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Antitumor activity of halogen analogs of phosphoramide, isophosphoramide, and triphosphoramide mustards, the cytotoxic metabolites of cyclophosphamide, ifosfamide, and trofosfamide

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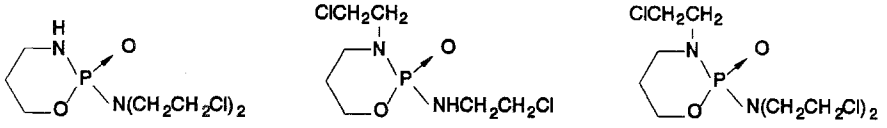

Cyclophosphamide Ifosfamide Trofosfamide

Fig. 1. Chemical structures of cyclophosphamide, ifosfamide, and trofosfamide

Abstract. A series of halogen analogs of phosphoramide mustard, isophosphoramide mustard, and triphosphoramide mustard, the cytotoxic metabolites of the antitumor drugs cyclophosphamide, ifosfamide, and trofosfamide, respectively, was evaluated in vitro against human tumor cell lines and in vivo against experimental tumors to investigate the effect of replacement of chlorine with bromine or fluorine on the antitumor activity of the parent phosphoramide mustards. In the experimental tumors L1210 leukemia, B16 melanoma, mammary adenocarcinoma 16/C, and ovarian sarcoma M5076, the antitumor activity of the analogs was observed to be generally comparable with that of the parent mustards when chlorine was replaced by bromine but uniformly lower when chlorine was replaced by fluorine. Furthermore, the monobromo analog of isophosphoramide mustard displayed equal or somewhat greater activity in comparison with cyclophosphamide when evaluated against subcutaneously implanted L1210 leukemia with intraperitoneal drug treatment and against mammary adenocarcinoma 16/C.

Key words: Phosphoramide mustards – Chemotherapy

Introduction

Cyclophosphamide (CPA) is an established clinical antitumor agent that has been in use for many years, and ifosfamide, an isomer of cyclophosphamide, has recently been approved by the Food and Drug Administration (FDA) of the United States for treatment of testicular cancer and soft-tissue sarcomas. Trofosfamide is a congener that has received only a limited amount of clinical interest in Europe [1, 14]. The chemical structures of these agents are shown in Fig. 1.

Because CPA was not cytotoxic in vitro, its metabolism was investigated in many laboratories to discover its activation pathway. These investigations led to the establishment of its metabolic pathway as shown in Fig. 2, and related studies on ifosfamide and trofosfamide indicated generally similar metabolism ([11] and references cited therein). Determination of the cytotoxity of the stable metabolites in vitro, coupled with their alkylating activity, confirmed that only the corresponding phosphoramide mustards represent the ultimate cytotoxic metabolites derivable from the parent drugs [3, 13]. The chemical structures of these metabolites are illustrated in Fig. 3.

Because it is possible that the cytotoxic form of a prodrug may be a superior antitumor agent in comparison with the prodrug form, an investigation of this possibility is being pursued in our laboratories. Because the phosphoramide mustards are cytotoxic as such, possible patient variability in drug activation is eliminated by direct treatment, whereas this variability could be responsible for less effective treatment in patients receiving the parent drugs. In addition, exposure to the toxic metabolite acrolein, gener-

