Anti-Cancer Drugs 2001, **12,** pp. 453–458

Report

(*S*)-(−)-Bromofosfamide (CBM-11): synthesis and antitumor activity and toxicity in mice

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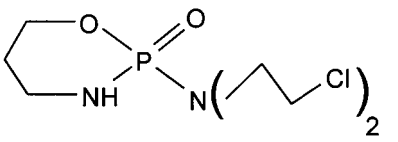
(*S*)-(−)-Bromofosfamide (CBM-11), an enantiomerically pure bromo analog of ifosfamide, was found to be potent against several model tumors in mice. Therapeutic indices of CBM-11 were more favorable as compared to those received for ifosfamide. [© 2001 Lippincott Williams & Wilkins.]

Key words: Alkylating agents, anticancer drugs, bromofosfamide, oxazaphosphorine.



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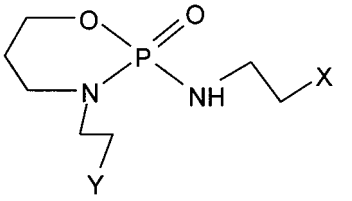

2a, X = Y = Cl
2b, X = Cl, Y = Br

Introduction

Cyclophosphamide (CP, **1**), an oxazaphosphorine-type alkylating agent, has been the most frequently used cytotoxic drug exhibiting activity against numerous common solid tumors as well as leukemias and lymphomas.[1] Its positional isomer, ifosfamide (**2a**), introduced into clinical practice in the 1980s, was found to be particularly useful in the treatment of soft tissue sarcomas and variety of pediatric tumors.[2,3] Among numerous congeners of cyclofosfamide and ifosamide synthesized to date, only their levorotatory enantiomers and 2-bromoethylamino analog of ifosfamide revealed meaningful increase of therapeutic index in several experimental murine tumor models.[4,5]

Continuing our structure–activity relationship studies on the bromo-substituted analogs of **2**, new methods of their synthesis, both in racemic and enantiomerically pure forms, were designed, and preliminary evaluation of their antitumor activity and toxicity in mice was performed.[6] In parallel, studies in rats showed considerable stereodependent differences in pharmacokinetic and bioavailability of those compounds.[7] Based upon obtained results, (*S*)-(−)-3-(2-bromoethyl)-*N*-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-amine 2-oxide (CBM-11, **2b**) has been selected for phase I clinical trials in several hospitals in Poland.

In this report we describe the synthesis of CBM-11, and present results of its toxicity and antitumor activity evaluation in comparison with racemic ifosfamide as the reference drug.

This project was in part financially assisted by the State Committee for Scientific Research, grant no 4 P05F 023 15.

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Materials and methods

(*S*)-(−)-Bromofosfamide (CBM-11, **2b**)

Starting from (S_C,S_P)-3-(α-methylbenzyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-chloro 2-oxide (**3**),[8,9] a key intermediate (*R*)-(−)-*N*-(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-amine 2-oxide (**4**) was obtained by the previously published[8] and recently improved procedure. Compound **4** [19.85 g, 0.1 mol, m.p. 107–108°C, δ_P 11.8 ($CDCl_3$), $[\alpha]_D^{20}$ −15.4 (CH_3OH)] and dry triethylamine hydrobromide (45.50 g, 0.4 mol) were dissolved in dry chloroform (400 ml) and bromoacetyl bromide (9.0 ml, 0.1 mol) in dry chloroform (100 ml) were added drop-wise to the stirred solution. The reaction mixture was left for 24 h at room temperature, washed with water (3 × 250 ml), dried over anhydrous. $MgSO_4$ and concentrated to dryness. Resulting solid residue was re-crystallized from the mixture of ethyl acetate and *n*-hexane (1:2) providing the bromoacetyl derivative **5** [(24.9 g, m.p. 87–88°C, δ_P 2.6 ($CDCl_3$), $[\alpha]_D^{20}$ −44.5 (CH_3OH), 78% yield)]. To the solution of compound **5** (19.17 g, 0.06 mol) in dry THF (150 ml) sodium borohydride (3.32 g) was added in one portion. Into the resulting slurry a solution of boron trifluoride etherate (13.2 ml) in dry THF (30 ml) was added drop-wise with stirring. The stirring was continued for 4 h and then the reaction was quenched by careful addition of water (180 ml). The obtained solution was concentrated *in vacuo* to half of its initial volume and then extracted with chloroform (3 × 180 ml). Chloroform solutions were combined, dried ($MgSO_4$) and concentrated to dryness. The obtained solid was re-crystallized from the mixture of ethyl acetate and *n*-hexane (1:1) to give the desired product **2b** [15.0 g, 82% yield, m.p. 84–85°C, $[\alpha]_D^{20}$ −42.3 (c 2.0, CH_3OH)]. Purity of obtained compound CBM-11 was examined by thin-layer chromatography and high-performance liquid chromatography (HPLC) analysis. Enantiomeric purity of CBM-11 was analyzed by recording its ^{31}P-NMR spectra in the presence of $Eu(tfc)_3$[8] and by HPLC using the chiral stationary phase.[10] The structure of CBM-11 was proved by ^{1}H-, ^{13}C- and ^{31}P-NMR, and mass spectroscopy and elemental analysis.

