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Isophosphoramide mustard analogues as prodrugs for anticancer gene-directed enzyme-prodrug therapy (GDEPT)★✪

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Two types of prodrugs, benzyl analogues of isophosphoramide mustard (iPAM), activated by cytochrome P450, and acylthioethyl analogues, activated by esterases, were designed. In contrast to iPAM that hydrolyse rapidly, the examined compounds are stable in phosphate-buffered saline and Tris buffer. Benzyl analogues of iPAM are poor substrates for cytochrome P450, are not cytotoxic and posses no antitumour activity. Acylthioethyl analogues of iPAM are good substrates for pig liver esterase, are cytotoxic and exert antitumour activity against L1210 leukaemia in mice. The observed correlation for iPAM analogues between their susceptibility to hydrolysis and cytotoxicity and antitumour activity suggests possible application of these compounds as the prodrugs in gene-directed enzyme-prodrug therapy.

The toxic side-effects of a conventional anticancer therapy are mostly caused by the lack of selectivity of cytostatic drugs against tumour target cells. This selectivity can be achieved, among others, by the use of gene therapy. One of the methods of such therapy is the enzymatic activation of a prodrug in genetically transformed cancer cells, e.g. gene-directed enzyme-prodrug therapy (GDEPT) [1]. In recent years many prodrug/enzyme

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; PBS, phosphate-buffered saline.

systems developed, some of them are already being tested in the clinical trials [2, 3].

Isophosphoramide mustard (iPAM) [4] is the active cytotoxic metabolite of ifosfamide, a cytostatic alkylating drug, widely used in the clinic. The cancerotoxic selectivity of iPAM is low due to its high reactivity towards biomolecules, e.g. DNA [5]. Esters of iPAM, such as ifosfamide, are less cytotoxic and to exert biological activity they require, hydrolytic, spontaneous or enzymatic activation. Recently, glufosfamide, a β-D-glucopyranosyl ester of iPAM, requiring transmembrane glucose transport for its activity, has been developed as a promising anticancer agent [6, 7].

The paper presents studies on the use of iPAM esters as prodrugs for GDEPT. Two activation systems were examined: cytochrome P450 [8] that is known to activate ifosfamide and was already used to increase canceroselectivity of this drug by GDEPT [9], and digestion by esterases, shown to be efficient in activation of several types of pronucleotides [10], e.g. AZT analogues [11].

