binds to metal ions such as zinc or cadmium (Fitzgerald and Coleman, 1991).

In this study we examined 37 metal compounds for their inhibitory activities against infection with HIV-1 and found that zinc group metal compounds, namely, zinc, cadmium and mercury compounds, have anti-HIV-1 activities. These compounds more specifically inhibited HIV-1 transcription than cellular RNA synthesis, while they did not inhibit HIV-1-induced syncytium formation and HIV-1 viral DNA synthesis following HIV-1 infection.

2. Materials and methods

2.1. Cells and virus

The human T-cell lines MT-4 (Miyoshi et al., 1981), C8166 (Salahuddin et al., 1983) and Molt-4 clone 8 (Kikukawa et al., 1986) were cultivated in RPMI1640 medium containing 10% fetal calf serum. Peripheral blood lymphocytes (PBLs) were stimulated with PHA and cultured as described elsewhere (Haraguchi et al., 1997). Stocks of HIV-1 were prepared from the culture supernatants of Molt-4/IIIB cells and Molt-4/GUN1 cells, which are Molt-4 cells persistently infected with IIIB (Popovic et al., 1984) and GUN1 strains (Takeuchi et al., 1987) of HIV-1, respectively.

2.2. Compounds

The thirty-seven metal compounds examined in this experiment are shown in Table 1. These compounds were dissolved in distilled water at 5–10 mg/ml and diluted tenfold with the cell culture medium. They were grouped into four by their cytotoxities. Anti-HIV-1 activities were judged at concentrations at which marked cytotoxity was not observed microscopically (more than 10% cells were living). Manganese(III)meso-tetraphenylporphyrin chloride (Mn(TPP)Cl) was prepared according to the method reported (Adler et al., 1970). Metal-acetylacetonate complexes ($Mn(AA)_2$, $Mn(AA)_3$ and $Co(AA)_3$) were purchased from Dojindo Laboratories (Kumamoto, Japan). The other metal compounds were obtained from Wako Pure Chemical Industries, (Osaka, Japan).

Table 1
Compounds examined in this study[a]

Group	Concentration	Compound		
I	1 mg/ml	LiCl	NaCl	$MgCl_2$
		KCl	$CaCl_2$	$FeCl{\cdot}4H_2O$
		$FeNH_4(SO_4)_2{\cdot}12H_2O$	$Fe(ClO_4)_2{\cdot}6H_2O$	RbCl
		$SrCl2{\cdot}6H_2O$	CsCl	$BaCl_2{\cdot}2H_2O$
		$CeCl_3{\cdot}7H_2O$	$Ce(SO_4)_3{\cdot}8H_2O$	
II	100 μg/ml	NH_4VO_3	VCl_3	$VOSO_4$
		$Mn(AA)_2$	$Mn(AA)_3$	$KMnO_4{\cdot}2H_2O$
		$MnSO_4{\cdot}4–5H_2O$	$Co(AA)_3$	$(CH_3COO)_2Ni{\cdot}4H_2O$
		$(CH_3CO0)_2Zn{\cdot}2H_2O$	$ZnCl_2$	$Zn(NO_3)_2{\cdot}6H_2O$
		$(NH_4)_6MO_7O_{24}{\cdot}4H_2O$	$Na_2WO_4{\cdot}2H_2O$	$Pt(NH_3)_2Cl_2$
III	10 μg/ml	Mn(TPP)Cl	$Cu(CH_3COP)_2{\cdot}H_2O$	$CuCl_2$
		$AgNO_3$	$(CH_3COO)_2Cd{\cdot}2H_2O$	$HgCl_2$
		CH_3COOTl		
IV	1 μg/ml	H_2SeO_3		

[a] All compounds were dissolved in water, diluted 10-fold and examined for their anti-HIV-1 activities at concentrations at which they primed little toxic to C8166 cells. Highest concentrations of each group of compounds tested are shown.