Toxicity studies

Subacute toxicity (9-week observation period) of CBM-11 and ifosfamide was evaluated in mice of first generation of interstrain crosses: BALB/c × DBA/2 (CDF1) or C57BL/6 × DBA/2 (BDF1). Healthy females, 12–16 weeks old and weighing 23–27 g were injected once i.p. or i.v., or were given one oral gavage (p.o. route) of the tested agent. Mortality distribution was cumulated from four to six separate experiments in which usually four to six dose levels with at least five mice per cohort were used. The Litchfield–Wilcoxon procedure was used for estimation of doses lethal for 50% of treated animals (LD_{50}) with confidence limits (CL) as well as maximally tolerated doses (MTD = LD_5). All computations were performed using CSS Statistica software (StatSoft).

Antitumor activity evaluation

Antitumor effects of compound CBM-11 and IF were evaluated *in vivo* after single-dose treatment (day 1 only) of mice with L1210 leukemia, B16 melanoma or Lewis lung carcinoma. The experiments were conducted and activity of tested compound estimated according to the NIH/NCI standard screening protocols *in vivo*.[11]

L1210 leukemia. CDF1 mice were inoculated i.p. with 10^5 ascitic tumor cells suspended in 0.2 ml of PBS. Observation was carried out for 2 months and tumor-free mice surviving that period were regarded as long-term survivors (LTS) or cures. Doses curative for 50% of treated animals (CD_{50}) were estimated graphically from dose–effect curves according to Litchfield–Wilcoxon procedure.[12]

B16 melanoma tumors derived from s.c. passages were pressed through a plastic sieve into the serum-free Hank's balanced salt solution (Hank's medium) using a glass syringe plunger. BDF1 mice were injected i.p. with 0.25 ml of the obtained 20% w/v tissue suspension. The evaluated parameter of activity was increase in life-span (ILS), calculated from average survival times (AST) of treated and control mice. Effective doses (ED_{50}) providing 50% increase in life span were estimated directly from least-square-fitted dose–response curves.

Lewis lung carcinoma BDF1 mice were inoculated s.c. with 2×10^6 viable tumour cells in 0.2 ml of Hank's medium. Cell suspension was prepared similarly as described for B16 melanoma and cell viability was assessed by Trypan dye exclusion. Curative effects were estimated after 2 months of observation.

All mice were purchased from the Inbred Animal Breeding Centre, Institute of Immunology and Experimental Therapy, Wrocław. They were kept under conventional hygienic conditions (minimal disease standard) of the experimental animal house with free access to balanced rodent diet and filtered tap water.

Animal experiments were approved by the institutional Animal Care Committee according to USPHS

guidelines and were performed with regard to the International Laboratory Animal Care Convention.

