Antiviral Research, 5 (1985) 67–73
Elsevier

AVR 00156

Thio-analogues of inorganic pyrophosphate inhibit the replication of influenza virus A in vitro

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(Received 14 May 1984; accepted 6 August 1984)

Summary

Mono- and bisthiopyrophosphate can inhibit the replication of influenza virus A/X49 in Madin–Darby canine kidney (MDCK) cells at concentrations at which no cytotoxic effect is observed after 3 days. The thiopyrophosphate analogues inhibit the RNA transcriptase activity of this virus possibly by chelating with an essential metal ion in the transcriptase complex. [^{31}P]NMR spectroscopy indicates that bisthiopyrophosphate coordinates to zinc through sulphur and magnesium through oxygen which may influence the inhibitory properties of this compound with metal-containing enzymes.

influenza; inhibition; monothiopyrophosphate; bisthiopyrophosphate; transcriptase

Introduction

The antiviral activity of pyrophosphate analogues such as phosphonoacetic (1) [1], phosphonoformic (2) [2] and substituted methylene bisphosphonic (3) [3] acids is well known. We have recently observed that the inhibitory activity of these compounds on the RNA transcriptase activity of influenza virus A/X49 appears to be related to their metal-chelating properties, and, in particular, to their ability to complex with zinc ions. On the Pearson 'hard and soft' acid and base scale, zinc ions are classified as 'intermediate' and hence should form complexes with both hard and soft ligands [4]. We now report on the effect of thiopyrophosphates (4) and (5) which contain a potential soft ligand (sulphur) on the replication of influenza virus A/X49 and on its RNA transcriptase.

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$(HO)_2P(O)CH_2COOH$ (1)

$(HO)_2P(O)COOH$ (2)

$(HO)_2P(O)CCl_2P(O)(OH)_2$ (3)

$(HO)_2P(S)OP(O)(OH)_2$ (4)

$(HO)_2P(S)OP(S)(OH)_2$ (5)

Materials and Methods

Monothiopyrophosphate (4) and bisthiopyrophosphate (5) were prepared as described previously [5,6] and the determination of zinc ion stability constants ($K_{d'}$) were carried out as described [3].

Virus type and preparation

The influenza virus A/X49 was a cross between A/England/864/75 and A/PR/8/34 with the H3N2 surface antigens of the A/England strain. The virus was grown in the allantoic sac of fertile hens' eggs and was isolated essentially as described [7]. Eleven-day-old embryonated hens' eggs were inoculated with infected allantoic fluid (0.1 ml of a 10^{-3} dilution in phosphate-buffered saline (PBS)), the eggs were incubated (33°C/48 h) and then chilled (–20°C/2 h). The allantoic fluid was collected and centrifuged (3000 r.p.m./20 min) to remove unwanted egg membranes; from this point onwards all procedures were carried out at 0–4°C. The supernatant was removed and the virus was pelleted by centrifugation (21 000 r.p.m./90 min, 6 × 250 ml rotor). The supernatant was discarded and the virus pellet was allowed to soak overnight in PBS. The pellet was then resuspended in PBS and layered onto a velocity gradient of 10–40% (w/v) sucrose in buffer (30 ml, 10 mM Tris-HCl, pH 7.4) and centrifuged (22 000 r.p.m./1 h, 3 × 65 ml swing-out rotor). The diffuse virus band was collected by bottom puncture and the sucrose was diluted out with PBS to a final volume of 30 ml. The virus suspension was layered onto an equilibrium gradient of 30–70% (w/v) sucrose in buffer (30 ml, 10 mM Tris-HCl, pH 7.4) and centrifuged (20 000 r.p.m./overnight, 3 × 65 ml swing-out rotor). The virus band was collected, diluted with PBS and the virus pelleted by centrifugation (30 000 r.p.m./2 h, 8 × 50 ml rotor). The supernatant was discarded and the pellet was allowed to soak overnight in PBS. The virus was then resuspended in buffer (3 ml, 400 μM Tris-HCl, pH 8.0) and frozen as aliquots at –70°C and thawed once prior to use.

RNA transcriptase assay

The standard reaction mixture contained in 200 μl: 50 mM Tris-HCl, pH 8.0; 5 mM magnesium acetate; 150 mM potassium chloride; 5 mM dithiothreitol; 0.4 mM ApG; 0.25% (v/v) Nonidet P-40; 0.4 mM each of ATP, CTP, GTP and [^{3}H]UTP (5 μCi) and purified virus (10 μl, 2000 HAU). Mixtures were kept at 4°C until zero time of

reaction, polymerisation being initiated by addition of virus. The mixture was kept at 30°C for 12 h during which time the incorporation of tritium into acid-precipitable material increased in a linear fashion. After 1 h, cold saturated sodium pyrophosphate solution (200 µl) followed by cold TCA (2 ml, 10% w/v) was added and the mixture kept on ice for 15 min after thorough agitation. Precipitated material was collected on Whatman GF/C discs which were washed several times with 10% TCA, once with ethanol and dried. The radioactivity of material precipitated on the discs was then determined by scintillation counting using a toluene-based medium. Pyrophosphate analogues were added to the reaction mixtures before addition of virus. The concentration of analogue which inhibited by 50% the incorporation of [^{3}H]uridine into acid-insoluble material was derived from the dose–response curve for each compound.