2.3. Assay for anti-HIV-1 activity and cytotoxity

The measurement of activities of compounds against HIV-1 replication was based on the inhibition of viral protein expression in C8166 or MT-4 cells infected with the IIIB or GUN1 strain of HIV-1. Briefly, C8166 or MT-4 cells were seeded at 1.0×10^5 cells/ml, and compounds were added at a concentration of 0.1–1000 μg/ml. After incubation for 1 h, the cells were infected with HIV-1. Four days later HIV-1 antigen-positive cells were detected by indirect immunofluorescence assay (IFA), as described elsewhere (Haraguchi et al., 1997). The 50% inhibitory concentration (IC_{50}) was defined as a concentration of a compound that was estimated to reduce the percentage of HIV-1 antigen-positive C8166 and MT-4 cells to half of that of the control cell cultures.

Anti-HIV-1 activities of metal compounds examined using PBLs were evaluated by the quantitative detection of HIV-1 p24 antigen in the culture supernatants of HIV-1-infected PBLs as described previously (Haraguchi et al., 1997). That is, PHA-stimulated PBLs were treated with metal compounds at 37°C for 1 h and then infected with HIV-1 strain IIIB at 37°C for 1 h. After inocula were removed by centrifugation, fresh medium containing one of the metal compounds and recombinant human IL-2 (100 U/ml) was added to the PBLs. Culture supernatants were harvested 6 days later. The culture supernatants were lysed with 0.2% Triton X-100 and heated for 1 h at 60°C. The amounts of HIV-1 p24 were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using a mouse monoclonal antibody against HIV-1 p24 antigen and HIV-1-seropositive human serum as described elsewhere (Haraguchi et al., 1997). IC_{50} was calculated as a concentration of each metal compound that would reduce the amount of HIV-1 p24 antigen in the culture supernatants of metal compound-treated cultures to 50% of that of the mock-treated control.

The cytotoxities of zinc group metal compounds to C8166 and MT-4 cells were assayed by the trypan blue dye exclusion test. The cytotoxities of these compounds to PBLs were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described elsewhere (Ikeda et al., 1996). The 50% cytotoxic concentration (CC_{50}) was calculated as the concentration of each metal compound that would reduce viable cell number (C8166 and MT-4 cells) or absorbance (OD 560, PBLs) to 50% of that of mock-treated control.

2.4. Syncytium formation assay

Syncytium formation assay was performed as follows: 5.0×10^4 C8166 cells were co-cultured with 5.0×10^3 Molt-4/IIIB cells in the presence or absence of the metal compounds for 8 h. The number of syncytia was counted using an inverted microscope. Ratios of viable cells were determined by the trypan blue dye exclusion test.

2.5. Polymerase chain reaction to detect HIV-1 DNA synthesis

The effects of the metal compounds on HIV-1 DNA synthesis after infection were examined by the cell-free HIV-1 polymerase chain reaction (PCR) assay. Briefly, C8166 cells were treated with metal compounds for 1 h and infected with HIV-1 strain IIIB. The cells were incubated for 24 h in medium containing these compounds. DNA was then extracted from the cells and HIV-1 DNA was amplified by PCR. PCR primers SK38-out (5′-AAGGGGAAGTGACATAGCAG-3′) and SK39-out (5′-GGACCAACAAGGTTTCT-GTC-3′) were used to amplify the gag region of the HIV-1 genome. The length of amplified DNA was calculated to be 278 bp. After 28 cycles of PCR amplification and subsequent electrophoresis through gels containing 2% NuSieve agarose (FMC Bioproducts, Rockland, ME) and 1% TAKARA H-14 agarose (TAKARA shuzo, Kyoto, Japan), the gels were photographed under ultraviolet light.