Results

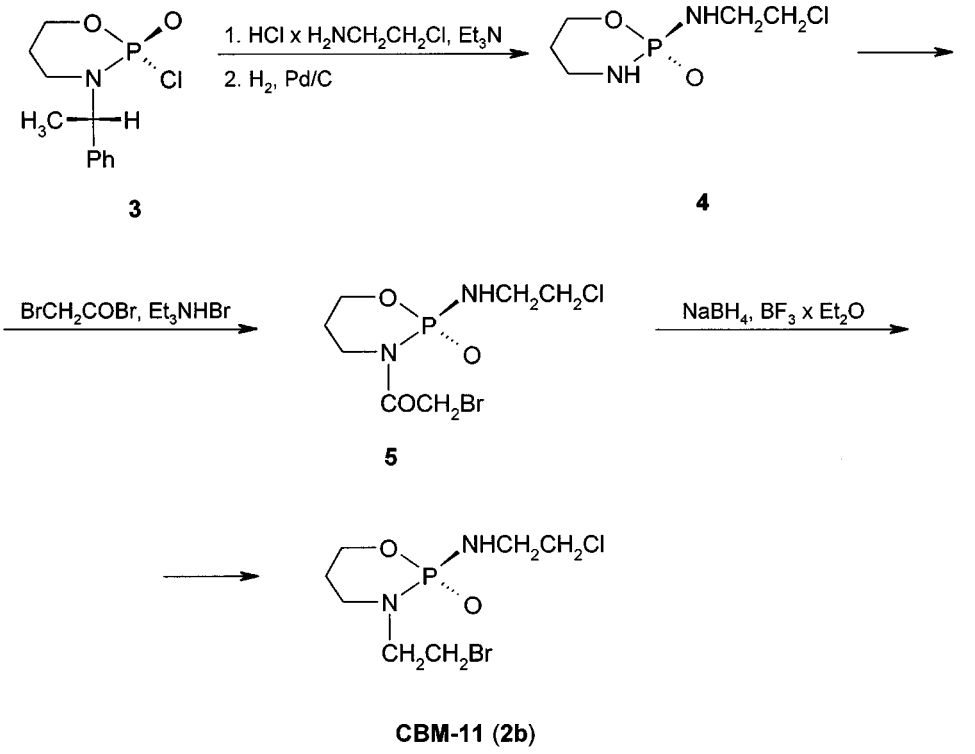
Chemistry

The method of preparation of CBM-11 (**2b**) was based upon the use of chiral α-methylbenzyl auxiliary attached to the nitrogen atom of tetrahydro-2H-1,3,2-oxazaphosphorinane moiety, which enabled stereoselective synthesis of diastereomerically pure 3-(α-methylbenzyl)tetrahydro-2H-1,3,2-oxazaphosphorine-2-chloro 2-oxide (**3**). This method was used for the first time in the synthesis of enantiomers of cyclophosphamide, ifosfamide and trofosfamide.[8,9] Its modification has been elaborated for efficient preparation of CBM-11 (Scheme 1).

Thus, instead of carcinogenic aziridine,[8] 2-chloroethylamine hydrochloride was used during synthesis of the key intermediate **4**. Such modification also allowed us to omit one synthetic step (ring-opening of aziridine moiety). Acylation of compound **4** with bromoacetyl bromide was performed in the presence of 4 M excess of triethylamine hydrobromide to trap liberated hydrogen bromide. The yield of compound **5** was 78%. Triethylamine, which is the base commonly used for trapping hydrogen halides formed in condensation reactions, cannot be used in the case of preparation of **5** for the reason of its reactivity towards halogenoacetyl halides.[13] Other bases like triethyl phosphate[14] used as a reaction medium are also useful, but the yields of product of acylation **5** were modest only. Reduction of the carbonyl group in compound **5** was performed using a reagent developed for this purpose: sodium borohydride in the presence of trifluoroboron etherate.[15] The total yield of CBM-11 was 25% in six steps. The absolute configuration of the resulting CBM-11 is *S*, as assigned by its chemical correlation with (*S*)-(−)-IF and confirmed by X-ray crystallography.[16]

Toxicity

It was found that CBM-11 is more toxic in mice than ifosfamide. MTDs and LD_{50}s of CBM-11 from i.p. and i.v. routes of administration were 2.2–2.5 times lower than those estimated for ifosfamide, similarly in CDF1 and BDF1 mice (Table 1).



3 **4** **5** **CBM-11 (2b)**

Scheme 1.

Both agents were found better tolerated when given p.o., although the difference between toxicities from i.p. and p.o. administrations was less pronounced in the case of ifosfamide. The exemplary dose–lethal effect curves from estimations in CDF1 mice are shown in Figure 1.

Antitumor activity

In all three tumor models (L1210 leukemia, B16 melanoma and Lewis lung carcinoma), CBM-11 was found to be more active and also more potent than ifosfamide (Figure 2). Even at the doses close to MTD the maximal therapeutic effect observed after ifosfamide treatment was significantly lower than after administration of CBM-11.

The observed higher antineoplastic activity of CBM-11 corresponds with the increase in therapeutic index (TI), calculated here as MTD:CD_{50} ratio. It can be seen in Table 2 that TI of CBM-11 was significantly increased as compared to IF in two model systems, i.e. L1210 leukemia and B16 melanoma. The latter tumor is particularly insensitive to treatment with oxazaphosphorine drugs such as cyclophosphamide or ifosfamide.