Antiviral activity in vitro

The haemagglutinin titre of the virus grown at low multiplicity on MDCK cells was determined with and without added pyrophosphate analogue in the following manner. Glass vials (1 cm diam.) were seeded with 2×10^5 MDCK cells and 1 ml of maintenance medium (Dulbecco's modified Eagle's medium; DMEM) containing glutamine (200 µM), 5% foetal calf serum and penicillin/streptomycin (50 units/ml) was added. The cells were incubated overnight at 37°C in a CO_2 incubator, then the maintenance medium was removed and the cell sheet washed twice with a solution of 0.1% BSA in PBS. The vials were inoculated with influenza virus A/X49 at a m.o.i. of 0.01 infectious virus particles/cell in 100 µl volume. After the virus had been allowed to absorb onto the cells for 1 h at room temperature, the inoculum was removed and 500 µl of maintenance medium (DMEM + glutamine + 0.1% BSA + penicillin/streptomycin + 25 µg trypsin/ml) which contained 0, 10, 50 or 100 µM of test compound were added. The cells were incubated at 33°C for 3 days in a gassed incubator after which time the medium was removed and the haemagglutinin assay carried out (Table 1).

A plaque assay was also carried out in the following manner. MDCK cells were seeded onto 5 cm plastic dishes at 3.0×10^6 cells/plate. These were grown at 37°C until confluent in maintenance medium. The confluent monolayers were washed with PBS and aspirated to dryness. Virus suspension (100 µl) was added to the cell sheets and left for 1 h at room temperature. The inoculum was removed and the cells were overlaid with an agar medium which contained 0.1% BSA, 2.5 µg/ml crystalline trypsin and 0.1% DEAE Dextran. The plates were incubated at 33°C for 4–6 days, stained with neutral red and the plaques counted. The results are presented in Table 2. Samples from the last wash of the cell monolayers after absorption of the virus were retained and plaques to show that haemagglutinin and infectious virus yields were not due to residual non-absorbed virus.

No cytotoxic effects could be observed with either thiopyrophosphate at 100 µM after 3 days.

TABLE 1

Effect of mono- and bisthiopyrophosphates on haemagglutinin titre of influenza virus A/X49 grown on MDCK cells (mean of two assays)

Compound	Concentration (μM)	HAU (log_{10} units/ml)
Cell control		<2.0
Virus control	0	3.65
Monothiopyrophosphate	100	2.64
	50	3.10
	10	3.45
Bisthiopyrophosphate	100	2.10
	50	2.70
	10	3.60

TABLE 2

Yield reduction assay by measurement of infectious influenza A/X49 virus grown on MDCK cells (mean of two assays)

Compound	Concentration (μM)	PFU/ml
Cell control		⩽10
Virus control	0	4.77×10^7
Monothiopyrophosphate	100	1.05×10^6
	50	1.5×10^7
	10	4.25×10^7
Bisthiopyrophosphate	100	4.2×10^5
	50	9.7×10^6
	10	5.0×10^7

[^{31}P]NMR measurements

These were carried out at 308 K and 36.44 MHz on a Bruker WH90 spectrometer, chemical shifts are recorded relative to H_3PO_4 (0 ppm). Solutions of tetrasodium pyrophosphate or tetra(triethylammonium)bisthiopyrophosphate (15 mg/ml) were prepared in 0.1 M triethanolamine hydrochloride buffer, pH 8.2, the latter being made up in water which had previously been passed down a small Chelex 100 chelating column (Na^+ form, Biorad Inc.). To the pyrophosphate or bisthiopyrophosphate solution (1 ml, 1 mol) was added a solution in deuterium oxide (also treated with Chelex 100, 1 ml) which contained 0.8 mol of magnesium chloride, zinc chloride or cadmium bromide. After mixing the [^{31}P]NMR spectra were recorded (Table 3).

Results

Both mono- and bisthiopyrophosphate inhibit the replication of influenza virus

TABLE 3

[^{31}P]NMR chemical shifts (ppm) of solutions of pyrophosphate or bisthiopyrophosphate at pH 8.2 after the addition of metal ions

Metal	P_2O_7	$P_2O_5S_2$
None	-6.045	29.693
Mg^{2+}	-5.254	32.418
Zn^{2+}	-4.839	21.849
Cd^{2+}	-5.643	20.714