2.6. HIV-1 production and cell growth assays

The effects of the metal compounds on HIV-1 production were examined by detecting HIV-1 *Gag* protein p24 in the culture supernatants of HIV-1-infected cells using the p24 sandwich

ELISA. Namely, Molt-4/IIIB cells were seeded at 3×10^5/ml and metal compounds were added at a concentration of 0.1–100 μg/ml. After incubation for 1 day the cells were collected after low-speed centrifugation, resuspended in medium containing metal compounds, and incubated for another 1 day. Then culture supernatants were harvested and mixed with Triton X-100 at a final concentration of 0.2% and heat-inactivated at 60°C for 1 h. The amounts of HIV-1 p24 in the culture supernatants were determined by the sandwich ELISA as described above.

The effects of metal compounds on the viability and growth of Molt-4/IIIB cells were assayed by the trypan blue dye exclusion test. The cells were seeded at 2×10^5 cells/ml and the compounds were added at a concentration of 0.1–1000 μg/ml. After incubation for 2 days, numbers of viable cells were counted using trypan blue. CC_{50} was calculated as the compound concentration that would reduce the viable cell number to 50% of that of the control.

2.7. Reverse transcriptase-polymerase chain reaction

The effects of metal compounds on the transcription of HIV-1 RNA were examined by reverse transcriptase-polymerase chain reaction (RT-PCR). Namely, Molt-4/IIIB cells were seeded at 3×10^5/ml and metal compounds were added at concentrations of 0.1–100 μg/ml. After two days, the cells were harvested and the total cellular RNA was extracted using as RNA extraction kits (Sanko Jun-yaku, Tokyo, Japan). cDNA was synthesized using an oligo(dT) primer and reverse transcriptase from avian myeloblastosis virus. PCR was performed with the SK38-out and SK39-out primer pair under conditions described above.

3. Results and discussion

3.1. Anti-HIV-1 activities of mercury, cadmium and zinc compounds

Over 90% of C8166 cells acutely infected with HIV-1 were positive for viral antigens after cultivation for four days in the absence of the test compounds. In this study we examined anti-HIV-1 activities of 37 metal compounds: alkaline metals (Li, Na, Rb and Cs), magnesium, alkaline earth metals (Ca, Sr and Ba), lantanoid (Ce), 5A, 6A and 7A transition elements (V, Mo and W), iron group metals (Fe, Co and Ni), platinum, copper group metals (Cu and Ag), zinc group metals (Zn, Cd and Hg) and 3B and 6B typical elements (Tl and Se) (Table 1). These metal compounds were grouped into Groups I-IV by their cytotoxities (Table 1). Their anti-HIV-1 activities were examined at concentrations where they were not severely toxic to C8166 cells (more than 10% cells were living).

Compounds containing the zinc group elements, zinc, cadmium and mercury, showed anti-HIV-1 activities. Namely, zinc acetate, zinc chloride, zinc nitrate, cadmium acetate and mercury chloride inhibited the expression of HIV-1 antigens in C8166 cells (Fig. 1A): their IC_{50}s for inhibition of HIV-1 infection were calculated to be 8, 8, 13, 0.18 and 0.12 μg/ml, respectively. Cadmium acetate and mercury chloride showed marked cytotoxities at 10–100 μg/ml (Fig. 1B). Zinc compounds demonstrated severe cytotoxity only at a concentration of 1000 μg/ml. These compounds showed also anti-HIV-1 activities in other cells (MT-4 and PBL), and against other HIV-1 strains, i.e. an SI-type clinical isolate, i.e. GUN1 (Table 2). The other 32 metal compounds hardly affected HIV-1 infection at non-cytotoxic concentrations.

