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Synthesis and antitumour activity of stereoisomers of 4-hydroperoxy derivatives of ifosfamide and its bromo analogue

Konrad Misiura [a,*], Daria Szymanowicz [a], Halina Kusnierczyk [b], Joanna Wietrzyk [b], Adam Opolski [b]

[a] *Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Sienkiewicza 112, 90-363 Lodz, Poland*

[b] *Department of Tumour Immunology, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wroclaw, Poland*

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Abstract

Racemic mixtures and laevorotatory enantiomers of *cis*- and *trans*-4-hydroperoxyifosfamide and 4-hydroperoxybromofosfamide possess high antitumour activity both in vitro and in vivo. However, no major differences in biological activity were observed among these stereoisomers.

Keywords: Ifosfamide; Bromofosfamide; 4-Hydroperoxyifosfamide; Antitumour activity

1. Introduction

Ifosfamide (IF) like its structural isomer cyclophosphamide is an anticancer alkylating agent widely used for the treatment of variety of human tumours [1]. IF is a chiral molecule and both of its enantiomers have been synthesized [2]. We have found that laevorotatory enantiomer of ifosfamide [(−)-IF] is more active than the parent racemic drug against several experimental tumours in mice [3]. Within the process of search for new and more active congeners of IF, bromo analogues have been prepared [4]. Also, among stereoisomers of bromo analogues of IF stereo-differentiation in antitumour activity [5] and pharmacokinetics [6] was observed. Based on these results (*S*)-(−)-3-(2-bromoethyl)-*N*-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazphosphorine-2-amine 2-oxide [7] [compound CBM-11, (−)-BF] became subject to the Phase I clinical trials in Poland. Therapeutic application of high-dose IF [8,9] or (−)-BF [10] is limited by several side effects; among them neurotoxicity and nephrotoxicity are of the greatest concern. It was postulated [8] that these side effects are connected with metabolic transformation of IF and a release of chloroacetaldehyde following the hydroxylations at C-1 atoms of 2-chloroethyl groups. IF is a pro-drug activated in vivo via cytochrome P-450 mediated oxidation of C-4 atom of 1,3,2-oxazaphosphorine ring [11]. It is conceivable that the use of pre-activated, 4-hydroperoxy derivatives of IF and (−)-BF could diminish these side effects.

Racemic *cis*- and *trans*-4-hydroperoxyifosfamide were obtained previously and their antitumour activity proved [12,13]. In this study we examined the influence of the configuration, on phosphorus atom in 4-hydroperoxy derivatives, on their antitumour activity. Obtained results shed some additional light on the problem of stereo-differentiation of biological activity of IF and BF enantiomers.

2. Experimental

2.1. Chemistry

2.1.1. General synthetic procedure

Racemate or laevorotatory enantiomer of IF or BF (20 mmol) was dissolved in a mixture of acetone (80 ml), water (40 ml), and 30% hydrogen peroxide (12 ml).

* Corresponding author.
E-mail address: kmisiura@bio.cbmm.lodz.pl (K. Misiura).

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Obtained solution was bubbled with ozone (rate 0.5 g/h) at 0 °C. After 4 h reaction mixture was concentrated up to ca. 50 ml and obtained suspension was extracted with chloroform (3 × 50 ml). Organic layers were combined, dried with $MgSO_4$, and solvent evaporated. Resulting mixture was separated on silica gel using chloroform/acetone 1:1 as an eluent providing 4-keto derivatives (R_f 0.52), trans (R_f 0.45), and *cis* isomers (R_f 0.34) of the appropriate 4-hydroperoxy compounds.

2.1.2. HPLC analysis conditions

Reverse-phase C8 column (Alltech Econoshere, 5 μm, 250 mm); eluent, isocratic 25% acetonitrile; flow 1.5 ml/min; λ 200 nm.

2.1.3. ^{31}P NMR analysis conditions

Spectra of 100 mM racemic 4-ketoifosfamide and 50 mM $Pr(tfc)_3$ showed a presence of two signals of 8.7 and 7.2 ppm with 1:1 ratio. Spectra of (−)-4-ketoifosfamide obtained from a mixture of isomers coming from (−)-*cis*- and (−)-*trans*-4-HOO-IF, performed as above, revealed the presence of only one signal of 8.7 ppm.

