Synthesis and Properties of Bis(2,2-dimethylaziridinyl)phosphinic Amides: A Series of New Antineoplastic Agents

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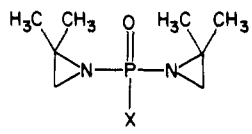
In continuation of efforts to improve the antitumor selectivity of the 2,2-dimethylaziridine class of alkylating agents, a series of N-substituted bis(2,2-dimethyl-1-aziridinyl)phosphinic amides has been synthesized and evaluated. All of these compounds (3–15) were tested in vivo against leukemia P-388 in mice, where most of them caused significant increase of survival time at nontoxic dose levels. Some of the most active compounds were also tested against leukemia L1210, B16 melanoma, and colon 26 carcinoma; in the latter tests, the parent unsubstituted amide 3 appeared to show the highest antitumor activity. Since the dose-limiting toxicity of the clinically tested prototypes of this class of anticancer agents AB-132 (1) and AB-163 (2) had been found to be CNS toxicity attributable mainly to the inhibition of cholinesterase, the compounds were tested in vitro against the cholinesterases from horse serum, electric eel, and bovine erythrocytes, as well as in vivo for the inhibition of the cholinesterases present in the whole blood of mice. In all of these assays, the various members of the present series showed a wide range of anticholinesterase activities, ranging from almost zero (for 3) to even higher potency than that of the prototype 2. A similarly wide range of stability was observed toward hydrolytic ring opening of the 2,2-dimethylaziridine moieties. Several of the compounds, particularly 3, deserve further study.

Earlier studies of various antitumor agents incorporating the bis(2,2-dimethyl-1-aziridinyl)phosphinoyl moiety[1-5] have shown that this class of compounds exhibits pharmacologic properties that are markedly different from those of the conventional C-unsubstituted aziridine type alkylating agents. Representative members of this group AB-132 (1)[3] and AB-163 (2)[6] (Chart I), have undergone extensive animal[2,7,8] and clinical[9-11] testing that has demonstrated the effectiveness of both of these agents in causing tumor regression in a variety of neoplasms. Noted for these compounds was a relatively low hematologic toxicity,[2,7,9-11] as compared with that of most other alkylating agents; but instead, they exhibited significant CNS toxicity attributed to the inhibition of cholinesterases[13] as their dose-limiting side effect. In addition, in both animal and clinical studies, these compounds appear to potentiate the antitumor effect of X-irradiation.[2,9,11-15]

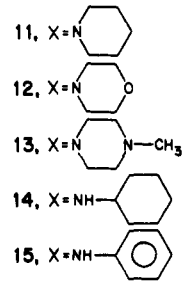
In the continuing study of this interesting class of agents, our efforts have been directed at the synthesis of analogues possessing enhanced antitumor effectiveness, but lower cholinesterase inhibitory activity. Presently, a series of bis(2,2-dimethyl-1-aziridinyl)phosphinic amides has been prepared and investigated.

Chemistry. Most of the derivative compounds (3–13) were prepared (Scheme I) by first adding 2 equiv of 2,2-dimethylaziridine to $POCl_3$ in the presence of an appropriate proton acceptor such as triethylamine and then adding the appropriate amine to the bis(2,2-dimethyl-1-aziridinyl)phosphinyl chloride, again in the presence of a proton acceptor. Purification of the final product by vacuum distillation or sublimation was possible in most cases. Compound 14 was prepared by the addition of 2,2-dimethylaziridine to the cyclohexylphosphoramidic dichloride generated in situ from cyclohexylamine and $POCl_3$ in the presence of triethylamine. Because of the low nucleophilicity of aniline, compound 15 was formed by the initial addition of aniline to $POCl_3$ followed by the addition of the 2,2-dimethylaziridine moieties. Purification of 15 was by crystallization from ether. In all cases, contact with acids or moisture was scrupulously avoided, since this would lead to hydrolytic ring opening and/or polymerization of the aziridine moieties.

Chart I

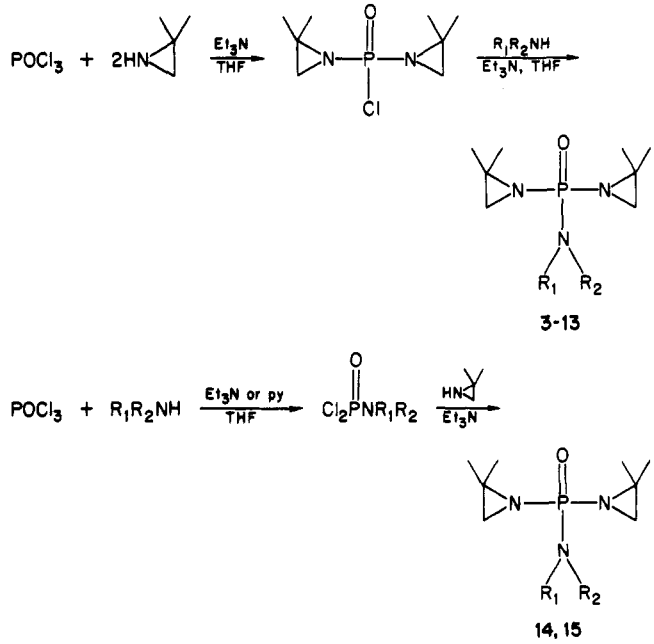


1, X = NHC(O)OCH$_2$CH$_3$ (AB-132)
2, X = OCH$_2$CH$_3$ (AB-163)
3, X = NH$_2$
4, X = NHCH$_3$
5, X = N(CH$_3$)$_2$
6, X = NHCH$_2$CH$_3$
7, X = NHCH$_2$CH$_2$CH$_3$
8, X = NHCH$_2$CH$_2$CH$_2$CH$_3$
9, X = NHCH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$
10, X = NHCH$_2$CH$_2$CH$_2$OCH$_3$