MATERIALS AND METHODS

iPAM analogues. To a stirred solution of phosphorus oxychloride (0.93 ml, 10 mmol) in dry chloroform (50 ml) at –20°C a solution was added of an appropriate hydroxy compound **1a–g** (10 mmol) and dry triethylamine (1.53 ml, 11 mmol) in dry chloroform (10 ml). After 30 min at –20°C 2-chloroethylamine hydrochloride (2.55 g, 20 mmol) was added in three portions into the reaction mixture. Then, dry triethylamine (3.06 ml, 22 mmol) was added again and the mixture was kept at room temperature for 1 h. The obtained suspension was filtered and solution washed with water (3 × 20 ml) and dried with anhydrous sodium sulphate. The solvent was evaporated and the crude product was purified by column chromatography on silica-gel 60 (30–40 μm) using a mixture of chloroform and ethanol 19:1 (v/v) as an eluent. The obtained compounds were crystallised from a mixture of ethyl ether and n-hexane. The following analogues of iPAM were obtained: benzyl-*N, N′*-bis(2-chloroethyl)phosphorodiamidate (**2a**) [mp. 42–43°C; ^{1}H NMR ($CDCl_3$), δ: 2.94–3.14 (m, 2H), 3.14–3.32 (m, 4H), 3.54 (t, J = 5.3 Hz, 4H), 5.04 (d, J = 8.0 Hz, 2H), 7.35–7.44 (m, 5H); elemental analysis, calc./found: C 42.26/42.31, H 5.51/5.60, N 9.00/8.90, P 9.95/9.87, Cl 22.76/22.98], *p*-methoxybenzyl-*N, N′*-bis(2-chloroethyl)-phosphorodiamidate (**2b**) [mp. 62–63°C; ^{1}H NMR ($CDCl_3$), δ: 2.47 (s, 3H), 2.84–3.05 (m, 2H), 3.16–3.32 (m, 4H), 3.52 (t, J = 5.7 Hz, 4H), 4.97 (d, J = 8.7 Hz, 2H), 7.14–7.30 (m, 4H); elemental analysis, calc./found: C 44.32/44.45, H 5.89/5.89, N 8.61/8.56, P 9.53/9.55, Cl 21.81/22.08], *p*-fluorobenzyl-*N, N′*-bis(2-chloroethyl)phosphorodiamidate (**2c**) [mp. 48–49°C; ^{1}H NMR ($CDCl_3$), δ: 3.04–3.44 (m, 6H), 3.56 (t, J = 5.5 Hz, 4H), 5.00 (d, J = 8.6 Hz, 2H), 6.97–7.10 (m, 2H), 7.34–7.45 (m, 2H); elemental analysis, calc./found: C 40.14/40.65, 4.90/4.84, N 8.51/8.63, P 9.41/9.50, Cl 21.54/21.64], *p*-nitro-*N,N′*-bis(2-chloroethyl)phosphorodiamidate (**2d**) [mp. 74–75°C; ^{1}H NMR ($CDCl_3$), δ: 3.02–3.14 (m, 2H), 3.20–3.39 (m, 4H), 3.60 (t, J = 5.6 Hz, 4H), 5.12 (d, J = 8.1 Hz, 2H), 7.48–7.61 (m, 2H), 8.19–8.31 (m, 2H); elemental analysis, calc./found: C 37.10/37.29, H 4.53/4.55, N 11.80/11.79, P 8.70/8.75, Cl 19.91/19.91], acetylthioethyl-*N,N′*-bis(2-chloroethyl)phosphorodiamidate (**2e**) [mp. 46–47°C; ^{1}H NMR ($CDCl_3$), δ: 2.36 (s, 3H), 3.04–3.45 (m, 8H), 3.61 (t, J = 5.5 Hz, 4H), 4.00–4.14 (m, 2H); elemental analysis, calc./found: C 29.73/29.44, H 5.30/5.29, N 8.67/8.42, P 9.58/9.41, Cl 21.94/22.52], pivaloylthioethyl-*N,N′*-bis(2-chloroethyl)phosphorodiamidate (**2f**) [mp. 57–58°C; ^{1}H NMR ($CDCl_3$), δ: 1.23 (s, 9H), 3.07–3.35 (m, 8H), 3.60 (t, J = 5.4 Hz, 4H), 4.02–4.15 (m, 2H), elemental analysis, calc./found: C 36.16/36.26, H 6.35/6.40, N 7.67/7.56, P 8.48/8.43, Cl 19.41/19.38], benzoylthioethyl-*N,N′*-bis(2-

chloroethyl)phosphorodiamidate (**2g**) [mp. 63–64°C; ^{1}H NMR ($CDCl_3$), δ: 3.08–3.21 (m, 2H), 3.24–3.33 (m, 4H), 3.37 (t, J = 6.4 Hz, 2H), 3.60 (t, J = 5.5 Hz, 4H), 4.19 (dt, J = 6.4, 8.0 Hz, 2H), 7.42–7.50 (m, 2H), 7.55–7.63 (m, 1H), 7.99–7.94 (m, 2H); elemental analysis, calc./found: C 40.53/40.54, H 4.97/4.84, N 7.27/7.32, P 8.04/8.08, Cl 18.41/18.76].

Hydrolytic stability. A 5 mM solution of iPAM analogue **2a–g** (1 ml) in 100 mM Tris/HCl, pH 7.5, or in PBS was incubated at 37°C for 8 and 24 h. HPLC analysis was performed under the following conditions: LDC Analytical system; column Alltech Econosphere C-8, 5 μ, 4.6 × 250 mm; solvent 40% acetonitrile, isocratic; flow 1.5 ml/min; λ 260 nm for **2a–d** and 240 nm for **2e–g**.

Stability in foetal calf serum. A 10 mM solution of iPAM analogue **2a–g** (0.5 ml) in PBS was mixed with foetal calf serum (Gibco, Grand Island, U.S.A., 0.5 ml) and incubated at 37°C for 2, 4, 8, and 24 h. After each incubation time a 50 μl sample was heated at 95°C for 2 min, centrifuged, and analysed by HPLC (conditions as above).