Table 1. Toxicity[a] of CBM-11 and ifosfamide

Route	CBM-11		Ifosfamide	
	MTD (mg/kg)	LD_{50} (CL) (mg/kg)	MTD (mg/kg)	LD_{50} (CL) (mg/kg)
i.p.[b]	226	290 (275–305)	519	687 (649–727)
i.p.[c]	233	264 (251–278)	510	667 (625–694)
i.v.	255	316 (301–337)	472	644 (601–689)
p.o.[b]	427	496 (457–538)	ND[d]	~1250
p.o.[c]	353	541 (485–604)	552	982 (824–1169)

[a]Lethality from single injection of tested compounds was evaluated after 2 months of obseration.
[b]In CD2F1 mice.
[c]In B6D2F1 mice.
[d]Not determined.

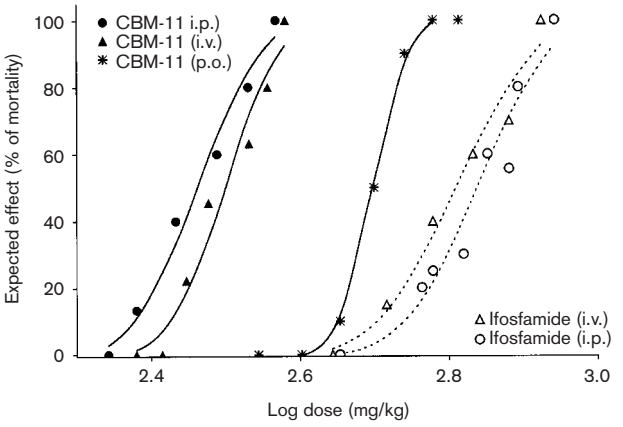


Figure 1. Lethality evaluation of CBM-11 and ifosfamide in CDF1 mice. Expected percent mortality curves are shown with original (uncorrected) observed points, each representing cumulated mortality from several evaluations in healthy females (10–40 mice per dose-level).

Discussion

The rationale for the synthesis of bromo analogs of ifosfamide was based upon the assumption that such modification should influence the rate of alkylation of target DNA by the final, active metabolite of these bromo analogs. Indeed, we found that *N*-(2-bromoethyl)-*N*-(2-chloroethyl)phosphorodiamidic acid, a probable active metabolite of CBM-11, produced a higher amount of DNA interstrand cross-links in HeLa cells as compared to that obtained with isophosphoramide mustard, an active metabolite of ifosfamide.[17] In the 1,3,2-oxazaphosphorine series bromo analogs of *cis*-4-hydroperoxyifosfamide have been synthesized and anticancer activity proved.[18] Very recently a new potent analog of tallimustine possessing a bis(2-bromoethyl)amino alkylating moiety has been also reported.[19] Among halogenoacrylic derivatives of distamycin A, the bromo analog was found to be the most potent.[20]

Marked differences in anticancer activity and toxicity have been displayed by enantiomers of cyclophosphamide, ifosfamide and bromo analogs of ifosfamide.[4,5] Among all tested agents (−) enantiomers were more potent than (+) enantiomers or racemates against several experimental tumors in mice. It was also proved in our studies that metabolism of ifosmide in humans is stereoselective with a considerable increase of the (−) enantiomer content in the excreted 2-dechloroethylifosfamide metabolite.[21] These findings were later confirmed independently by Wainer[22] and Boss.[23] On the other hand, based on the observations of a slower clearance of (+)-ifosfamide as compare to (−)-ifosfamide and on a lower level of chloroacetaldehyde (the metabolite is suspected of neurotoxic[24] and nephrotoxic[25] side effects manifested during ifosfamide treatment) released from (+) enantiomer, Wainer recommended dextrarotatory ifosfamide, (+)-ifosfamide for clinical use.[26,27]

In addition to conventional anticancer chemotherapy, CBM-11 may be also considered as useful in combination with biotherapeutic approaches. For example, we have demonstrated recently that administration of CBM-11 in mice bearing advanced colon 38