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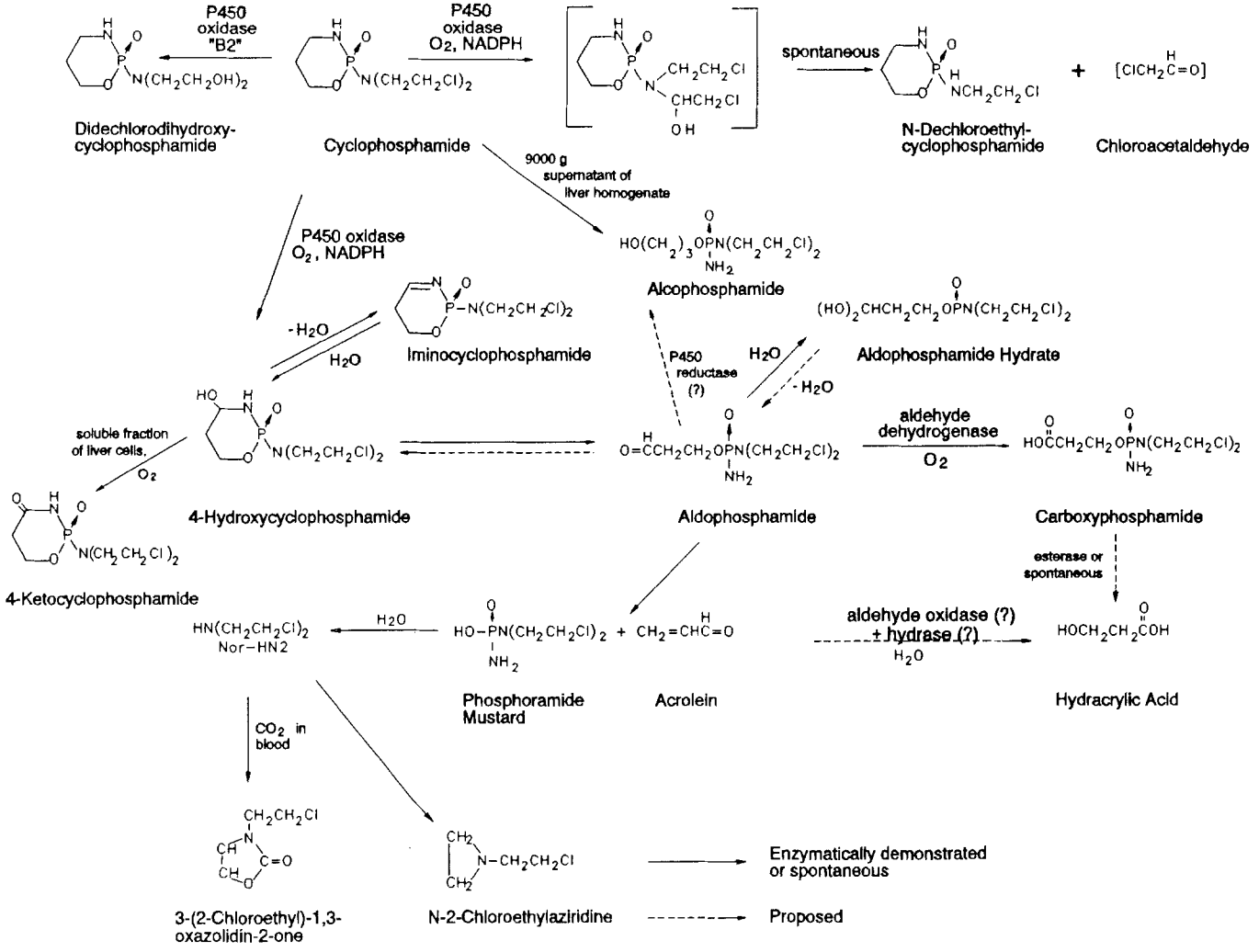

Fig. 2. Metabolism of cyclophosphamide

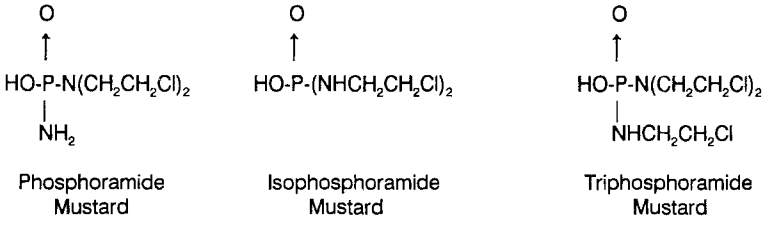

Fig. 3. Chemical structures of phosphoramide mustard, isophosphoramide mustard, and triphosphoramide mustard

ated on the activation pathway, is eliminated by direct treatment with the phosphoramide mustards.

We report herein the results of evaluation of the in vitro and in vivo anticancer activity of congeners of the phosphoramide mustards derivable from the parent prodrug forms. Details of chemical synthesis will be reported elsewhere, and a preliminary account of these studies has previously been presented [12].