Microsomal digestion. A 0.4 mM solution of benzyl analogue **2a–d** (10 ml) in 0.1 M Tris/HCl, pH 7.5, containing: 5 mM $MgCl_2$, 6 mM glucose-6-phosphate, 0.3 mM NADP, glucose-6-phosphate dehydrogenase (0.8 U/ml) and active or inactive (inactivated at 95°C for 4 min) microsomes [12], equivalent to 1 g of rat liver, were incubated in oxygen atmosphere at 37°C for 10, 20, 40, and 60 min. After each incubation time a sample was removed (2 ml) and chilled on ice. After adding a 0.4 mM solution of **2b** (for **2a**, **2c**, and **2d** digestion) or **2a** (for **2b** digestion) in 0.1 M Tris/HCl, pH 7.5 (0.2 ml), each sample was extracted with ethyl acetate (3 × 3 ml). Organic solutions were combined, concentrated and redissolved in 40% acetonitrile (0.5 ml). The samples obtained were analysed by HPLC under the conditions described above.

As a positive control, microsomal digestion of ifosfamide was performed using cyclophosphamide as an internal standard. HPLC analysis was performed using λ 200 nm [13].

Carboxylesterase and lipase digestion. Acylthioethyl analogue **2e–g** (5 mM, 1 ml) in 0.1 M Tris/HCl, pH 7.5, was incubated at 37°C in the presence of pig liver esterase (Sigma E-3019, 10 U/ml) or lipase from *Candida rugosa* (Sigma L-8525, 10 000 U/ml) for 15, 30 min, 1, 2, 4, 8, and 24 h. After each incubation time a 50 μl sample was heated at 95°C for 2 min, centrifuged, and analysed by HPLC.

***Cytotoxic activity assay* in vitro**. The following established human cancer cell lines were used: KB (oral carcinoma), HL60 (leukaemia), HCV29T (bladder cancer), SW707 (rectal adenocarcinoma), and MOLT4 (leukaemia).

SRB test. The cytotoxicity assay was performed after a 72-h exposure of the cultured cells to varying concentrations (from 0.1 to 100 μg/ml; each concentration was tested in triplicate) of the agents. The cells attached to the plastic wells were fixed by gently layering cold 50% trichloroacetic acid on the culture medium in each well. The plates were incubated at 4°C for 1 h and then washed five times with tap water. Background absorbance was measured in wells filled with cell-free culture medium. The cellular material fixed with trichloroacetic acid was stained with 0.4% sulphorhodamine B dissolved in 1% acetic acid for 30 min. Unbound dye was removed by rinsing (4 ×) with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of absorbance (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Multiskan RC photometer. Each experiment was repeated 3–5 times.

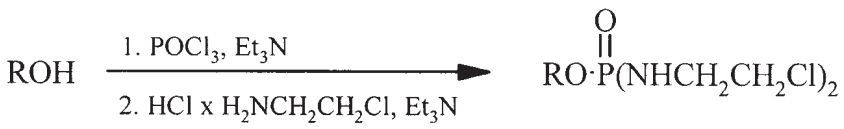
MTT test. This technique was applied for cytotoxicity screening against leukaemia cells growing in suspension culture. The assay was performed after a 72-h exposure to varying concentrations (from 0.1 to 100 μg/ml) of the agents. For the last 3–4 h of incubation 20 μl of MTT solution was added to each well (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide (Sigma, St. Louis, MO, U.S.A.); stock solution: 5 mg/ml). Viable cells reduce in their mitochondria the pale yellow MTT to a navy blue formazan, so the more viable cells are present in the well, the more MTT will be reduced to formazan. When incubation time was completed, lysing mixture (80 μl) was added to each well (lysing mixture: 225 ml dimethylformamide, 67.5 g sodium dodecyl sulphate, both from Sigma (St. Louis, MO, U.S.A.) and 275 ml of distilled water). After 24 h, after formazan crystals had been dissolved, the absorbances of the samples were read on a Multiskan RC photometer (Labsystems) at a 570 nm.

