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In Vitro Activity of Zinc Salts against Human Rhinoviruses

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The antiviral activity of zinc salts against rhinovirus types 1A and 39 was assayed by yield reduction and inhibition of cytopathic effect in cell culture. The findings indicate that the zinc salts tested have low in vitro therapeutic indices and suggest that the possible beneficial effects of zinc lozenges in reducing cold symptoms may not be related to selective antirhinovirus activity.

Zinc ions inhibit the in vitro replication of diverse viruses, including rhinovirus (4, 5), herpes simplex virus (7), and vaccinia virus (3), and other pathogenic organisms, including *Chlamydia trachomatis* (2). Zinc chloride (0.1 mM) inhibited plaque formation by eight of nine human rhinoviruses in HeLa cells and reduced the yield of rhinovirus 1A when added as late as 6 to 8 h after infection (5). The antirhinovirus activity of this zinc salt appears to be mediated by inhibition of posttranslational cleavage of precursor polypeptides (4). Zinc gluconate lozenges were reported to be effective in reducing the duration of symptoms in natural colds of undefined viral etiology (1). In contrast, another study, using a somewhat lower dosage of zinc acetate lozenges, found no clinical benefit compared with a placebo in the treatment of natural colds (R. Douglas, personal communication). Unpalatable taste, mouth irritation, and nausea have been reported side effects of zinc lozenge administration (1; B. M. Farr and J. M. Gwaltney, Jr., J. Chronic Dis., in press).

The present studies were undertaken to determine the in vitro antiviral activity of a variety of zinc salts against two human rhinoviruses representing serotypes previously shown to be inhibited by zinc chloride (5). Serotype 1A (passage history: MRC-5/5, WI-38/1, fetal tonsil/1) and serotype 39 (passage history: WI-38/2, MRC-5/4) were used. The following zinc salts (molecular weight) were provided by R. G. Achari, Bristol Meyers Products, Inc., Hillside, N.J.: acetate (243.5), carbonate (125.4), chloride (136.3), gluconate (455.7), lactate (183.5), sulfate (161.4), and oxide (81.4). The salts were dissolved in sterile distilled water or 0.1 N HCl to make stock solutions of 10 mM concentration. Serial $\log_{10}$ or 0.5 $\log_{10}$ dilutions of the stock solutions were made in cell culture maintenance medium appropriate to the cell type. MRC-5 and WI-38 human embryonic lung fibroblast cells (M.A. Bioproducts, Inc., Walkersville, Md.) at passage levels 23 to 25 were maintained in Eagle minimal essential medium supplemented with glutamine, 2% fetal bovine serum (HyClone Laboratories, Logan, Utah), vancomycin (20 μg/ml), gentamicin (50 μg/ml), and amphotericin (1 μg/ml). HeLa-M (R19 strain) cells were provided by R. Colonno, Merck Sharp & Dohme Research Laboratories, West Point, Pa., and passaged serially in our laboratory. McCoy 5A medium containing 5% fetal bovine serum and 30 mM $MgCl_2$ served as the medium for the HeLa-M-rhinovirus experiments.

For cytopathic effect (CPE) inhibition and infectious virus yield reduction assays, monolayers of a particular cell type were inoculated with 0.2 ml of Hanks balanced salt solution (HBSS) containing 30 to 1,000 50% tissue culture infective doses ($TCID_{50}$) of virus. After incubation at 35°C for 1 h, the inoculum was decanted, and the monolayers were rinsed once or twice with Hanks balanced salt solution. The monolayers were overlaid with medium containing the appropriate zinc salt concentration, incubated at 33°C on a roller drum apparatus, and scored daily for evidence of rhinoviral CPE (6). Duplicate tubes of uninfected cell monolayers were exposed to the zinc salt and observed for cytotoxicity. Quadruplicate monolayers and supernatant fluids were harvested at 24 and 48 h, pooled, and frozen at −70°C. Virus yields were determined by inoculating serial 10-fold dilutions onto duplicate or quadruplicate MRC-5 or WI-38 fibroblast monolayers; they are expressed as $TCID_{50}$. In some experiments, the monolayers were preincubated with the zinc salt for 1 h before viral inoculation.

