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Cellular toxicity of pyrophosphate analogues

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As a result of the reported antiviral effects of the pyrophosphate analogues, PFA* [1, 2] and PAA [3], systematic efforts have been initiated to find similar structures with improved antiviral activity and low toxicity. Structure–activity relations have been reported for the effect of several pyrophosphate analogues on viral nucleic acid polymerase [4–6] and virus multiplication [4, 5, 7, 8]. In addition to PFA and PAA, methylene hydroxydiphosphonate (MHDP) and hypophosphate have been inhibitory to viral polymerases and virus multiplication [4, 6]. A high concentration of PFA is required to affect macromolecular synthesis and cell proliferation and this inhibition is reversible [9]. PAA is also reported to be non-toxic at concentrations where herpes virus replication is inhibited [3] but is cytotoxic at higher concentrations [10]. This investigation was undertaken in order to eliminate cytotoxic pyrophosphate analogues from further evaluation as antiviral drugs. A comparison between cell and animal toxicity tests is made.

Material and methods

Chemical and isotopes. The source of the pyrophosphate analogues tested has been described previously [4].

[Methyl-^{3}H]thymidine (20.0 Ci/mmole), [^{3}H(G)]-L-proline (5.0 Ci/mmole), [^{3}H(G)]-L-trypophan (5.96 Ci/mmole), [4,5-^{3}H(N)]-L-lysine (72.1 Ci/mmole), [2,3-^{3}H]-L-aspartic acid (17.3 Ci/mmole), [2,3,4-^{3}H]-L-valine (11.18 Ci/mmole), [^{32}P]pyrophosphate were from New England Nuclear, Boston, Mass, U.S.A. and [5-^{3}H]uridine (25 Ci/mmole) was from The Radiochemical Centre, Amersham, England.

Cell cultures. AGMK cells were grown in MEM supplemented with 10% foetal calf serum, 20 mH HEPES buffer pH 7.2, penicillin (70 μg/ml) and streptomycin (100 μg/ml) from Gibco Bio-Cult, Paisley, Scotland. Normal embryonic lung fibroblasts, WI-38 (Flow Laboratories, Ayrshire, Scotland), was grown in the same medium with the addition of 1% non-essential amino acids. All cell-cultures were grown in a humidified 5% CO_2: air mixture at 37° and were routinely screened for mycoplasma contamination by the uridine/uracil incorporation method [11].

Macromolecular synthesis. 1 ml (10^5 cells) of a trypsinized cell suspension was added to each well of a 24-well tissue culture cluster dish (Costar, Cambridge, Mass, U.S.A., 16 mm in diameter) and preincubated for about 15 hr. Each substance was added to two wells and pulse-labelling was carried out with 5 μCi of radioactive precursor during the last 20 min of each incubation period as indicated in figures and tables. The incorporation was terminated by rapid removal of medium with a six-pipe suction head and the cells were washed five times with ice-cold 5% trichloroacetic acid. The cells were then incubated in 0.5 ml of 0.3 M NaOH per well at 80° for 15 min. Samples were counted in 10 ml Biofluor (New England Nuclear, Boston, Mass, U.S.A.) scintillation solution. The DNA, RNA and protein synthesis was determined as the incorporation of labelled precursor into the trichloroacetic acid insoluble product as compared to control cells treated with solvent. Calculated percentages are derived from the mean of two values when the test substance was added to two duplicate wells in two or more different experiments.

Cell proliferation. Three ml of a cell suspension (0.5×10^5 cells/ml) was seeded in each well of a 6-well tissue culture cluster dish (Costar, Cambridge, Mass, U.S.A., 35 mm in diameter) and incubated overnight to assure that the growth rate was normal. Incubation with hypophosphate started at a cell number of 2×10^5 cells per well. At specified times duplicate wells were each washed with 3 ml of PBS and mildly trypsinized in trypsine-EDTA solution (Gibco Bio-Cult) for 5 minutes at 37°. The trypsinized cells were diluted to 5 ml in particle-free buffer (Baker Diagnostics, Winchester, U.S.A.) and counted in a multi-channel electronic cell counter (model 134, Analysinstrument AB, Stockholm, Sweden). When reversal was studied the inhibited cells were washed once with 3 ml of prewarmed PBS per well, fresh medium with serum was added and incubation was continued up to 56 hr after addition of substance. The presented cell numbers are the mean of two different experiments.