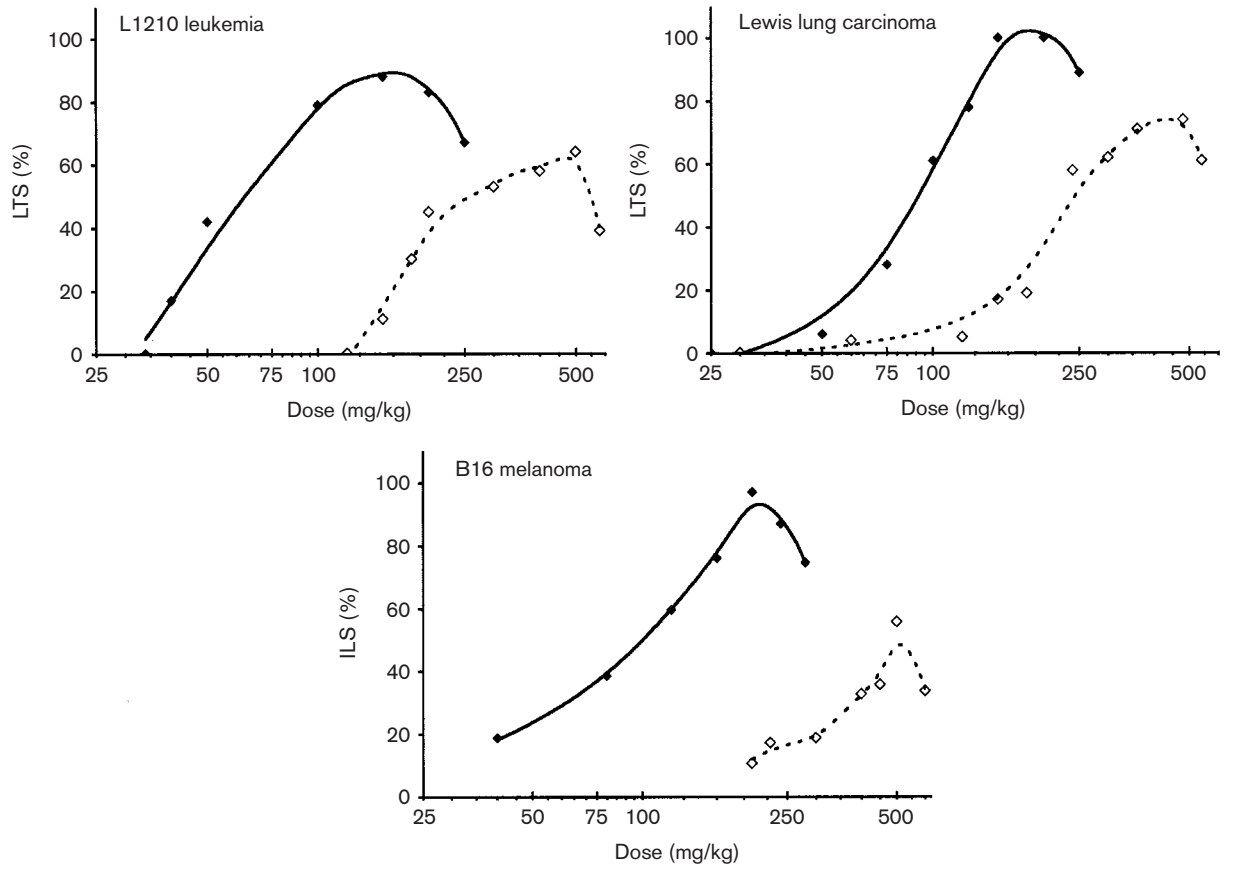


Figure 2. Antineoplastic activity of CBM-11 (◆) and ifosfamide (◇). The chart shows least-square-fitted curves of the dose–therapeutic effect relationship in the three transplantable tumor models: L1210 leukemia, Lewis lung carcinoma and B16 melanoma. Each point represent data cumulated from four to six experiments (24–40 mice per dose level), all conducted in females.

Table 2. Curative doses[a] and therapeutic indices of CBM-11 and ifosfamide

Tumor system	Compound CBM-11		Ifosfamide	
	CD_{50} (CL)	TI	CD_{50}	TI
L1210 leukemia	57.6 (48.9–67.6)	4.0	221 (199–245)	2.3
Lewis lung carcinoma	90.5 (78–105)	2.6	232 (197–274)	2.2
B16	91.8	2.5	483	1.1

[a]Curative doses were estimated 2 months after a single injection of tested compounds in tumor-bearing mice.

tumor prior to multiple peritumoral injections of IL-2-secreting cells was curative for a high percentage of treated animals and that the observed effects were clearly synergistic.[28]

Results of studies on antitumor activity of CBM-11 in advanced stages of tumor growth in mice and phase I clinical trial evaluations will be published soon.

Conclusions

(*S*)-(−)-Bromofosfamide (CBM-11), a new of bromo analog of ifosfamide in an enantiomeric form, was obtained employing an optimized acylation–reduction method.[8] CBM-11 was found to be more potent than ifosfamide against several experimental tumors in mice.

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(Received 27 February 2001; accepted 10 March 2001)