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ACYLOXYMETHYL ESTERS OF ISOPHOSPHORAMIDE MUSTARD AS NEW ANTICANCER PRODRUGS

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Abstract: A series of new prodrugs: [bis(2-chloroethylamino)phosphoryloxy]methyl acetate, [bis(2-chloroethylamino)phosphoryloxy]methyl pivalate and [bis(2-chloroethylamino)phosphoryloxy]methyl benzoate, was obtained in the reaction of isophosphoramide mustard (iPAM) with the corresponding acyloxymethyl halides. The cytotoxic activity of these new compounds is also shown. All compounds were highly active in the inhibition of cancer cell proliferation against the human lung (A594), prostate (PC-3) and breast (MCF-7) cancer cell lines.

Keywords: isophosphoramide mustard, prodrug, carboxyesterase, anticancer activity

The design and synthesis of prodrugs have shown intense interests to overcome pharmacodynamic and pharmacokinetic barriers in clinical drug application. Typical problems include lack of site specificity, chemical instability, toxicity, low oral absorption and poor patient compliance due to bad taste and odor (1, 2). A prodrug should meet many criteria. Among them are: selectivity and susceptibility for activating targeted enzyme, the stability in the undesired processes of biotransformation in the body, lipophilicity (whereby the prodrug can freely pass through the biological membrane), neutrality to the system before activation. Prodrugs are composed of two parts, a one unit that is the substrate for the activating enzyme, and the second unit that is activated by this metabolic process. These two elements are sometimes joined by a definable linker. In the case of anticancer prodrugs, the active form, produced during the reaction with the enzyme, should demonstrate a high efficiency of cytotoxic activity, and trigger off so-called the bystander effect (3). Furthermore, another desirable feature of the prodrug is the fact that the effect mentioned above is triggered irrespective of the cell cycle development. Prodrug therapy has become useful due to the poor selectivity of the currently used cytostatic drugs in conventional cancer chemotherapy.

One group of the enzymes involved in the activation of prodrugs are the carboxyesterases, (4) that hydrolyze esters and some carbamate derivatives (5). Carboxyesterases are known as activators of many currently used anticancer drugs, such as irinotecan, (6) paclitaxel (7) or KW-2189 (water-soluble duocarmycin derivative) (8-10). Irinotecan is metabolized by carboxyesterase to the active metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38) (11, 12). Carboxyesterases can hydrolyze esters of carboxylic and some inorganic acids. However, the simple carboxylic acid ester prodrugs are rapidly digested by carboxyesterases (13), but phosphate (14) and phosphonate esters are metabolitically more stable (1, 15), that is why acyloxyalkylesters of phosphoric and phosphorus acids are one of most commonly used prodrug. In this class of compounds there are the prodrugs for antiviral therapy, for example, adefovir dipivoxil (for hepatitis type B) (16) (Fig. 1) and tenofovir disoproxil (for HIV) (17). Such prodrugs are also used for hypercholesterolemia, for example, the bis(pivaloyloxymethyl) squalene synthase

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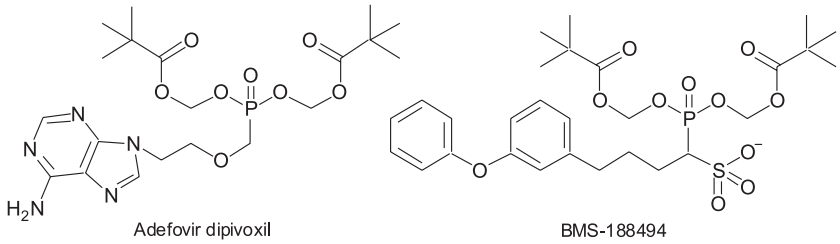


Figure 1.

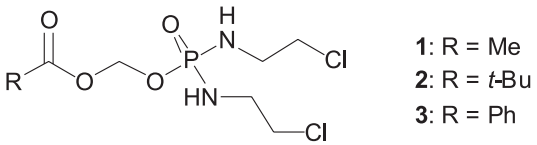


Figure 2. The structure of the synthesized compounds **1**-**3**

inhibitor (BMS-188494) (18) (Fig. 1) and ER-27856 (19).

The isophosphoramide mustard (iPAM) is an active, cytotoxic metabolite (20) of ifosfamide (IF), an alkylating anticancer drug widely used in the clinic against a wide range of human cancers (21), including breast cancer, endometrial cancer, lung cancer and various leukemia and lymphomas (22-24). Recently, the isophosphoroamide mustard (iPAM) has been examined as an anticancer agent. Preclinical trials are very promising since iPAM retains high activity against L1210 and P388 leukemias (25, 26).