Materials and methods

Phosphoramide mustards

The phosporamide mustard congeners were prepared by reaction of phenyl phosphorodichloridate with a mono- or bis-(2-haloethyl)amine followed by reaction with ammonia or a 2-haloethylamine. Reduction of the phenyl phosphorodiamidates gave the phosphoramide mustards, which were typically purified by crystallization and characterized by melting point (m.p.) and by mass spectral, [^{1}H]-nuclear magnetic resonance ([^{1}H]-NMR), and elemental analysis. For example, the findings for *N,N'*-bis(2-bromoethyl)phosphorodiamidic acid (BB-IPM) were as follows: m.p., 134° – 135° C; mass spectrum (FAB): *m/z* 309 $(M+1)^+$ (2 Br); ^{1}H-NMR (ppm): 3.04–3.14 (4 H, quint., $-NHCH_2$), 3.40–3.46 (4H, trip, $-CH_2Br$), 6.14 (3H, broad singlet, -NH-, -OH); CHN analysis: C, 15.58; H, 3.57; N, 9.09 (theory); C, 15.54; H, 3.76; N, 8.94 (found).

Cytotoxicity evaluation in vitro

Cell lines. ACHN, a human renal adenocarcinoma cell line, was generously provided by E. C. Borden (Medical College of Wisconsin, Milwaukee, Wis.). The remaining human tumor lines (DLD-1 colon

adenocarcinoma, NCI-H23 non-small-cell lung adenocarcinoma, SK-MEL-28 melanoma, and SNB-7 glioblastoma) were provided by the Division of Cancer Treatment Tumor Repository [National Cancer Institute (NCI), Frederick, Md.]. The cell lines were propagated as monolayer cultures in RPMI 1640 with 9% fetal bovine serum, 1% iron-supplemented calf serum, and 2 m*M* glutamine. Cell monolayers were subcultured using 0.05% trypsin dispersement and seeding in fresh media. The lines were passaged one to two times weekly and incubated at 37° C in an atmosphere containing 5% CO_2 at 95% relative humidity.

Anticellular assays. The anticellular effects of compounds were assessed using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt] vital dye-conversion assay [9]. Tumor cells were dispersed with trypsin and a uniform suspension was prepared in complete media. The cells were seeded in 96-well tissue-culture plates (Costar 3595) at 1×10^3 cells/well in 100 µl media. The compounds were dissolved in saline and then diluted in complete fresh media to various concentration levels. For each concentration level, 16 replicate wells were treated with 100 µl, and the plates were incubated for 72 h. On the day of an assay, XTT was weighed and dissolved (1 mg/ml in media without serum). Phenazine methosulfate (PMS, Sigma) was prepared once per month (5 mg/ml in PBS) and stored light-protected at 4° C until used in the assay. The PMS was added to the XTT solution to a final concentration of 0.05 m*M*. The wells were treated with 20 µl XTT/PMS solution and incubated for 4 h at 37° C in an atmosphere containing 5% CO_2 at 95% relative humidity. The absorbance was read at 450 nm (620 nm reference) on an Anthos/Denley Microplate reader.

Antitumor evaluation in vivo

All of the phosphoramide mustards were evaluated in murine tumor models in accordance with protocols established by the NCI [5]. For L1210 leukemia, tumors were maintained in DBA/2 mice and passaged weekly by i.p. transplantation of known amounts of ascitic leukemia cells into healthy 8- to 10-week-old mice. Cyclophosphamide-resistant L1210 cells were obtained from Microbiological Associates (Bethesda, Md.), and the cell line was developed by Dr. William DeWys. For the chemotheratpy evaluations, 5- to 7-week-old $CD2F_1$ (BALB/c X DBA/2) mice were used. Drugs were dissolved in physiological saline and injected i.p. 24 h after i.p. or s.c. implantation of tumor cells. The tumor implantation day was designated as day 0. Mice were observed for increase in life span (ILS). Antitumor activity was assessed on the basis of the median percentage of ILS (%ILS) and net $\log_{10}$ cell kill. Calculations of net $\log_{10}$ cell kill were made from the tumor-doubling time that either was determined from an internal tumor titration consisting of implants from serial 10-fold dilutions or was based on historical data [8]. Long-term (45- to 60-day) survivors were excluded from calculations of %ILS and tumor cell kill. For assessment of the tumor cell kill at the end of treatment, the survival time difference between treated and control groups was adjusted to account for the regrowth of tumor cell populations that may occur between individual treatments [7]. The net $\log_{10}$ cell kill was calculated as follows:

$$\text{Net } \log_{10} \text{ cell kill} = \frac{\text{(T-C)} - \text{(duration of treatment in days)}}{3.32 \times T_d},$$

where (*T-C*) is the difference in the median day of death between the treated (T) and the control (C) groups and T_d is the mean tumor-doubling time (days) calculated from a log-linear least-squares fit of the implant sizes and the median days of death of the titration groups.