2.2. Cytotoxic activity in vitro

The following established in vitro human cancer cell lines were employed: SW707 (rectal adenocarcinoma), MCF-7 (breast carcinoma), KB (cervical carcinoma), and HCV29T (bladder cancer). The cytotoxicity assay was performed after 72-h exposure of the cultured cells to varying concentrations (from 0.1 to 100 μg/ml) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% trichloroacetic acid on top of the culture medium in each well. The plates were incubated at 4 °C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA 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 the determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtitre plate reader Multiskan RC photometer. Each compound in given concentration was tested in triplicates in each experiment, which was repeated 3–5 times.

2.3. Antitumour activity against L1210 leukaemia

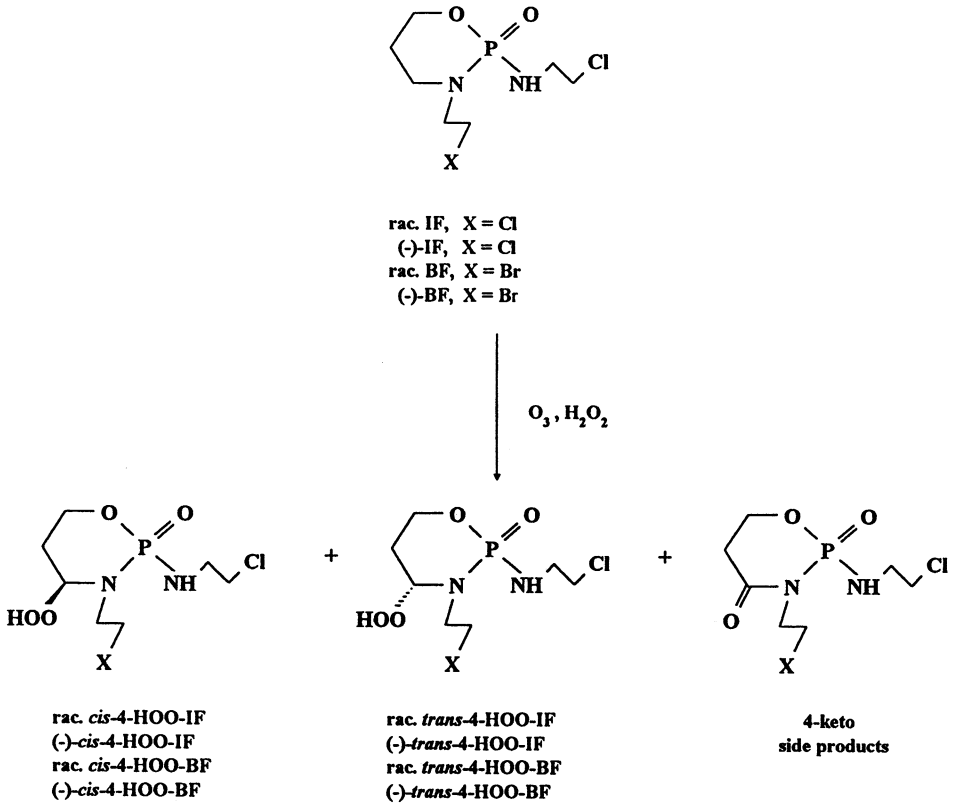
Leukaemia cells were implanted i.p. in CD2F1 mice at 10^5 cells/mouse. Each experimental group was composed of six mice. Tested compounds were administered i.p. 24 h after tumour implantation (day 1). Antitumour activity was determined according to In vivo Cancer Models (NIH Publication No. 84-2635) by comparing the mean survival time of the treated group with that of the control group, and expressed as percentage of increased life span over control (ILS%). Doses providing 50% ILS (ED_{50}) were estimated graphically from the least-square-fitted dose–effect curves. Similarly, doses curative for 50% of treated animals were estimated from curves where the effect was expressed as the percentage of long-term survivors (in 2-month observation period).

3. Results

Stereoisomers of 4-hydroperoxyifosfamide (4-HOO-IF) and 4-hydroperoxy-bromofosfamide (4-HOO-BF) were obtained by the ozonolysis of racemic IF, (−)-IF, racemic BF, and (−)-BF performed in the presence of hydrogen peroxide (Scheme 1).

From each substrate both *cis*- and *trans*-isomers of 4-hydroperoxy compound together with 4-keto side-product, were formed in nearly equimolar ratio. Obtained products were separated by careful silica gel column chromatography and were characterized by ^1H- and ^{31}P NMR, and CI-MS. Their purity was examined by TLC, HPLC, and ^{31}P NMR. Yields of products and selected physicochemical data are presented in Table 1.