Results and discussion

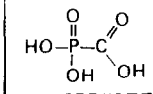
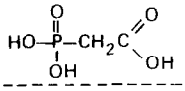
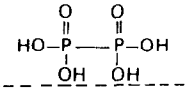
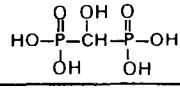
Inhibition of cellular metabolism by pyrophosphate analogues. PAA has been shown to interact with the pyrophosphate binding site of herpes virus DNA polymerase [12] thereby exerting the antiviral activity. The inhibition by pyrophosphate analogues could therefore be directed towards processes dependent on release of pyrophosphate, such as polymerization of nucleoside triphosphates and protein synthesis. The effects of PFA, PAA, MHDP and hypophosphate on cell macromolecular synthesis is shown in Table 1(a). These analogues, which previously have been reported to inhibit virus multiplication [4, 7, 8] showed different effects on DNA, RNA and protein synthesis in uninfected cells. PFA and PAA did not inhibit DNA, RNA or protein synthesis at a concentration of 500 μM. In contrast, hypophosphate and MHDP were strong inhibitors of macromolecular synthesis with ID_{50} of 4–5 μM for hypophosphate and 35–50 μM for MHDP. Several pyrophosphate annalogues not active on virus multiplication were, at 500 μM without effect on cell macromolecular synthesis (Table 1(b)). The inhibition by hypophosphate and MHDP was not restricted to AGMK cells while DNA synthesis of the normal human fibroblast, WI-38, was also inhibited to the same extent by the compound (data not shown).

Irreversible inhibition of cell proliferation by hypophosphate. Hypophosphate rapidly reduced the growth rate of AGMK cells and after 24 hr, proliferation was completely stopped (Fig. 1). Although both 10 and 100 μM of hypophosphate were able to stop the cells from dividing, 10 μM seemed to allow more cells to divide before the inhibition was complete. The lesser effect of 10 μM was accompanied with an increase in mean cell volume (data not shown), which indicates an uncoupling between the normal cell volume growth and cell division. This increase in cell volume has been described for several chemotherapeutic agents except for specific inhibitors of protein synthesis [13].

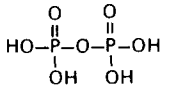
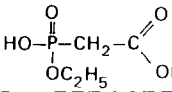
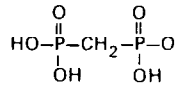
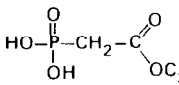
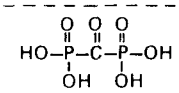
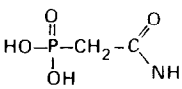
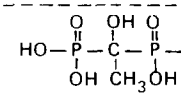
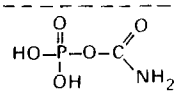
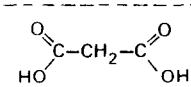
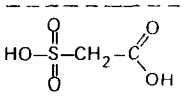
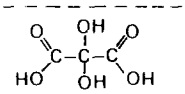
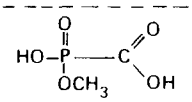
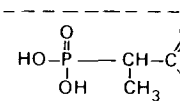
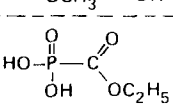
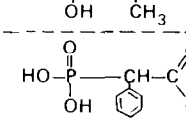
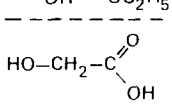
* The abbreviations used are: AGMK cells: African green monkey kidney cells; EDTA: ethylenediaminetetraacetic acid; HEPES: *N*-2-hydroxyethyl piperazine-*N'*-2-ethane-sulfonic acid; ID_{50}: dose giving 50% inhibition compared to control; LD_{50}: dose giving 50% mortality; MEM: minimal essential medium (Eagle); MHDP: methylene hydroxydiphosphonate; PAA: phosphonoacetic acid; PBS: phosphate buffered saline; PFA: phosphonoformic acid; INN: foscarnet sodium.

Table 1. Effects of pyrophosphate analogues on cell metabolism and virus multiplication. (a) Cell toxicity of pyrophosphate analogues with antiviral activity. (b) Pyrophosphate analogues with no antiviral activity and no cell toxicity

a

Compounds with antiviral activity	ID_{90} (μM) HSV-1 plaque reduction	ID_{50} (μM) DNA synthesis	RNA synthesis	Protein synthesis
 PFA	100	>500	>500	>500
 PAA	100	>500	>500	>500
	100	4	5	5
 MHDP	100	35	50	40

b

Compounds without antiviral- and celltoxic activity	
	
	
	
	
	
	
	
	

Cell toxicity was measured as incorporation of labelled precursor into DNA, RNA and protein as described in Methods. ID_{90} is the concentration where HSV-1 plaque formation was reduced by 90% [4].