3.2. Effects of metal compounds on HIV-1-induced syncytium formation, HIV-1 DNA synthesis, HIV-1 production and cell growth

Next we examined the possible mechanism of the anti-HIV-1 activity of the zinc group compounds. First we tested whether they affected an adsorption or penetration step of HIV-1. Thus, we performed the syncytium formation assay, which has been used to examine effects of drugs on an early step of HIV-1 infection (Nakashima et al., 1988). Zinc chloride hardly affected syncytium formation induced by HIV-1 even at 100 μg/ml, while cadmium acetate and mercury chloride

Table 2
Cytotoxities and anti-HIV-1 activities of the metal compounds

Compound	Cytotoxity[a] (CC_{50})			Anti-HIV-1 activity[b] (IC_{50})			Selectivity index[c] (CC_{50}/IC_{50})		
	C8166	MT-4	PBLs	C8166/IIIB	MT-4/GUN1	PBLs/IIIB	C8166/IIIB	MT-4/GUN1	PBLs/IIIB
$ZnCl_2$	280	120	550	8.0	3.5	9.3	35	34	59
$(CH_3COO)_2Cd{\cdot}2H_2O$	2.2	1.0	2.9	0.18	0.052	0.30	12	19	10
$HgCl_2$	1.5	0.88	3.1	0.12	0.033	0.42	13	27	7.0

[a] Cytotoxities of the metal compounds to indicator cells were determined as CC_{50} (μg/ml) by the trypan blue dye exclusion test (C8166 and MT-4 cells) or MTT assay (PBLs).

[b] Anti-HIV-1 activities were determined as IC_{50} (μg/ml) by IFA (C8166/IIIB and MT-4/GUN1) or ELISA (PBLs/IIIB). C8166 cells and PBLs, and MT-4 cells were infected ith IIIB and GVN1 strains, respectively, and anti-HIV-1 activities were determined.

[c] Selectivity indies were calculated as CC_{50}/IC_{50} for each combination of the indicator cells and HIV-1 strains.