B16 melanoma and M5076 sarcoma were maintained in C57BL/6 mice, whereas mammary 16/C tumor was maintained in C3H mice. For B16 melanoma evaluations, C57BL/6 mice were used, whereas $B6C3F_1$ mice were used for M5076 and mammary 16/C tumor evaluations. All tumors were passaged every 2–3 weeks. Treatment was begun 2–3 days after s.c. implantation of 30- to 60-mg tumor fragments. Tumors were measured with calipers twice weekly and the following formula was used to calculate mass, assuming unit density: $(axb^2)/2$ where *a* is the length in millimeters and *b* is the width in millimeters. Antitumor activity was assessed on the basis of net $\log_{10}$ cell kill and delay in tumor growth (T-C). For early drug treatment (e.g., beginning on day 1 or 3), the delay in tumor growth is the unweighted average of the differences in the median times (days) required postimplantation for tumors in the treated (T) and control (C) groups to attain two evaluation sizes. For delayed drug treatment, the delay in tumor growth is the difference in the median times required postimplantation for tumors in the treated and control groups to double in mass. Drug-related deaths, tumor-free survivors, and any other animal whose tumor failed to attain the evaluation size(s) were excluded.

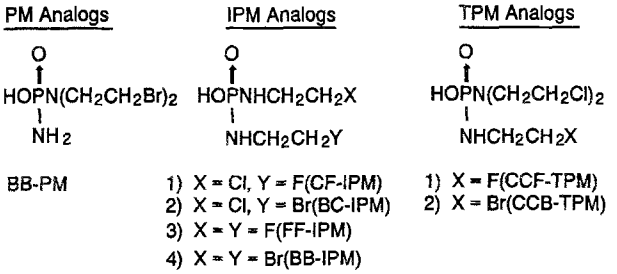

Fig. 4. Chemical structures of halogen analogs of phosphoramide mustards

Results

A series of halogen analogs of phosphoramide mustard (PM), isophosphoramide mustard (IPM), and triphosphoramide mustard (TPM) was prepared for the evaluation of antitumor activity in vitro and in vivo. The structures of the new analogs evaluated are shown in Fig. 4.

Several of the analogs were evaluated in vitro for their inhibition of human tumor cell lines representing some of the major human tumor types. The data given in Table 1 indicate that BC-IPM is uniformly cytotoxic to all of the cell lines tested and that its cytotoxicity is superior to that of 4-hydroperoxycyclophosphamide, a synthetically activated form of cyclophosphamide, against two of the cell lines, although the former analog is less cytotoxic against three of the lines.

Initial antitumor evaluation of the analogs in vivo utilized L1210 murine leukemia. The four IPM analogs were evaluated against an i.p. inoculum of 10^5 cells, with drug treatment being initiated 24 h later (day 1), using CPA and IPM as positive controls. The results, which are shown in Table 2, indicate comparable activity for BC-IPM, IPM and CPA for administration of doses equal to or less than their LD_{10} (the dose producing 10% lethality). Similar activity was observed for BB-IPM at a dose of 100 mg/kg, but at the expense of some toxicity (LD_{17}). Both of the fluorine-substituted analogs were inactive. In a confirmatory test in the same model, BB-IPM gave 4/6 long-term survivors and 84% ILS at a dose of 50 mg/kg, whereas BC-IPM gave 5/6 long-term survivors and 173% ILS at a dose of 100 mg/kg and 3/6 long-term survivors and 194% ILS at a dose of 50 mg/kg. The IPM control gave 3/6 long-term survivors and 78% ILS at a dose of 100 mg/kg, and CPA gave 1/6 long-term survivors and 63% ILS at a dose of 250 mg/kg.