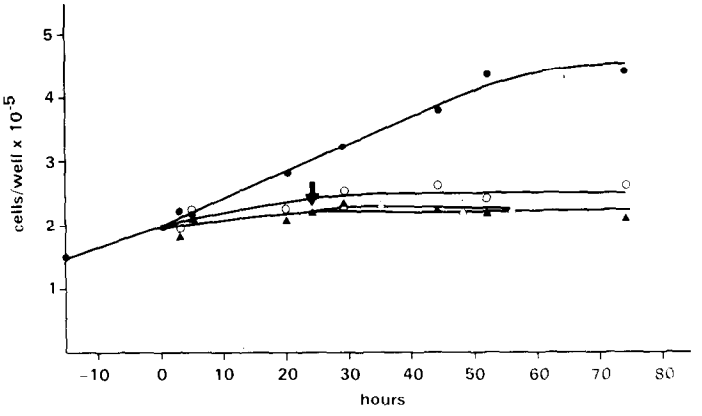


Fig. 1. Effect of hypophosphate on cell proliferation and the effect of removing the compound. The multiplication of GMK cells was followed after incubation with 10 μM and 100 μM of hypophosphate. At 24 hr after addition, hypophosphate was removed (arrow) and cell multiplication was followed for another 32 hr. Number of cells was measured in an electronic cell counter as described in Methods. Symbols: (●) control; (○) 10 μM hypophosphate; (▲) 100 μM hypophosphate; (△) 100 μM hypophosphate removed after 24 hr.

When cell division had stopped after 24 hr with 100 μM hypophosphate, the medium was changed and incubation was contained without hypophosphate for another 32 hr. Even after this long time of recovery no increase in cell number could be observed (Fig 1), which indicates that one or more of the processes necessary for cell division had been irreversibly damaged. This is different from the reversible inhibition observed at the high (10 mM) concentration of PFA [9].

Comparison between cellular and animal toxicity. Cellular toxicity for the 4 pyrophosphate analogues with antiviral activity were compared to the dose giving 50% mortality of mice after intraperitoneal (i.p.) treatment. The comparison was made on a molar basis between ID_{50} concentration for DNA synthesis in AGMK cells and the LD_{50} concentration for the same compound. The molarity of the compounds in mice were calculated from the LD_{50} values in mg/kg from a body weight of 20 g and assuming an accesible body volume of 8.5 ml. The LD_{50} and ID_{50} values for pyrophosphate analogues were highly correlated (Table 2). This correlation could possibly be even higher if pharmacokinetic parameters for each compound were known. It is not certain that such a correlation between *in vitro* toxicity will be found for other compounds. It is, however, likely that compounds toxic, and especially irreversibly toxic, in *in vitro* experiments will also have toxic preperties *in vivo*.

The mechanism of inhibition by hypophosphate and MHDP on cell functions cannot be determined conclusively from the present results. It has been reported that pyrophosphate analogues are inhibitory to succinic dehydrogenases [14]. Since PAA and hypophosphate had the same inhibition constant for succinic dehydrogenase [14] it was unlikely that the toxic effect observed for hypophosphate, but not for PAA, was due to an inhibition of this enzyme. Hypophosphate is also reported to inhibit phosphate esterification [15] but at concentrations (1000–3000 μM) almost three logs higher than the ID_{50} for DNA synthesis (4 μM). An increase in alkaline phosphatase activity has been observed for cells treated with diphosphonates [16] and this inhibits cell growth. The rate of this inhibition is, however, much slower [16] than that observed for MHDP (data not shown).

Table 2. Comparison of LD_{50} and ID_{50} values for pyrophosphate analogues

	ID_{50} (mg/kg)	LD_{50} (μM)	ID_{50} (μM)
Hypophosphate	1	8	4
MHDP	37.5	300	35
PFA	>1000	>4000	1000
PAA	>1000	>4000	1000

The LD_{50} concentration in mice and the ID_{50} for DNA synthesis aftter 24 hr was determined as described in Methods.