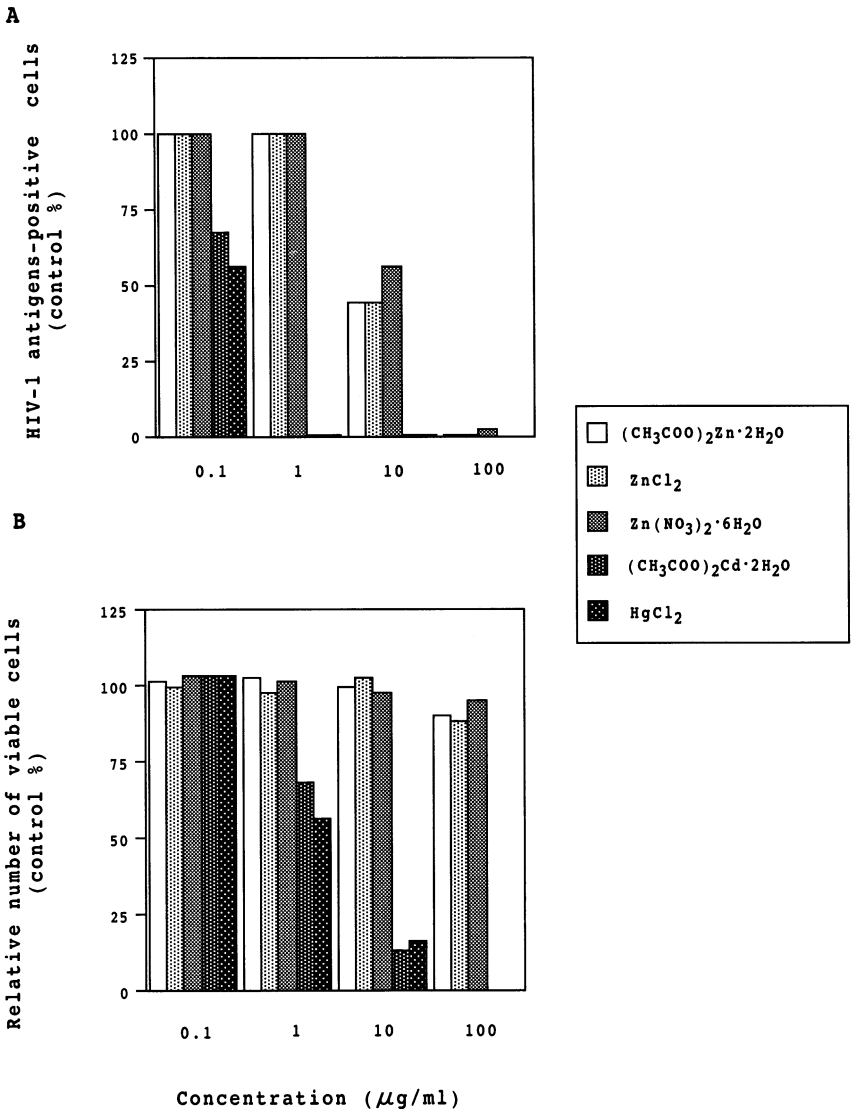

Fig. 1. Anti-HIV-1 activities and cytotoxities of metal compounds. C8166 cells were infected with HIV-1 and cultured in the presence of metal compounds. After incubation for 4 days, the percentage of HIV-1 antigen-positive C8166 cells was determined by IFA (A). The viability of C8166 cells was determined by the trypan blue dye exclusion test after incubation with the metal compounds for 4 days (B).

strongly inhibited syncytium formation at 100 μg/ml and weakly at 10 μg/ml (Table 3). Because mercury and cadmium compounds showed cytotoxities at 10–100 μg/ml (Table 3), the inhibition of syncytium formation by these compounds could be attributed mainly to their cytotoxities. These metal compounds did not affect syncytium formation at non-cytotoxic concentrations, suggesting that they had little if any effect on the adsorption or entry of HIV-1.

Next we examined whether the zinc group metals affected the reverse transcription step of HIV-1. Therefore, we determined the amount of HIV-1 DNA formed through reverse transcription, by using PCR. Cadmium acetate and mercury chloride hardly inhibited the synthesis of HIV-1 DNA at 0.1–10 μg/ml (Fig. 2). Zinc chloride also did not inhibit the synthesis of HIV-1 DNA at 1–100 μg/ml (Fig. 2). These results suggest that the zinc group metals did not affect the reverse transcription step.

To examine the possibility that these compounds inhibit virus production by HIV-1-infected cells, their effects on HIV-1 production was evaluated by sandwich ELISA. Namely, HIV-1 p24 antigen production in the culture supernatants of Molt-4/IIIB cells treated by the compounds was determined by ELISA. Cadmium

Table 3
Effect of the metal compounds on syncytium formation induced by HIV-1

Compound	Drug concentration					IC_{50}[a] (μg/ml)	CC_{50}[c] (μg/ml)
	100	10	1	0.1	0		
$ZnCl_2$	33[b] (101)[c]	38 (98)	35 (101)	42 (96)	39 (100)	>100	(>100)
$(CH_3COO)_2Cd{\cdot}2H_2O$	0 (0)	18 (53)	33 (92)	37 (98)		10	(12)
$HgCl_2$	0 (0)	10 (61)	36 (95)	35 (99)		5	(15)

[a] Fifty percent inhibitory concentration of syncytium formation. Syncytia were detected 8 h after cocultivation of C8166 cells with Molt-4/IIIB cells.

[b] Number of syncytia per well (mean of duplicate assays).

[c] Numbers of viable C8166 cells were counted by the trypan blue dye exclusion test and the relative numbers of viable cells were calculated. The cell number of mock-treated control was 5.5×10^4 cells. CC_{50} values were calculated as described in Section 2.