In our current studies, we report the synthesis of three new compounds as potential prodrugs for anticancer therapy. The proposed compounds are diaminophosphoester prodrugs containing the isophosphoramide mustard moiety. We chose to use an oxymethyl linker since compounds possessing direct connection of carboxylic acid residue with phosphorus one (carboxylic phosphate anhydrides) are unstable and difficult to obtain. Additionally, these acyloxymethyl esters of iPAM might serve as the useful (i.e., orally administrative and able to cross cellular membranes) prodrug.

For this reason, to obtain higher selectivity of cytostatic drugs, we synthesized new ester analogues of iPAM with oxymethyl linker, which can be activated by carboxyesterase. These esters could be easily hydrolyzed by various and ubiquitous esterases. An ester as a prodrug alters lipophilicity of the drug and consequently, enhances the cellular adsorption and distribution. The potential prodrugs **1**-**3**, analogues of N,N'-bis(2-chloroethyl)diamidophosphoric acid, are shown in Figure 2.

These prodrugs should exhibit good stability in human plasma and should be substrates for the activating enzyme. After penetration to the cancer cells, the acyloxymethyl esters of isophosphoramide mustard are expected to be converted into isophosphoramide mustard (iPAM) by intracellular enzymatic cleavage by carboxyesterases (27) to give the corresponding hydroxymethyl analogues. This intermediate, in turn, is chemically labile and spontaneously loses one cytotoxic molecule of formaldehyde (28) to yield the cytotoxic iPAM.

EXPERIMENTAL

Chemistry

All reagents were commercially available or synthesized following the procedures described in the literature. All NMR spectra were recorded on a Bruker Avance III 700 MHz and Bruker Avance III 400 MHz spectrometer, using $CHCl_3$ as solvents, with TMS as an internal standard. The UV spectra were recordered on a T 60U Spectrometer (PG Instruments). Chromatography was performed with Merck silica gel 60 (0.040-0.063 mm). TLC was performed with Merck fluorescent F_{254} glass plates. HPLC was performed on Shimadzu LC-10AD system, column Phenomenex C-18.

Preparation of isophosphoramide mustard (**4**) (29)

White solid, m.p. 104-105°C. ^{1}H NMR (200 MHz, D_2O, δ, ppm): 3.25 (dt, 4H, J_1 = 8.6 Hz, J_2 = 5.8 Hz, 2 × CH_2), 3.62 (t, 4H, J_1 = 5.8 Hz, 2 × CH_2). ^{13}C NMR (D_2O, δ, ppm): 40.55 (CH_2), 41.06 (CH_2). ^{31}P NMR (D_2O, δ, ppm): 4.58 (100%).

Preparation of (bis(2-chloroethylamino)phosphoryloxy)methyl esters (**1**-**3**)

To a suspension of the isophosphoramide mustard (**4**) (3 mmol, 0.663 g) in dry acetonitrile (15

mL) a chloromethyl esters (3 mmol, for synthesis of **3** 4.5 mmol) and DIPEA (3 mmol, 0.51 mL) were added. After the addition of DIPEA, the solid of iPAM was dissolved. The reaction mixture was stirred at room temperature and monitored by TLC (chloroform/methanol 9:1, v/v) for 2 days. The solvent was evaporated. The obtained crude product was purified by silica gel column chromatography (chloroform/methanol 9:1, v/v).

(Bis(2-chloroethylamino)phosphoryloxy)methyl acetate (**1**)

Yellow oil, 38% yield. ^{1}H NMR (200 MHz, $CDCl_3$, δ, ppm): 2.15 (s, 3H, CH_3), 3.29 (m, 6H, 2 × CH_2, 2 × NH), 3.62 (t, 4H, J_1 = 5.6 Hz, 2 × CH_2), 5.62 (d, 2H, J_1 = 12.6 Hz, CH_2). ^{31}P NMR ($CDCl_3$, δ, ppm): 13.79 (100%).

(Bis(2-chloroethylamino)phosphoryloxy)methyl pivalate (**2**)

Yellow oil, 32% yield. ^{1}H NMR (200 MHz, $CDCl_3$, δ, ppm): 1.24 (s, 9H, 3 × CH_3), 3.29 (m, 6H, 2 × CH_2, 2 × NH), 3.62 (t, 4H, J_1 = 5.4 Hz, 2 × CH_2), 5.60 (d, 2H, J_1 = 6.0 Hz, CH_2). ^{31}P NMR ($CDCl_3$, δ, ppm): 13.67 (100%).