In summary, the present results show that PFA and PAA are non-toxic to cells at concentrations where an antiviral effect is observed and therefore selective antiviral inhibitors. The antiviral effect of hypophosphate and MHDP could, on the other hand be explained by their cell-toxicity. Hypophosphate is also shown to irreversibly inhibit cell division in contrast to the reversible effect of PFA [9]. Results from the present *in vitro* tests were highly correlated to the results from the *in vitro* toxicity tests which indicate that cell culture tests may reduce the amount of animal experiments necessary for the evaluation of drug toxicity.

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Marked differences in the inductive effects of two symmetrical hexachlorobiphenyls and the corresponding unsymmetrical isomer on hepatic monooxygenases

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Polychlorinated biphenyls (PCBs) produce a mixed type of induction of hepatic mixed-function oxidases similar to that produced by a mixture of the two classical types of inducers, phenobarbital and 3-methylcholanthrene (3-MC) [1]. The cytochromes induced by PCBs are catalytically, spectrally, electrophoretically, and immunologically identical to those induced by combination of phenobarbital and 3-MC (generally referred to as cytochromes P-450 and P-448 respectively) [2]. Individual isomers, however, have been separated into two groups: those which induce cytochrome(s) P-450 (phenobarbital-type), and those which induce cytochrome P-448 (3-MC-type induction) [3, 4].

Isomers that induce cytochrome P-448 are isosteric with the very potent inducer, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). They contain halogens in the *meta* and *para* positions of both rings (3,4,3′,4′-tetra-; 3,4,5,3′,4′-penta-; and 3,4,5,3′,4′,5′-hexa-) but not in the *ortho* positions. These compounds interact with the cytosolic protein believed to be the receptor for 3-MC and TCDD [4]. From this data, it has been hypothesized that the optimum configuration for binding to the TCDD receptor is a planar rectangle 3×10 Å, with halogens in at least three of the four corners. Presumably, *ortho* halogenation inhibits binding because it increases the energy barrier to rotation [5], which must be overcome for the biphenyl rings to assume a coplanar configuration.

Most PCB isomers are phenobarbital-type inducers, or they are inactive [3]. The structural requirements for phenobarbital-type induction are not clear; however, we noted earlier that, in general, potency seems to be inversely related to the rate of metabolism [3]. Highly chlorinated congeners are more potent than less chlorinated congeners [3], and they are metabolized more slowly than the less chlorinated congeners [6]. Moreover, the presence of two adjacent, unsubstituted carbon atoms decreases potency, possibly by increasing the rate of metabolism. *Para* substitution increases potency among the less chlorinated isomers, but it is not important for highly chlorinated isomers since 2,3,5,2′,3′,5′-hexachlorobiphenyl is as active as 2,4,5,2′,4′,5′-hexachlorobiphenyl [7]. *Para* substitution also decreases the rate of metabolism of the less chlorinated isomers, but would not be expected to have much effect when the compound does not have two adjacent unsubstituted carbon atoms [6].

Most of this work has involved symmetrical PCB isomers. Recently, Dannan *et al.* [8] reported that 2,4,5,3′,4′,5′-hexabromobiphenyl was a mixed inducer, suggesting that the presence of only one *ortho* halogen did not completely block interaction with the TCDD receptor. More recently, Parkinson *et al.* [9, 10] synthesized a number of unsymmetrical congeners containing a 3,4- or 3,4,5-halogenation pattern in one ring and a 2,3,4-; 2,4,5-; or 2,3,4,5-pattern in the second ring. With the exception of 2,4,5,3′,4′,5′-hexachlorobiphenyl, which was a phenobarbital-type inducer in their hands, several of these unsymmetrical isomers (2,4,5,3′,4′-penta-; 2,3,4,3′,4′-penta-; 2,3,4,3′,4′,5′-hexa-; 2,3,4,5,3′,4′,-hexa; and 2,3,4,5,3′,4′,5′-heptachlorobiphenyls) were mixed inducers. The discrepancy between the effects reported for 2,4,5,3′,4′,5′-hexachlorobiphenyl [10] and the corresponding brominated analog [8] has not been explained.

In the present study, we synthesized and examined the inducing properties of 2,3,5,3′,4′,5′-hexachlorobiphenyl (HCB), which contains a 3,4,5,-halogenation pattern in one ring and a 2,3,5-pattern in the other ring. 2,3,5,2′,3′,5′-HCB is a phenobarbital-type inducer [7] whereas 3,4,5,3′,4′,5′-HCB is the most potent of the 3-MC type inducers [3]. This compound, therefore, differs from most other PCB isomers tested in that it is: (1) unsymmetrical; (2) contains one-half of the phenobarbital-induc-