Stability of 4-hydroperoxy compounds (conc. 10 mM) in 0.1 M phosphate buffer, pH 7.4 and 0.1 M Tris–HCl buffer, pH 7.5 was measured using HPLC method. It was found that all tested compounds undergo fast *cis*↔*trans* isomerization. For each compound this isomerization was completed within 15 min at room temperature. By prolonging incubation time it was also found that 4-HOO-IF and 4-HOO-BF are chemically unstable in these buffers and collapse with spontaneous release of most probably 4-hydroxy derivatives (HPLC analysis, T_R 2.77, 3.12 min), which decompose further to several products. The above mentioned isomerization was earlier observed [13] for racemic *cis*- and *trans*-4-hydroperoxyifosfamide dissolved in chloroform in the presence of *p*-toluenesulfonic acid. It was postulated [13,14] that this process proceeded with epimerization at phosphorus atom and retention of configuration at C-4 atom. However, by using optically active form of 4-HOO-IF we proved that *cis*↔*trans* isomerization did not change the configuration at P-atom. (−)-*cis*-4-HOO-IF (conc. 50 mM) was epimerized in 0.1 M Tris–HCl buffer with pH 7.5 within 1 h and, without isolation, reduced to 4-ketoifosfamide by adding 0.1 M iron(II) sulphate. Obtained 4-ketoifosfamide was isolated by extraction with ethyl acetate and its enantiomeric purity was examined by ^{31}P NMR spectroscopy performed in the presence of a chiral shift reagent $Pr(tfc)_3$. It was found



Scheme 1.

Table 1
Yields of products and selected physicochemical data of 4-hydroperoxy derivatives

Comp.	Yield (%)	$[\alpha]_D$	R_T (RP-HPLC) (min)	δ (^{31}P NMR) (ppm)
rac. *cis*-4-HOO–IF	8		4.81	8.82
rac. *trans*-4-HOO–IF	16		5.44	8.93
(−)-*cis*-4-HOO–IF	9	−39.1	4.81	8.82
(−)-*trans*-4-HOO–IF	10	−38.6	5.44	8.93
rac. *cis*-4-HOO–BF	9		5.32	8.66
rac. *trans*-4-HOO–BF	21		5.98	8.82
(−)-*cis*-4-HOO–BF	6	−38.9	5.32	8.66
(−)-*trans*-4-HOO–BF	12	−36.2	5.98	8.82

that obtained (−)-4-ketoifosfamide possessed 100% enantiomeric purity. Earlier, we have proved [15] by chemical correlation that (−)-4-ketoifosfamide has the same S_p configuration as (−)-(*S*)-IF. The same analysis of stereochemistry at P-atom was performed for a mixture of isomers obtained from (−)-*trans*-4-HOO-IF and no epimerization at phosphorus was found. These results of stereochemical analysis of *cis*↔*trans* isomerization of 4-HOO-IF is consistent with the kinetic data obtained by Borch for *cis*↔*trans* isomerization of 4-hydroperoxycyclophosphamide [16], for which elimination–addition of hydrogen peroxide mechanism was proposed.

Cytotoxic activity of obtained 4-hydroperoxy compounds was analysed on several human tumour cell lines (Table 2).

Table 2
Cytotoxic activities of 4-hydroperoxy derivatives

Sample	Cell line/ID_{50} [a] (μg/ml)			
	KB	SW707	HCV29T	MCF-7
rac. *trans*-4-HOO-IF	32.6 ± 1.2	4.1 ± 1.4	3.2 ± 1.1	4.4 ± 1.4
rac. *cis*-4-HOO-IF	37.9 ± 1.1	3.3 ± 1.0	2.9 ± 1.3	3.5 ± 1.0
(−)-*trans*-4-HOO-IF	33.7 ± 1.0	3.3 ± 1.1	3.0 ± 1.2	3.2 ± 1.0
(−)-*cis*-4-HOO-IF	39.0 ± 1.0	3.4 ± 1.1	3.3 ± 1.1	4.5 ± 1.4
rac. *trans*-4-HOO-BF	32.3 ± 1.4	6.4 ± 1.6	5.5 ± 1.2	17.6 ± 1.1
rac. *cis*-4-HOO-BF	40.4 ± 1.0	27.1 ± 1.2	12.3 ± 1.9	30.0 ± 1.1
(−)-*trans*-4-HOO-BF	38.0 ± 1.1	26.0 ± 1.0	26.8 ± 1.1	33.2 ± 1.1
(−)-*cis*-4-HOO-BF	49.8 ± 1.0	31.3 ± 1.2	22.9 ± 1.5	33.3 ± 1.1

[a] The dose of compound that inhibits proliferation rate of the tumour cells by 50% as compared to the control untreated cells.