***Antitumour activity* in vivo**. The antitumour effect of the agents was evaluated *in vivo* after single administration into mice bearing advanced L1210 leukaemia (day 3). CDF1 mice were inoculated i.p. with 10^5 ascitic tumour cells suspended in 0.2 ml of PBS. The experiments were conducted and the activity of the compounds tested evaluated according to the NIH/NCI *in vivo* standard screening protocols. The effective doses (ED_{50}) causing 50% increase of lifespan of treated animals over control were estimated graphically from least square-fitted dose–effect curves.

RESULTS AND DISCUSSION

Benzyl analogues of iPAM **2a–d** were obtained in one-pot reaction of appropriate *para* substituted benzyl alcohols with phosphorus oxychloride in the presence of triethylamine (Scheme 1). The obtained dichlorophosphate intermediate was reacted, without isolation, with 2-chloroethylamine hydrochloride in the presence of triethylamine. This method was previously established by us for the synthesis of isotopically labelled iPAM [14]. In the same way acylthioethyl analogues of iPAM **2e–g** were prepared with good yields (Table 1). Appropriate acylthioethyl alcohols were synthesized by two methods, one using thioacids and 2-iodoethanol [15] and another using acyl chlorides and 2-mercaptoethanol [16]; the second method proved to be more convenient.



1a-g **2a-g**

1a, **2a** R = $C_6H_5CH_2$
1b, **2b** *p*-$CH_3C_6H_4CH_2$
1c, **2b** *p*-$FC_6H_4CH_2$
1d, **2d** *p*-$NO_2C_6H_4CH_2$
1e, **2e** $CH_3COSCH_2CH_2$
1f, **2f** $(CH_3)_3CCOSCH_2CH_2$
1g, **2g** $C_6H_5COSCH_2CH_2$

Scheme 1.

One of the crucial requirements for a prodrug to be used in GDEPT is good stability under physiological conditions providing enough time to penetrate the cells. HPLC analysis showed that all iPAM analogues **2a–g** are completely stable when incubated for up to 24 h in Tris/HCl, pH 7.5, and PBS buffers. Moreover, incubation in the presence of foetal calf serum showed no digestion of compounds **2a–d** and some hydrolysis of **2e**

Table 1. Yields of synthesis and selected physicochemical data of the synthesized iPAM analogues

Compound	Yield [%]	t_R[a] [min]	λ_{max} ($\varepsilon \times 10^{-3}$)[b] [nm]	δ_{31P}[c] [ppm]	m/z[d]
2a	64	9.50	257.6 (0.02)	15.02	311.4 (M+H)
2b	43	13.46	261.7 (0.12)	14.90	325.1 (M+H)
2c	39	10.43	262.8 (0.34), 269.0 (0.32)	15.24	329.0 (M+H)
2d	45	10.02	272.2 (3.5)	15.17	356.0 (M+H)
2e	47	5.51	230.4 (4.2)	14.79	323.1 (M+H)
2f	63	15.10	232.5 (3.6)	14.75	365.4 (M+H)
2g	84	15.33	240.7 (9.8), 265.8 (9.1)	14.80	385.1 (M+H)

[a]HPLC, for conditions see Materials and Methods; [b]UV, solution in H_2O; [c] ^{31}P NMR spectra in $CDCl_3$; [d]chemical ionization mass spectrometry.

($t_{1/2}$ 6 h 20 min), **2f** ($t_{1/2}$ 12 h 40 min), and **2g** ($t_{1/2}$ 10 h 20 min). This limited degradation was presumably caused by non-specific esterases present in the serum. A similar pattern of stability in serum was found for acylthioethyl analogues of 3′-azido-2′,3′-dideoxythymidine 5′-monophosphate [15].