Table 1. Cytotoxicity of phosphoramide mustards in an in vitro human tumor panel

Name	IC_{50} (g/ml)				
	ACHN (renal)	DLD-1 (colon)	NCI-H23 (lung)	SK-MEL-28 (melanoma)	SNB-7 (CNS)
BB-IPM	3	>19	15	11	9
bis(2-Bromoethyl) amine hydrobromide	NT	>30	10	14	5
BB-PM	NT	>30	8	13	5
BC-IPM	0.40	> 7	0.02	5	5
CF-IPM	>17	>20	>20	>14	>18
CCF-TPM	2	>16	>16	>17	10
4-Hydroperoxycyclophosphamide	2	2	1	1	2

Cytotoxicity studies involved a 72-h period of exposure to the compounds tested in an XXT assay. NT, Not tested

Table 2. Evaluation of the antitumor activity of BB-IPM, BC-IPM, CF-IPM, and FF-IPM against i.p. implanted L1210 leukemia

Agent	Dose[a] (mg/kg)	60-day survivors	% ILS (in dying mice)	Net $\log_{10}$ cell kill
BB-IPM	100 (LD_{17})[b]	3/6	122	5.9
	50	0/6	94	5.9
BC-IPM	100	4/6	388	5.9
CF-IPM	50	0/6	12	0.8
FF-IPM	100	0/6	18	1.3
IPM	100	4/6	111	5.9
CPA	250	2/6	133	5.9

A total of 10^5 cells were implanted i.p. and the compounds were injected i.p. on day 1 only

[a] Highest nontoxic dose ($\leq LD_{10}$) in a range of doses

[b] Dose level producing 17% lethality

The leukemia-active analogs BB-IPM and BC-IPM were next compared with IPM and CPA in an s.c. implanted L1210 model using 10^5 cells, with i.p. drug treatment being given on day 1 following tumor implantation. The results indicated comparable activity for BC-IPM and CPA in this tumor model, with the analog and CPA giving 3/6 and 4/6 60-day survivors, respectively, whereas BB-IPM and IPM failed to yield any long-term survivor in this experiment.

The results of the evaluation of CPA-resistant L1210 leukemia for cross-resistance to BB-IPM and BC-IPM are given in Table 3. Whereas the two analogs IPM and CPA were active against the CPA-sensitive tumor, only the phosphoramide mustards were active against the CPA-resistant tumor. All three mustards gave long-term survivors, whereas CPA, as expected, yielded no long-term survivor and no significant ILS.

The analogs were also evaluated against three murine solid tumors. The results obtained for BB-IPM and BC-IPM against s.c. implanted B16 melanoma with single-dose i.p. drug treatment are shown in Table 4. In this tumor model, the two analogs and IPM are clearly inferior to CPA. The T-C values for the three phosphoramide mustards represent a net cell kill of approximately 1 $\log_{10}$ unit, whereas the T-C value for CPA represents a net cell kill of 3 $\log_{10}$ units.

Against s.c. implanted mammary 16/C tumor, with i.p. drug being given on days 3, 7, 11, and 15 after tumor implantation, BC-IPM caused cytostasis and IPM gave a net cell kill of 1 $\log_{10}$ unit, whereas the tumor burden increased by approximately 1 $\log_{10}$ unit following treatment with BB-IPM or CPA. The results are summarized in Table 5.

Table 3. Evaluation of the activity of BB-IPM and BC-IPM against s.c. implanted L1210 leukemia and CPA-resistant L1210 leukemia

Agent	Dose (mg/kg)	L1210/0			L1210/CPA		
		45-d surv./ total	% ILS	Net $\log_{10}$ cell kill	45-d surv./ total	% ILS	Net $\log_{10}$ cell kill
BB-IPM	100	2/6	+52	4.4	1/6	+47	3.7
	50	0/6	+33	2.8	0/6	+13	1.0
BC-IPM	100	2/6	+42	3.6	2/6	+30	2.3
	50	0/6	+52	4.4	0/6	+43	3.3
IPM	100	0/6	+85	5.8	1/6	+56	4.3
CPA	250	6/6	~	>6.5	0/6	+ 4	0.3

A total of 10^5 cells were implanted s.c. and the compounds were injected i.p. on day 1 only. surv., Survivors