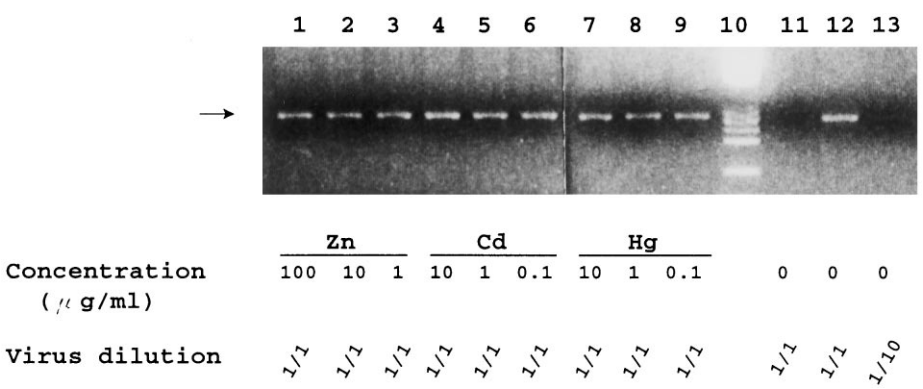

Fig. 2. Effects of the metal compounds on synthesis of HIV-1 viral DNA. C8166 cells were treated with the metal compounds for 1 h, infected with HIV-1 strain IIIB (lanes 1–9 and 12–13) or with heat-inactivated (at 60°C for 1 h) HIV-1IIIB (lane 11) for 1 h, and then incubated for another 24 h in medium containing the metal compounds. DNA of HIV-1-infected C8166 cells was amplified by PCR, resolved by electrophoresis through agarose gels, and stained with ethidium bromide. Dilutions of virus inocula are as follows: lanes 1–9 and 11–12, 1/1, lane 13, 1/10. Lanes 1–3, 4–6 and 7–9 represent C8166 cells treated with zinc chloride (Zn), cadmium acetate (Cd), and mercury chloride (Hg), respectively. Lane 1, 100 μg/ml; lanes 2, 4 and 7, 10 μg/ml; lanes 3, 5 and 8, 1 μg/m1; lanes 6 and 9, 0.1 μg/ml. Lanes 11–13 are compound-free controls. ϕX174 DNA digested with *Hae*III was the size maker (lane 10).