Toxicity of zinc salts for uninfected cells. All of the zinc salts at a concentration of 1.0 or 0.3 mM caused extensive cellular cytotoxicity with destruction of uninfected MRC-5 fibroblast monolayers within 1 day of exposure. A concentration of 0.1 mM caused generalized cell rounding and refractile change in most experiments within 1 day, although in some experiments this concentration did not cause visible cytotoxicity. MRC-5 fibroblast monolayers treated with concentrations of 0.03 mM and lower were consistently free of visible cytotoxity.

In HeLa-M cell monolayers, zinc lactate and zinc gluconate concentrations of 1.0 or 0.3 mM caused rapid development of cytotoxicity. At concentrations of ≤0.1 mM, cells appeared free of visible cytotoxic effects.

Inhibition of rhinovirus replication. In initial experiments, a range of zinc salts was tested for inhibition of rhinovirus type 1A CPE and virus yield in MRC-5 fibroblast monolayers. No clear antiviral effects were seen at zinc salt concentrations (0.01 and 0.001 mM) at which there was no cytotoxity (Table 1). Even a concentration of 0.1 mM, which caused various degrees of cell rounding in uninfected MRC-5 monolayers, was not associated with significant antiviral effects (≥1.0 $\log_{10}$ $TCID_{50}$ reduction), except for zinc oxide and zinc gluconate at 24 h (experiment 2).

Additional tests were conducted with zinc lactate and zinc gluconate in two different cell systems. These salts did not inhibit the development of rhinovirus 1A CPE at nontoxic concentrations in WI-38 or HeLa cell monolayers (Table 2).

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TABLE 1. Effect of zinc salts on rhinovirus type 1A replication in MRC-5 fibroblast monolayers

Salt	Concn[a] (mM)	Viral yield (log_{10} $TCID_{50}$/0.2 ml)[b]	
		Expt 1 (48 h)	Expt 2 (24 h)
Acetate	0.1	4.4	3.0
	0.01	5.5	3.5
	0.001	5.5	ND
Chloride	0.1	4.0	4.0
	0.01	4.5	4.0
	0.001	5.5	ND
Lactate	0.1	4.0	3.0
	0.01	5.0	3.5
	0.001	5.5	ND
Oxide	0.1	4.5	2.5
	0.01	6.0	4.0
	0.001	5.5	ND
Sulfate	0.1	4.5	3.0
	0.01	5.0	4.0
	0.001	5.5	ND
Carbonate	0.1	4.0	ND
	0.01	5.0	3.5
	0.001	5.0	ND
Gluconate	0.1	6.0	2.5
	0.01	5.0	3.5
	0.001	5.5	ND
Virus controls		4.5–5.5[c]	3.0–4.0[c]

[a] At 0.1 mM, all of the zinc salts except the sulfate caused cell rounding (cell toxicity) in uninfected monolayers within 24 h of exposure.
[b] The initial inoculum was 100 $TCID_{50}$s per monolayer in experiment 1 and 1,000 $TCID_{50}$s per monolayer in experiment 2. ND, Not determined.
[c] Range of three separate harvests.

In both HeLa and WI-38 cells, the lactate and gluconate salts delayed CPE development by rhinovirus type 39 by approximately 1 day compared with that in control monolayers.

In yield reduction experiments in WI-38 cells, the highest nontoxic zinc salt concentrations tested were associated with only modest ($\leq$0.75 log_{10} $TCID_{50}$) reductions in rhinovirus type 39 yield and no reductions in rhinovirus type 1A yield (Table 3). In HeLa cells the highest nontoxic concentration of zinc salts (0.1 mM) reduced rhinovirus type 39 yields by $\leq$1.25 log_{10} compared with that of the virus control at both 24 and 48 h after inoculation. Zinc gluconate also reduced rhinovirus serotype 1A yields by 1.0 log_{10} $TCID_{50}$ in HeLa cells at 24 h, and both salts reduced virus yield at 48 h. Lower concentrations of either salt were not inhibitory in either cell type.

TABLE 2. Effects of zinc gluconate and zinc lactate on inhibition of rhinovirus CPE

Cell type	Virus serotype	Salt (concn [mM])[a]	Time to rhinovirus CPE (days)[b]
WI-38	39	Control	3
		G (0.03)	4
		L (0.03)	4
	1A	Control	2
		G (0.03)	2
		L (0.03)	2
HeLa	39	Control	2
		G (0.10)	3
		L (0.10)	3
	1A	Control	3
		G (0.10)	3
		L (0.10)	3

[a] Maximum nontoxic concentration. G, Gluconate; L, lactate.
[b] Days after inoculation until occurrence of typical rhinovirus CPE involving $\geq$25% of the monolayers.