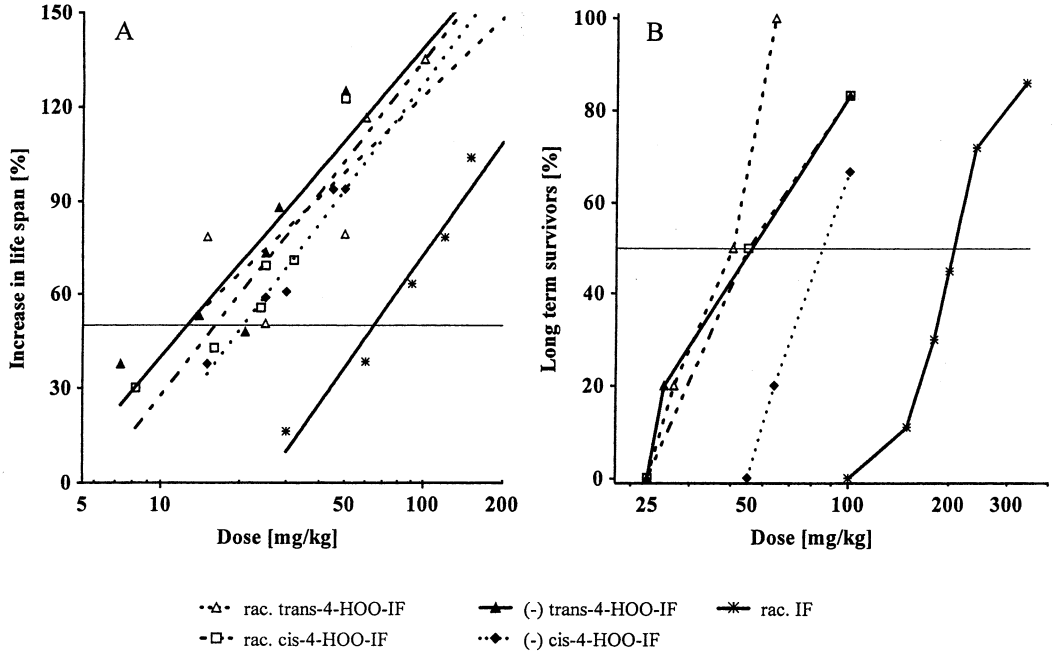


Fig. 1. Dose-dependent increase in life span (A) and percentage of long term survivors (B) in L 1210 leukaemia-bearing mice following i.p. administration of stereoisomers of 4-hydroperoxy derivatives of ifosfamide.

It was found that chloro analogues [IF and (−)-IF derivatives] are more active than bromo congeners. However, the differences in cytotoxicity between *cis*- and *trans*-isomers as well as between racemic and optically pure compounds were negligible.

4-Hydroperoxy derivatives of IF and (−)-IF were also active against L1210 leukaemia in mice (Fig. 1).

All examined compounds have similar therapeutic efficacy in this model. Effective doses (ED_{50}) were in a range of 14–22 mg/kg, while curative doses (CD_{50}) were 45–82 mg/kg, and they were 3–5 times lower when compared with the values obtained for IF.

4. Conclusions

It can be concluded that racemic and laevorotatory enantiomers of *cis*- and *trans*-4-hydroperoxyifosfamide possess high antitumour activity both in vitro and in vivo. However, no major differences in biological activity were observed among stereoisomers of 4-hydroperoxyifosfamide and 4-hydroperoxybromofosfamide. These results imply that differences in antitumour activity of stereoisomers of IF and BF are not related to the metabolic hydroxylation of C-4 atom of 1,3,2-oxazaphosphorine ring, but probably result from C-1 atoms hydroxylation of 2-chloroethyl chains. Indeed, in the studies on stereoselectivity of IF metabolism in humans [15] we found that 4-ketoifosfamide, a metabolite forming via ring hydroxylation, had a ratio 45:55 of (−)/(+) enantiomers, while for 3-*N*-dechloroethyl metabolite this ratio was 73:27. Similar stereoselectivities in *N*-dichloroethylation metabolic pathways of IF [17] and cyclophosphamide [18] were also recently observed.