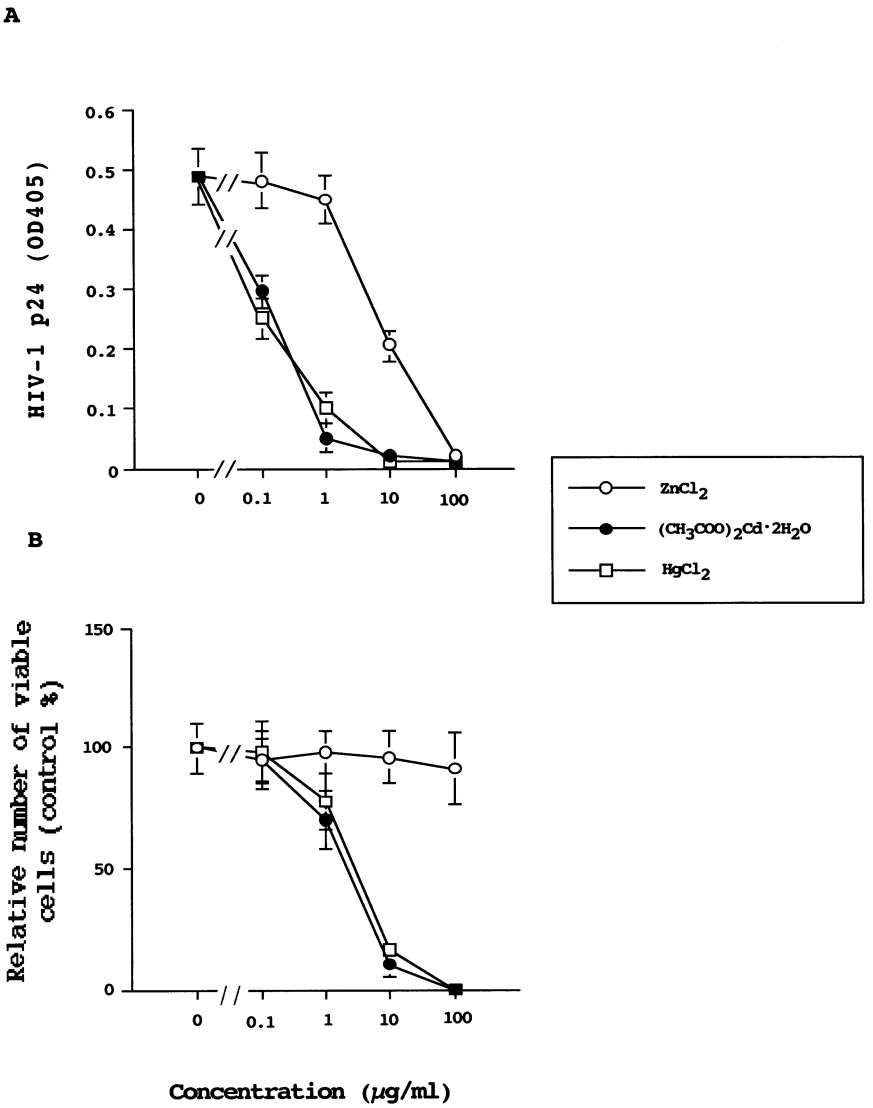

Fig. 3. Effects of the metal compounds on HIV-1 production and cell growth. (A) Molt-4/IIIB cells were cultivated for 2 days in the presence of zinc chloride, cadmium acetate or mercury chloride at concentrations of 0.1–100 μg/ml. The amounts of HIV-1 p24 in the culture supernatants were measured by p24 ELISA. Values represent the means ± S.D. of four independent experiments. Fresh medium was used as the control and its value was subtracted from each value. (B) Molt-4/IIIB cells were incubated for 2 days in the presence of zinc chloride (Zn), cadmium acetate (Cd) or mercury chloride (Hg) at concentrations of 0.1–100 μg/ml. Viable cell numbers were determined by the trypan blue dye exclusion test. Values represent the means ± S.D. for three independent experiments.

acetate and mercury chloride markedly inhibited viral production at 1–100 μg/ml (Fig. 3A), but they showed inhibitory effects on the cell growth at 10–100 μg/ml (Fig. 3B). Zinc chloride also markedly inhibited viral production at 10–100 μg/ml (Fig. 3A), but did not inhibit the cell growth at these concentrations: cytotoxity was observed only at 1000 μg/ml. Comparative effects of the metal compounds on HIV-1 production and cell growth were assessed as selectivity indices

(SIs) (Table 4). SIs of zinc chloride, cadmium acetate and mercury chloride were 93, 15 and 28, respectively, suggesting that the inhibitory effects of these compounds on HIV-1 production could be dissociated from their cytotoxities.

3.3. Effects of the metal compounds on HIV-1 transcription

Next we examined whether these metal compounds affected the transcription of HIV-1 RNA in Molt-4/IIIB cells, using RT-PCR. As shown in Fig. 4, cadmium acetate and mercury chloride at 1–10 μg/ml markedly inhibited the synthesis of HIV-1 RNA. These compounds did not affect cellular RNA synthesis: similar amounts of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) RNA were detected in the presence or absence of the compounds (Fig. 4). This finding also rules out an accelerated degradation of the RNA in the presence of the compounds. Zinc chloride at 100 μg/ml also markedly inhibited the synthesis of HIV-1 RNA, and did not inhibit the synthesis of G3PDH RNA at this concentration (Fig. 4). These findings indicate that the metal compounds more

Table 4
Effects of the metal compounds on HIV-1 production

Compound	CC_{50}[a] (μg/ml)	IC_{50}[b] (μg/ml)	Selectivity index (CC_{50}/IC_{50})
$ZnCl_2$	550	5.6	93
$(CH_3COO)_2Cd{\cdot}2H_2O$	2.2	0.15	15
$HgCl_2$	2.8	0.10	28

[a] Fifty percent inhibitory concentration for Molt-4/IIIB cell growth.
[b] Fifty percent inhibitory concentration for HIV-1 production by Molt-4/IIIB cells, determined by ELISA.