TABLE 3. Effects of various zinc salt concentrations on rhinovirus yields at 24 or 48 h after inoculation of cell monolayers with virus[a]

Cell type	Virus serotype	Time (h) to harvest	Zinc salt[b]	Virus yield (log_{10} $TCID_{50}$/0.2 ml) at salt concn (mM)[c]				
				0	0.1	0.03	0.01	0.003
WI-38	39	24	G	1.50	Toxic	0.75	1.75	0.75
			L		Toxic	1.25	1.75	1.25
	1A	24	G	3.50	Toxic	3.50	3.50	3.50
			L		Toxic	3.50	3.50	4.00
HeLa	39	24	G	2.50	1.25	2.25	2.00	ND
			L		1.50	2.25	2.50	ND
		48	G	5.00	4.00	5.00	6.00	ND
			L		4.00	5.00	4.75	ND
	1A	24	G	3.00	2.00	2.50	3.25	ND
			L		2.50	2.50	3.25	ND
		48	G	4.50	3.50	4.00	4.50	ND
			L		3.50	4.50	4.75	ND

[a] Monolayers were incubated with the salts 1 h before and for 24 or 48 h after virus inoculation.
[b] G, Gluconate; L, lactate.
[c] Concentrations of 1.0 and 0.3 mM caused visible cytotoxicity in HeLa cells with both salts. ND, Not determined.

In infectivity endpoint titration experiments, duplicate monolayers were inoculated with serial 0.5 log_{10} dilutions of the test virus. After a 1-h adsorption period, maintenance medium containing the zinc salt at a fixed concentration was added, and the monolayers were observed daily for rhinovirus CPE. No reductions in titer compared with that of nonexposed infected monolayers were seen in WI-38 fibroblast monolayers (Table 4). In some of these experiments, a zinc gluconate concentration (0.1 mM) which caused overt cytotoxicity in other experiments did not decrease the observed virus titer.

Comment. The results of these studies indicate that the tested zinc salts are neither particularly potent nor selective in their in vitro antirhinovirus activity. In most experiments,

TABLE 4. Endpoint titrations of rhinovirus in presence or absence of zinc gluconate in WI-38 human embryonic lung fibroblast monolayers[a]

Expt	Virus serotype	Salt concn (mM)	Virus titer (log_{10} $TCID_{50}$/0.2 ml)
1	1A	1.0	Toxic
		0.1	4.00
		0.01	4.25
		0	4.40
2	1A	0.5	Toxic
		0.1	4.00
		0	3.90
3	39	0.5	Toxic
		0.1	4.00
		0	4.00

[a] Endpoint virus titers were determined by inoculating serial 0.5 log_{10} dilutions of a virus suspension onto duplicate monolayers in the presence of zinc gluconate at the indicated concentration.

the ratio of the maximal nontoxic concentration to the minimal concentration demonstrating antiviral activity was very low. For zinc gluconate and zinc lactate, this ratio approached one for human embryonic lung fibroblasts and was less than five for HeLa cells. In other experiments, no particular advantage was found for a number of other zinc salts (Table 1). Previous studies describing the antirhinovirus activity of zinc chloride did not give comparable data regarding its therapeutic index or used short-term experiments, such as single-cycle yield reduction assays. Our findings suggest that the possible beneficial effects reported for zinc gluconate in treating colds may not be related directly to selective antirhinovirus activity.

The concentration of zinc salts which appeared to have inhibitory activity in some of our in vitro experiments (0.1 mM) exceeds the zinc concentration found in normal human serum (~0.02 mM), in which almost all zinc is protein bound (8). Zinc concentrations in the respiratory mucosa or in respiratory secretions have not been well characterized under normal conditions or after oral zinc lozenge use. These in vitro studies do not of course exclude the possibility of immunomodulating (8) or other effects unrelated to antiviral activity which could account for the reported beneficial effects of zinc gluconate lozenges in treating common colds. However, the findings emphasize the need for careful assessments of antiviral activity in future clinical trials using zinc salts for the prevention or treatment of colds caused by rhinoviruses.

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