(Bis(2-chloroethylamino)phosphoryloxy)methyl benzoate (**3**)

Yellow oil, 11% yield. ^{1}H NMR (200 MHz, $CDCl_3$, δ, ppm): 3.30 (m, 6H, 2 × NH, 2 × CH_2), 3.59 (t, 4H, J_1 = 5.4 Hz, 2 × CH_2), 5.87 (d, 2H, J_1 = 12.6 Hz, CH_2), 7.46 (m, 2H, $H_{arom.}$), 7.62 (m, 1H, $H_{arom.}$), 8.08 (m, 2H, $H_{arom.}$). ^{31}P NMR ($CDCl_3$, δ, ppm): 13.81 (100%).

Hydrolytic stability

A solution of iPAM analogue **1**-**3** (0.5%) in TRIS/HCl, pH 7.4, and PBS was incubated for 72 h at the temperature of 37°C. During this time, the HPLC analysis was performed under the following conditions: solvent - 50% acetonitrile/water, column - Phenomenex C-18, isocratic, flow 0.5 mL/min., l 238 nm for **1**, **2** and 265 nm for **3**.

Carboxyesterase digestion

iPAM analogues **1**-**3** in PBS (0.5%) was incubated for 3 h at the temperature 37°C in the presence of the pig liver esterase (Sigma, 130 U/mg). During this time, the HPLC analysis was performed every 20 min under the following conditions: solvent - 50% acetonitrile/water, column - Phenomenex C-18, isocratic, flow 0.5 mL/min., l 238 nm for **1**, **2** and 265 nm for **3**. This procedure was performed twice for every compound.

Anti-proliferative assay *in vitro*

The anti-proliferative tests were performed on human cancer lines MCF-7 (breast cancer), A549 (non-small cell lung carcinoma), PC-3 (prostate cancer) and mouse fibroblast cell line (Balb3T3). Twenty-four hours before the addition of the tested compounds, the cells were placed in the 96-well plates (Sarstedt, Germany) at the density of 10^4 cells per well and cultured in the mixture medium. The breast cancer cells (MCF-7) were cultured in the Eagle medium supplemented with 2 mM glutamine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), amino acids and insulin (Sigma-Aldrich). The lung (A549) and prostate (PC-3) cancer cells were cultured in the mixture of RPMI 1640 and Opti-MEM (1 : 1) medium. In this case, the RPMI 1640 and Opti-MEM (1 : 1) medium was supplemented with 2 mM glutamine. The fibroblast cells (Balb3T3) were cultured in Dulbecco medium supplemented with 4 mM glutamine and glucose. All media were supplemented with 100 mg/mL streptomycin (Polfa, Tarchomin, Poland), 100 µg/mL pencillin (Polfa, Tarchomin, Poland), 5% (A549, PC-3) or 10% (MCF-7, Balb3T3) fetal bovine serum (Sigma-Aldrich). The cells were cultured at 37°C in humid atmosphere saturated with 5% CO_2. The *in vitro* cytotoxic effect of all agents was examined after 72 h of exposure of the cultured cells to varying concentrations of the tested compounds, using the SRB assay for adherent cells (96 h after the cells were cultured on the 96-well plate). The results are presented as an IC_{50} (inhibitory concentration 50%), the dose (µg/mL or µM) of tested agent which inhibits the proliferation of 50% of the cancer cells population. The compound showing the activity lower than 50% measured for 100 µg/mL was considered as an inactive agent. IC_{50} values were calculated separately for each experiment. Each compound was tested at every concentration in triplicate in a single experiment, which was repeated 4 times.

RESULTS AND DISCUSSION

Chemistry

The synthesis of isophosphoramide mustard (**4**) (iPAM) was performed in a one-pot reaction between phosphorus oxychloride and 2-(chloroethylamine) hydrochloride in the presence of triethylamine. The product bis-2-(chloroethylamino)phosphoryl chloride reacted, without isolation, with water to afford iPAM (**4**) as a white crystalline solid. (29)

The chloromethyl acetate and chloromethyl pivalate were commercially available. The other

reagent, chloromethyl benzoate**,** was prepared by the reaction of benzoyl chloride with paraformaldehyde in the presence of catalytic amounts of zinc chloride (30).