Table 4. Response of s.c. implanted B16 melanoma to treatment with BB-IPM and BC-IPM

Agent	Dose (mg/kg)	Time (days) to reach		Days delay (T-C)	Tumor-free surv./total
		500 mg	1000 mg		
BB-IPM	100	12.4	12.8	2.0	0/10
	50	12.7	14.3	2.9	0/10
BC-IPM	100	14.5	15.6	4.5	0/10
	50	13.1	14.2	3.1	0/10
IPM	100	12.5	13.5	2.4	0/10
CPA	250	21.5	23.5	11.9	0/10

The compounds were injected i.p. on day 1 only. surv., Survivors

Table 5. Response of s.c. implanted mammary 16/C tumor to BB-IPM and BC-IPM

Agent	Dose (mg/kg)	Time (days) to reach		Days delay (T-C)	Tumor-free surv./total
		2000 mg	4000 mg		
BB-IPM	60	Toxic	Toxic	Toxic	0/10
	30	23.5	25.2	9.7	0/10
BC-IPM	60	Toxic	Toxic	Toxic	0/10
	30	26.9	28.6	13.1	1/10
IPM	60	28.8	31.2	15.3	0/10
CPA	60	21.5	24.5	8.3	0/10

The compounds were injected i.p. every 4 days x 4 starting on day 3. surv., Survivors

Table 6. Response of s.c. implanted M5076 sarcoma to BB-IPM and BC-IPM

Agent	Dose (mg/kg)	Time (days) to reach		Days delay (T-C)	Tumor-free surv./total
		500 mg	1000 mg		
BB-IPM	100	Toxic	Toxic	Toxic	0/10
	50	23.4	26.4	11.1	0/10
BC-IPM	100	Toxic	Toxic	Toxic	0/10
	50	21.6	24.7	9.4	0/10
IPM	100	22.8	25.9	10.6	0/10
CPA	250	24.7	28.7	12.9	0/10

The compounds were injected i.p. on day 1 only

Table 7. Response of s.c. implanted M5076 sarcoma to treatment with CCB-TPM and CCF-TPM

Agent	Dose (mg/kg)	Days to 2 doublings	Days delay (T-C)	Tumor-free surv./total
CCF-TPM	40	8.7	–0.6	0/6
CCF-TPM	27	9.1	–0.2	0/6
CCF-TPM	18	11.0	1.7	0/6
CCB-TPM	40	13.6	8.4	0/6
CCB-TPM	27	12.8	7.6	0/6
CCB-TPM	18	9.7	4.5	0/6
IPM	40	15.4	6.1	0/6
IPM	27	12.6	3.3	0/6
IPM	18	10.3	1.0	0/6

The compounds were injected i.p. daily on days 11–15

Evaluation of the analogs against M5076 sarcoma was accomplished with single-dose treatment for BB-IPM and BC-IPM and with daily treatment for 5 days for CCF-TPM and CCB-TPM. The results are shown in Tables 6 and 7, respectively. The T-C values of 11.1 and 9.4 days obtained for BB-IPM and BC-IPM, respectively, correspond approximately to a net cell kill of 2 $\log_{10}$ units, which is similar to the tumor reduction caused by IPM and CPA. Using the multiple-dose schedule, the activity of CCB-TPM was similar to that of IPM, whereas CCF-TPM was inactive.

Discussion

Antitumor evaluation in vivo of a series of halogen-substituted analogs of the ultimate alkylating agents derivable from the clinical antitumor drugs CPA, ifosfamide, and trofosfamide indicates that substitution of bromine for chlorine generally results in activity comparable with that of the parent phosphoramide mustards and of CPA, whereas substitution with fluorine generally results in lower activity. However, evaluation in vitro against a series of cell lines derived from solid tumors indicates that a structure containing at least two facile leaving groups, i.e., CCF-TPM, retains significant activity. The results of the evaluations in vitro are considered to be important in indicating generally comparable cytotoxicity against these solid tumor cell lines for the phosphoramide mustards in comparison with 4-hydroxyperoxy-CPA, a synthetic prodrug form of 4-hydroxy-CPA. In contrast, the phosphoramide mustards have generally been observed by other investigators to be significantly less cytotoxic in vitro than 4-hydroxy-CPA against cell lines derived from lymphoproliferative tumors, and such differences have been interpreted to suggest a major role for 4-hydroxy-CPA and 4-hydroxyifosfamide in mediating the antitumor activity of the parent drugs in vivo but only a minor role for extracellular phosphoramide mustards [11]. However, a comparison of the cytotoxicities of BC-IPM and 4-hydroperoxy-CPA as shown in Table 1 reveals a 5- to 50-fold greater cytotoxicity for BC-IPM against two of the cell lines and suggests a possible role for circulating phosphoramide mustards in contributing to the therapeutic effect of CPA and ifosfamide against certain solid tumors, particularly in consideration of the generally higher AUC values (areas under the concentration-time curve) observed for phosphoramide mustard in comparison with 4-hydroxy-CPA in patients receiving CPA [11].