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References

[1] J.E. Wright, in: B.A. Teicher (Ed.), Phosphoramide and Oxazaphosphorine Mustards, Cancer Therapeutics: Experimental and Clinical Agents, Humana Press, Totowa, NJ, 1997, pp. 23–79.
[2] K. Pankiewicz, R. Kinas, W.J. Stec, A.B. Foster, M. Jarman, J.M.S. Van Maanen, Synthesis and absolute configuration assignments of enantiomeric forms of ifosphamide, sulfosphamide, and trofosphamide, J. Am. Chem. Soc. 101 (1979) 7712–7718.
[3] H. Kuśnierczyk, C. Radzikowski, M. Paprocka, W. Budzyński, R.W. Kinas, K. Misiura, W.J. Stec, Antitumor activity of optical isomers of cyclophosphamide, ifosfamide and trofosfamide as compared to clinically used racemates, J. Immunopharmacol. 8 (1986) 455–480.
[4] K. Misiura, R.W. Kinas, W.J. Stec, H. Kuśnierczyk, C. Radzikowski, A. Sonoda, Synthesis and antitumor activity of analogues of ifosfamide modified in the *N*-(2-chloroethyl) group, J. Med. Chem. 31 (1988) 226–230.
[5] H. Glazman-Kuśnierczyk, J. Matuszyk, C. Radzikowski, Antitumor activity evaluation of bromine-substituted analogues of ifosfamide, immunopharmacol, Immunotoxicology 14 (1992) 883–911.

[6] A. Sloderbach, B. Hładoń, M. Sochacki, R. Kinas, H. Kuśnierczyk, H. Laskowska, Pharmacokinetic-stereoselective differentiation of some isomeric analogues of ifosfamide, Pol. J. Pharmacol. 49 (1997) 463–469.
[7] K. Misiura, R.W. Kinas, H. Kuśnierczyk, C. Radzikowski, W.J. Stec, (*S*)-(−)-Bromofosfamide (CBM-11): synthesis and antitumour activity and toxicity in mice, Anticancer Drugs 12 (2001) 453–458.
[8] L.D. Lewis, C.A. Meanwell, Ifosfamide pharmacokinetics and neurotoxicity, Lancet 335 (1990) 175–176.
[9] R. Skinner, I.M. Sharkey, A.O. Pearson, A.W. Graft, Ifosfamide, mesna, and nephrotoxicity in childern, J. Clin. Oncol. 11 (1993) 173–190.
[10] K. Kobylinska, P. Koralewski, B. Sobik, M. Gasiorek, M. Kobylinska, Pharmacokineticts and toxicity of oral (−)-(*S*)-bromofosfamide in lung cancer patients, Arzneimittelforschung 51 (2001) 600–603.
[11] A.V. Boddy, S.M. Yule, Metabolism and pharmacokinetics of oxazaphosphorines, Clin. Pharmacokinet. 38 (2000) 291–304.
[12] H.J. Hohorst, G. Peter, R.F. Struck, Synthesis of 4-hydroperoxy derivatives of ifosfamide and trofosfamide by direct ozonation and preliminary antitumor evaluation in vivo, Cancer Res. 36 (1976) 2278–2281.
[13] A. Takamizawa, S. Matsumoto, T. Iwata, I. Makino, Synthesis, stereochemistry and antitumor activity of 4-hydroperoxyifosfamide (NSC-227114) and related compounds, Chem. Pharm. Bull. 25 (1977) 1877–1891.
[14] A. Camerman, H.W. Smith, N. Camerman, Activated cyclophosphamide anticancer drugs: molecular structures of *cis*- and *trans*-4-hydroperoxyifosfamides, J. Med. Chem. 26 (1983) 679–684.
[15] K. Misiura, A. Okruszek, K. Pankiewicz, W.J. Stec, Z. Czownicki, B. Utracka, Stereospecific synthesis of chiral metabolites of ifosfamide and their determination in the urine, J. Med. Chem. 26 (1983) 674–679.
[16] R.F. Borch, K.M. Getman, Base-catalyzed hydrolysis of 4-hydroperoxy-cyclophosphamide: evidence for iminocyclophosphamide as an intermediate, J. Med. Chem. 27 (1984) 485–490.
[17] C.P. Granvil, J. Ducharme, B. Leyland-Jones, M. Trudeau, I.W. Wainer, Stereoselective pharmacokinetcs of ifosfamide and its 2- and 3-*N*-dechloretylated metabolites in female cancer patients, Cancer Chemother. Pharmacol. 37 (1996) 451–456.
[18] M.L. Wiliams, I.W. Wainer, C.P. Granvil, B. Gehercke, M.L. Berstein, M.P. Ducharme, Pharmacokinetics of (*R*)- and (*S*)-cyclophosphamide and their dechloroethylated metabolites in cancer patients, Chirality 11 (1999) 301–308.