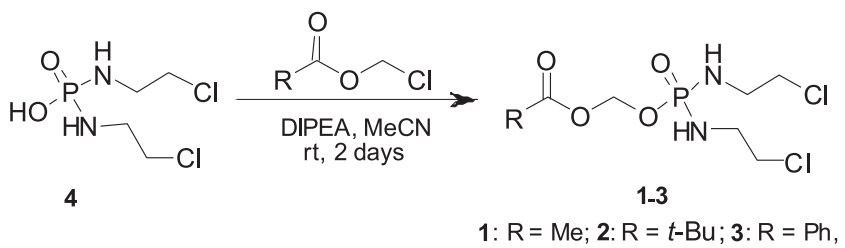
To obtain the new ester analogues of iPAM we employed a method described by Starrett and coworkers (16). The synthesis of compound **2** was attempted with four different conditions. The reaction of iPAM with chloromethyl pivalate in the presence of triethylamine and DMF gave product **2** in low yield (10%). In the presence of hindered base diisopropylethylamine, with higher basicity, the pivaloyloxymethyl ester of isophosphoramide mustard was obtained in only 27% yield. Using DMF as a solvent resulted in the difficulty to isolate the pure products. However, when acetonitrile was used instead of DMF, compound **2** was obtained after an easy purification step with 32% yield. In an attempt to increase the yields of this reaction, iodomethyl pivalate (the treatment of chloromethyl pivalate with sodium iodide in dry acetonitrile gave the iodomethyl pivalate) instead of chloromethyl pivalate was used. No improvement in the yield of the reaction was observed.

The analogues of isophosphoramide mustard **1** and **3** were obtained in the same procedure. (Scheme 1) The reaction was carried out in anhydrous acetonitrile, with equimolar ratio of the reactants. In the case of **3** the molar ratio was 1 : 1.5. The reactions were monitored by TLC analysis. Products **1**, **2** and **3** were isolated by column chromatography. The structure of the resulting products was confirmed by ^{1}H spectra and ^{31}P NMR.

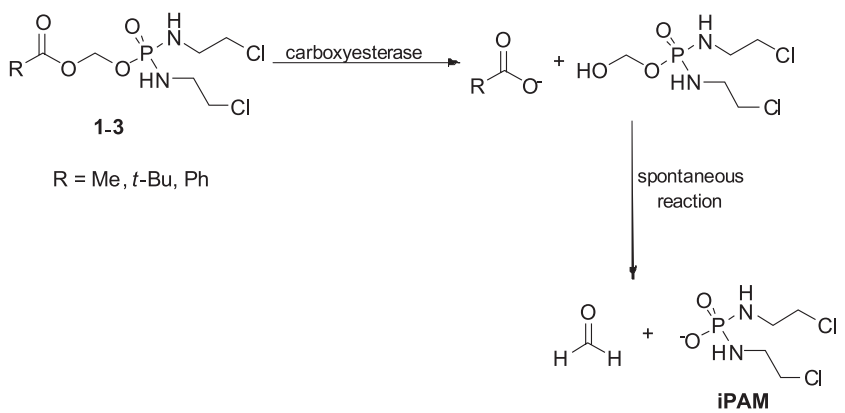
Hydrolytic stability

The stability of compounds **1**, **2**, **3** was studied in PBS buffer, pH 7.4, and TRIS-HCl buffer, pH 7.4, at 37°C. The tested derivatives were easily dissolved in aqueous solutions.

The examined compounds exhibited different stability. Ester **2** in PBS buffer had a good stability



Scheme 1. The synthesis of compounds **1**-**3**



Scheme 2. The proposed drug release pathway

Table 1. The *in vitro* antitumor activity of compounds **1**-**3**.

Compound*	IC_{50} (μg/mL) mean ± SD			
	A549	Balb3T3	MCF-7	PC-3
1 (M = 293.08 g/mol)	0.36 ± 0.02	0.24 ± 0.09	2.49 ± 1.35	0.69 ± 0.14
2 (M = 335.16 g/mol)	6.23 ± 8.14	0.17 ± 0.06	2.33 ± 0.98	0.80 ± 0.10
3 (M = 355.15 g/mol)	1.74 ± 0.69	0.19 ± 0.15	3.09 ± 0.39	-
Cisplatin	2.76 ± 0.06	3.17 ± 0.68	4.29 ± 0.47	5.12 ± 0.92

SD = standard deviation. * Compounds were tested in the following concentrations: 100, 10, 1 and 0.1 μg/mL.