The curative activity observed for the IPM analogs (Table 2) against L1210 leukemia indicates potent cytotoxicity in vivo and activity comparable with that of CPA, although the tumor model used (i.p. tumor and i.p. drug treatment) is known to be very sensitive. However, previous studies using this model have indicated that at 24 h after the inoculation of tumor cells, approximately 10% of the tumor burden is systemic [10], making the model more than a simple in vitro cytotoxicity assay conducted in vivo. The systemic activity of the analogs is illustrated more convincingly by their activity against s.c. implanted L1210, and the notable activity of BC-IPM in this model is indicated by its yielding approximately the same number of long-term survivors as CPA.

Since the resistance of CPA-resistant L1210 leukemia is believed to result from elevated levels of the 4-hydroxy-CPA-deactivating enzyme aldehyde dehydrogenase [11], the closely identical activity of the phosphoramide mustards against CPA-sensitive and -resistant tumors is not surprising (Table 3). All three phosphoramide mustards produced log-term survivors against the resistant tumor.

A more stringent test of the therapeutic efficacy of the phosphoramide mustards was given by their evaluation against three murine solid tumors. Although BB-IPM, BC-IPM, and IPM were inferior to CPA against B16 melanoma (Table 4), all were at least as effective as CPA against mammary 16/C tumor, with BC-IPM being slightly superior (Table 5). At the highest nontoxic doses tested, BB-IPM, BC-IPM, CCB-TPM, and IPM were essentially equal to CPA in activity when evaluated in the M5076 sarcoma model, whereas CCF-TPM was inactive (Tables 6, 7).

Substitution of bromine for chlorine in the various phosphoramide mustard analogs was investigated to compare the effect of this substitution on the activity of the analogs in vivo. Although the analogs, which exist in the anionic phosphorodiamidate form at physiologic pH, are clearly less lipophilic than the neutral, 4-hydroxylated, activated metabolite of cyclophosphamide and its clinically investigated congeners and, as a result, would be expected to enter tumor cells less readily, they nonetheless are in certain instances of comparable antitumor activity in vivo as shown in Tables 2–7. Pharmaceutically, the 4-hydroxy

metabolites are subject to oxidative and, possibly, reductive deactivation as well as to extracellular conversion to the mustards and disposition, whereas the mustards would appear to be chiefly subject to disposition. Consequently, the pharmacologic balance between tumor cell cytotoxicity and disposition appears to be comparable for the 4-hydroxy metabolite versus the mustards in vivo in some cases, resulting in comparable activity against certain tumors, although the 4-hydroxy metabolites are typically of greater potency as cytotoxic agents in vitro [11].

These studies indicate that chlorine and bromine but not fluorine analogs of phosphoramide mustards exhibit notable in vivo antitumor activity that is generally comparable with that of CPA and suggest the possible development of a suitable analog of this type as a clinical agent. Advantages in comparison with CPA and ifosfamide include the absence of acrolein as a metabolite, which is believed to be responsible at least in part for sometimes dose-limiting hemorrhagic cystitis [2, 4], the absence of chloroacetaldehyde as a metabolite, which is believed to be responsible for some of the neurotoxicity of the oxazaphosphorine drugs, particularly ifosfamide [6], and the lack of a requirement for metabolic activation, which could eliminate the possible variability in generation of the preactivated (4-hydroxy-CPA and -ifosfamide) and activated (phosphoramide mustard and IPM) metabolites of CPA and ifosfamide in some patients.

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