Another important factor determining the applicability of a prodrug in GDEPT is its enzymatic activation. Benzyl esters of iPAM **2a–d** were examined as potential substrates for cytochrome P450-mediated hydroxylation. Such hydroxylation on the benzylic carbon should produce a hemiacetal intermediate that should decompose with a release of iPAM and benzaldehyde. It was found that during 1 h of *in vitro* microsomal incubation [17] of **2a–d** these compounds were digested in less than 10%. Under the same conditions 59% of ifosfamide was digested. Such results suggest that benzyl analogues of iPAM **2a–d** are poor substrates for cytochrome P450. Much better microsomal activation was previously achieved for benzyl analogues of phosphoramide mustard, an active metabolite of cyclophosphamide [18]. One can assume that higher steric hindrance around the benzylic carbon in benzyl analogues of iPAM, compared with the steric hindrance in phosphoramide mustard derivatives, is responsible for the observed difference in reactivity of these two types of compounds. The acylthioethyl analogues of iPAM **2e–g** were exposed to hydrolysis by an esterase and lipase. Compounds **2e–g** were substrates for these enzymes (Table 2). The rate of hydrolysis depended on the type of acyl substituents; for **2e** the reaction was the fastest and for **2f** the slowest.

Table 2. Esterase and lipase digestion of acylthioethyl iPAM analogues

Compound	Hydrolysis by esterase (%)				Hydrolysis by lipase (%)			
	0.5 h	1 h	2 h	4 h	1 h	2 h	4 h	8 h
2e	29	39	55	64	12	23	40	52
2f	13	22	27	49	6	13	24	35
2g	16	26	41	55	6	15	27	39

The *in vitro* cytotoxic activity of the synthesized iPAM analogues **2a–g** was examined against human tumour cell lines – KB oral carcinoma and HL60 leukaemia (Table 3). iPAM was used as a reference compound in this study. The benzyl analogues **2a–d** and the pivaloylthioethyl compound **2f** did not reveal any cytotoxic activity against the KB cell

Table 3. Cytotoxic activity *in vitro* of iPAM analogues against human cancer cell lines

Compound	ID_{50} [μg/ml]				
	KB	HL60	HCV29T	SW707	MOLT4
iPAM	34.6 ± 1.1	2.4 ± 1.3	10.2 ± 1.7	40.6 ± 1.4	19.0 ± 1.9
2a	Neg[a]	Neg	ND[b]	ND	ND
2b	Neg	Neg	ND	ND	ND
2c	Neg	Neg	ND	ND	ND
2d	Neg	28.8 ± 1.2	ND	ND	ND
2e	16.9 ± 1.3	21.0 ± 1.2	0.12 ± 1.7	4.8 ± 1.2	0.34 ± 2.1
2f	Neg	35.0 ± 2.1	ND	ND	ND
2g	31.7 ± 1.0	35.0 ± 1.3	1.3 ± 1.2	12.5 ± 1.2	2.7 ±1.8

[a]No cytotoxicity was observed at concentrations up to 100 μg/ml; [b]not determined.

Table 4. Antitumour activity of iPAM analogues against L1210 leukemia in mice

Compound	Maximum dose [mg/kg]	ILS[a] (LTS[b])	ED_{50}[c] [mg/kg]
iPAM	30	103.9 (3/5)	11.4
2a	60	14.7	NA[d]
2b	60	10.2	NA
2c	60	14.7	NA
2d	60	0.5	NA
2e	30	103.9 (1/5)	<8
2f	60	56.0	39.0
2g	30	68.2	17.3

[a]ILS, increase in life span in percent over control at the maximum dose used. [b]LTS, long term survivors, the number of treated mice which survived tumour-free during two-month observation period; [c]effective dose causing a 50% of increase in life span; [d]not active at the maximum dose of 60 mg/kg.

line. The most active compounds, the acetylthioethyl analogue **2e** and the benzoylthioethyl analogue **2g,** were further tested against the following human cancer cell lines: HCV29T bladder cancer, SW707 rectal adenocarcinoma, and MOLT4 leukaemia. Both of them revealed an activity stronger than iPAM against the cell lines tested.

The *in vivo* antitumour activity of the iPAM analogues **2a–g** was evaluated in mice bearing L1210 transplantable leukaemia (Table 4). Similarly as in *in vitro* studies, it was found that **2e** and **2g** were the most active; **2e** was also more potent than iPAM.

For the iPAM analogues analysed, a correlation between the ability to undergo bioactivation and cytotoxic activity *in vitro* and antitumour activity *in vivo* was found. Particularly, the acylthioethyl analogues of iPAM **2e–g** with their significant antitumour activity may prove to be useful anticancer agents in GDEPT.

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