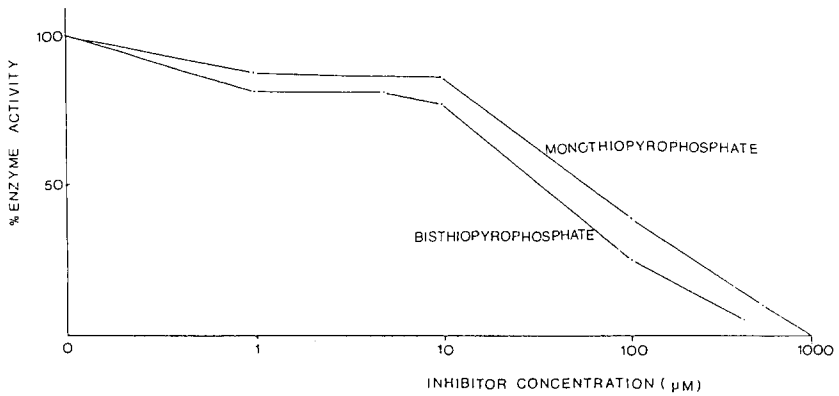

Fig. 1. Inhibition of RNA transcriptase of influenza virus A/X49 by mono- and bisthiopyrophosphate.

A/X49 in MDCK cells (Table 1). The haemagglutinin titre of 3.65 $\log_{10}$ units/ml for the control in the absence of thiopyrophosphate was reduced to 2.64 $\log_{10}$ units/ml for mono- and 2.10 $\log_{10}$ units/ml for bisthiopyrophosphate at 100 μM. Similarly a virus yield of 4.77×10^7 PFU/ml in the virus control was reduced to 1.05×10^6 PFU/ml for mono- and 4.2×10^5 PFU/ml for bisthiopyrophosphate at 100 μM. No cytotoxic effect was observed with either compound after 3 days in our assays. The compounds inhibit the RNA transcriptase activity of the virus (Fig. 1) producing a 50% inhibition of the transcriptase at 60 μM (monothiopyrophosphate) and 33 μM (bisthiopyrophosphate). Both compounds were good chelators of zinc ions and the $pK_{d'}$ at pH 8.2 [3] was 5.4 (monothiopyrophosphate) and 6.3 (bisthiopyrophosphate); under similar conditions inorganic pyrophosphate had a $pK_{d'}$ of 5.7. From the [^{31}P]NMR spectra of solutions of bisthiopyrophosphate after additions of metal ions it appears that zinc ions bind through sulphur and magnesium ions bind through oxygen (Table 3).

Discussion

Pyrophosphate analogues are of interest as antiviral compounds as they are product

inhibitors of polymerase/transcriptase enzymes and hence could inhibit viral nucleic acid synthesis without being metabolised. However, pyrophosphate is involved in many enzymic reactions in the host cells and hence pyrophosphate analogues may be cytotoxic as is the case with dichloromethylene bisphosphonate (3) (Cload, P.A. and Hutchinson, D.W., unpublished observations, 1983). Thus, it is significant that the mono- and bisthiopyrophosphates do not appear to be cytotoxic to MDCK cells after 36 h at concentrations at which they cause an appreciable reduction in the haemagglutinin titre of influenza virus A/X49. The thiopyrophosphates cause a 50% inhibition of the RNA transcriptase of this virus at concentrations similar to that for phosphonoformate (35 μM) and lower than that for inorganic pyrophosphate (125 μM).

We have suggested that the inhibitory effect on the RNA transcriptase of influenza virus A caused by pyrophosphate analogues is related to their ability to chelate zinc ions [3], and we believe that it is significant that both thiopyrophosphates are good chelators of zinc ions. The introduction of the 'soft' sulphur atom (on the HSAB scale) into the pyrophosphate residue might well alter the chelating properties of the molecule. It was, therefore, of interest to determine whether zinc ions coordinated to oxygen or sulphur in bisthiopyrophosphate. [^{31}P]NMR measurements (Table 3) show that when magnesium, zinc or cadmium ions were added to a solution of pyrophosphate in triethanolamine buffer at pH 8.2, the pH of the transcriptase assay, only small shifts (ca. 1 ppm) of the ^{31}P signal occurred, presumably due to the formation of complexes with the metal ions coordinated to the oxygen of the pyrophosphate.

In the case of bisthiopyrophosphate at pH 8.2, addition of a 'hard' metal ion (magnesium) produced a downfield shift of less than 2 p.p.m. On the other hand, addition of soft metal ions (zinc or cadmium) produced a large upfield shift of 8–9 ppm of the [^{31}P]NMR signal. Since cadmium ions have been shown to complex with sulphur in nucleoside thiophosphates [8], the [^{31}P]NMR results suggest that zinc ions probably coordinate to bisthiopyrophosphate through sulphur. If this method of coordination also occurs at the essential metal ion (probably zinc) in the transcriptase complex of influenza virus A, this may account for the difference in inhibitory activity between inorganic pyrophosphate and its thio-analogue.

Acknowledgements

We thank Miss L. McLain for the determination of the antiviral activity of the thio-analogues in vitro, and Mr. R.C.K. Jennings for the [^{31}P]NMR measurements.

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