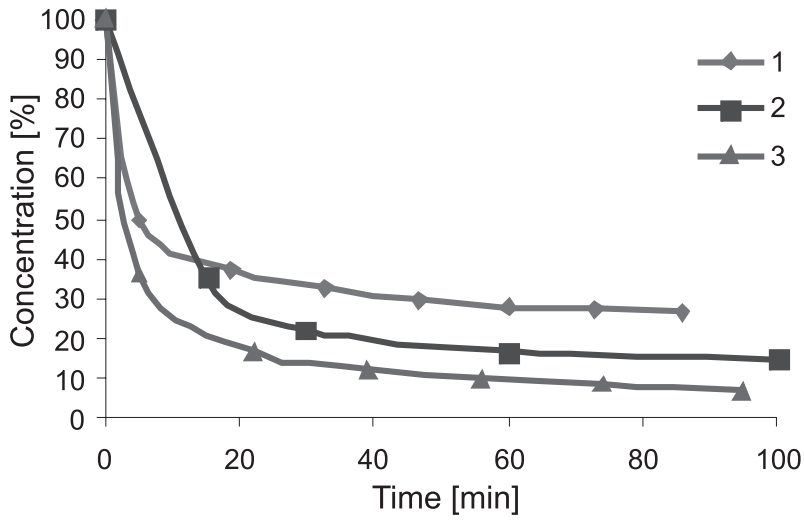


Figure 3. The progress of digestion of compounds **1**-**3** by carboxyesterase

and no decomposition was observed for 72 h. During the same analysis, derivative **1** was the least stable for only one hour (decomposition - up to 5%). Compound **3** was moderately stable showing decomposition (5%) after 6 h in PBS.

iPAM analogues **1**-**3** exhibit slightly lower stability in TRIS than in PBS. Different hydrolysis rate of esters is consistent with their structure. The most probably there occurs a hydrolysis of a carboxyester bond not phosphoric ester bond, since previously we made alkyl analogues of iPAM and they were stable in both buffers (unpublished data).

Enzymatic activation

Another important factor in determining the applicability of a prodrug is its enzymatic activation. The study of susceptibility to enzymatic activation was carried out to determine the production of the active forms of drugs **1, 2** and **3** as a result of the action of specific enzyme, carboxyesterase. The enzymatic hydrolysis of the ester should yield an acid and the corresponding hydroxymethyl derivative of iPAM, which, in the spontaneous reaction of division of the linker, produces the free (active) iPAM and cytotoxic formaldehyde (Scheme 2).

iPAM analogues **1**-**3** were incubated with esterase in PBS. HPLC analysis was performed to observe the progress of enzymatic digestion. Compound **2** shows the susceptibility to enzymatic activation, 50% of the ester linkages have been disrupted after a period of 11 min. Activation of the analogue **1** occurred in a shorter time than in the case of analogue **2**, as shown on the graph below. Half of the ester bonds was consumed within 5 min. The reduction of the amount of derivative **3** by a half occurred in 4 min. (Fig. 3). These results show that

derivative **3** is the most susceptible to enzyme digestion.

The presented analysis shows that each of the obtained derivatives easily undergoes enzymatic activation by carboxyesterase. The activation rates depend on the type of substituent and activation occurs fastest is in the case of benzoyl derivative **3**. Also in the case of compound **3**, the degree of hydrolysis of an ester bond in PBS buffer is satisfactory. The results from our work show that the rate of the enzymatic activation and of the hydrolytic stability depends on the substituent structure. In addition, the half-life of these compounds in PBS is suitable for them to be used as prodrugs.

Antiproliferative activity

The antiproliferative activity of all tested compounds against cancer cells shows the differences in IC_{50} values obtained for the three tested cell lines. Compound **1** was the most active in the proliferation inhibition against the human lung (A594) and prostate (PC-3) cancer cell lines. The IC_{50} results for breast (MCF-7) cancer cell line were similar to cisplatin value, but all compounds still meet the criterion of assumption of activity for chemical agents ($IC_{50} \sim 4$ μg/mL) (31). At the same time, all tested compounds revealed high antiproliferative activity against normal mouse fibroblast cell line Balb3T3. The IC_{50} values for all compounds (calculated as concentration in μg/mL) were over 10 times lower than the IC_{50} for cisplatin.

CONCLUSION

The above mentioned synthesis and testing methods constitute one of the alternatives in search of prodrugs in which the active form is an isophosphoramide mustard. The results of the hydrolytic stability tests and enzymatic activation show that the use of compounds **1** and **3** is questionable, but compound **2** can be used for further research as a prodrug. Taking into account the presented results for this study on antiproliferative activity, we can conclude that these compounds were highly active in inhibiting cancer cells proliferation, but they are also cytotoxic against mouse fibroblasts (a normal cell line). Probably, the introduction of another linker will influence the results of our study so we are currently working on the next derivatives of iPAM with different linkers to obtain better selectivity.

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