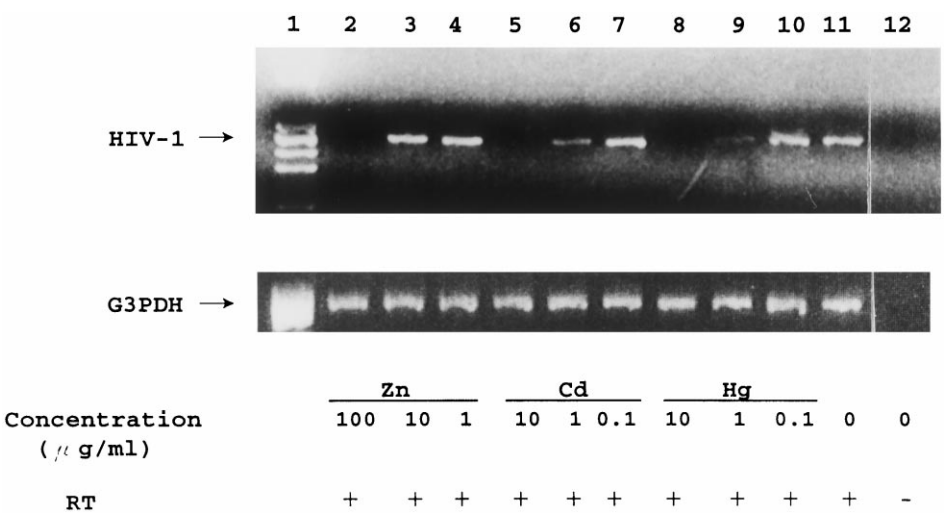

Fig. 4. Effects of metal compounds on the transcription of HIV-1 RNA. Molt-4/IIIB cells were incubated in the presence (lanes 2–10) or absence (lanes 11–12) of the metal compounds for 2 days. Then total RNA was isolated from Molt-4/IIIB cells and incubated in the reaction mixture with (lanes 2–11) or without (lanes 12) reverse transcriptase (RT). The samples were amplified by PCR using the HIV-1-specific primers and electrophoresed through an agarose gel. Lanes 2–4, lanes 5–7 and lanes 8–10 represent incubations of Molt-4/IIIB cells with zinc chloride (Zn), cadmium acetate (Cd) and mercury chloride (Hg), respectively, at the following concentrations: lane 2, 100 μg/ml; lanes 3, 5 and 8, 10 μg/ml; lanes 4, 6 and 9, 1 μg/m1; lanes 7 and 10, 0.1 μg/ml. Lane 11 is a compound-free control. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) RNA in each sample was amplified as a control. The size marker (lane 1) was the same as that in Fig. 2 (lane 10).

specifically inhibit HIV-1 RNA transcription than cellular RNA transcription.

In this study we showed that the zinc group metal compounds possess anti-HIV-1 activities and inhibit the HIV-1 RNA transcription and the production of HIV-1 into the cell culture medium. The Tat protein of HIV-1 transactivates genes that are transcribed from HIV-1 LTR. As zinc, cadmium or mercury have been found to account for the dimerization of the Tat protein (Frankel et al., 1988), they may bind to a metal-binding site of Tat protein and thus interfere with the functions of the Tat protein. However, it was also reported that zinc and cadmium do not inhibit the Tat-driven transcription in the CAT assay (Koken et al., 1994). Cellular transcriptional elements, such as NF-κB, also enhance HIV-1 transcription (Osborn et al., 1989) and may also serve as targets for the zinc group metal compounds. It remains to be examined whether these metal complexes affect HIV-1 transcription through interaction with HIV-1 Tat or other (i.e. cellular) factors.

It is obvious that cadmium or mercury, because of their toxic potential, cannot be used clinically. Zinc, however, is an essential element and is detected in serum at about 1 μg/ml. It is possible that metal complexes may be found with higher anti-HIV-1 potency and/or lower toxicity than those reported here, and that this lead may yield useful drug candidates for the treatment of HIV-1 infections.

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