11, X = N
12, X = N O
13, X = N N—CH$_3$
14, X = NH
15, X = NH

Scheme I



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14, 15

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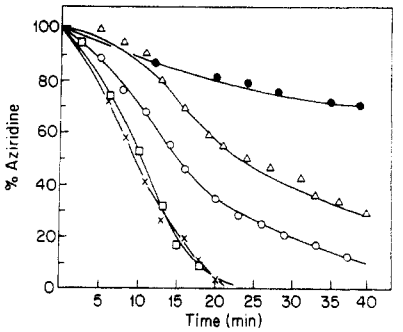

Figure 1. Hydrolysis in D_2O, at 35 °C and 0.2 M concentration, of phosphoric *N*-(*n*-alkyl)amides: **3** (●); **4** (○); **6** (×); **7** (△); **8** (□).

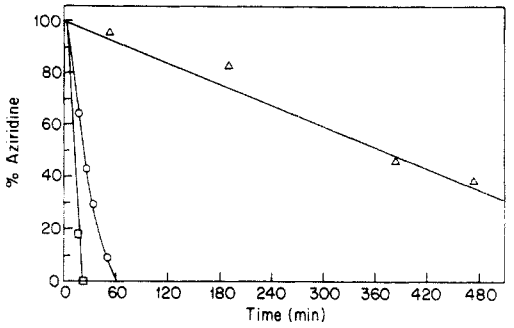

Figure 2. Hydrolysis of cyclic amine derivatives **11** (□), **12** (○), and **13** (△), 0.2 M in D_2O, 35 °C.

An interesting feature of the proton NMR spectra of most of these compounds (**3** does not show this phenomenon) in the solvent $CDCl_3$ is the appearance of the aziridine geminal (2,2) methyl groups as a set of two singlets near δ 1.40, separated by about 0.05 ppm, and the complex splitting of the aziridine geminal (3,3) proton signal. For some bis(2,2-dimethyl-1-aziridinyl)phosphoryl compounds such as AB-163 (**2**) this methylene signal appears as a neat doublet, J being about 14 Hz, due to three-bond coupling of the protons to the phosphorus atom. In compounds **4**–**14** it has the appearance of an irregular doublet of broadened doublets. This pattern is interpretable as the result of nonequivalence of the two protons of each aziridine methylene, such that one is shifted upfield from the other by approximately 0.02 ppm and each methylene proton is coupled to P by a different coupling constant: J = 12.0–13.0 Hz for the upfield doublet centered at about δ 2.10 and J = 14.0–14.5 Hz for the downfield doublet centered at about δ 2.12. The pattern is further complicated by geminal coupling of about 2 Hz. It is proposed that in this set of compounds the usual free rotation around the P–N bonds to the aziridine rings is impeded by either steric or electronic effects, causing nonequivalence of the faces of the attached aziridines at ambient temperature. This effect disappears when D_2O is used as the NMR solvent.

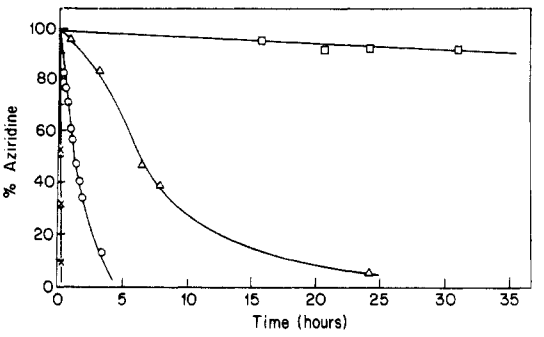

Figure 3. Wide range of hydrolysis rates for a selection of the phosphoraziridines (0.2 M concentration, in D_2O at 35 °C): **3** (○); **6** (×); **9** (□); **13** (△).

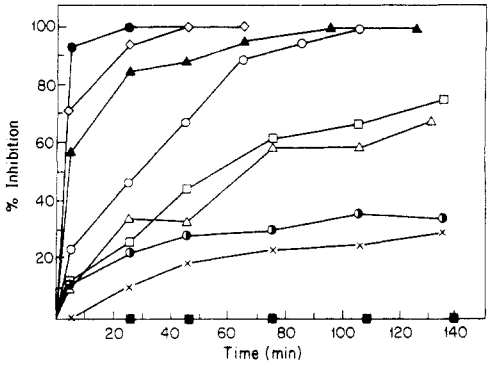

Figure 4. Inhibition of horse serum cholinesterase by the phosphoraziridines: **2** (○); **3** (■); **4** (×); **5** (◑); **6** (●); **7** (□); **8** (◇); **9** (▲); **10** (△).

Hydrolysis Studies. An estimate of the compounds stabilities in aqueous media is significant to the understanding of their pharmacodynamic properties as well as for assessing their limitations regarding parenteral drug formulations. The rates of the hydrolyses of the aziridine rings in several of the bis(2,2-dimethyl-1-aziridinyl)phosphinic amides (Figures 1–3) were studied primarily by means of the NMR spectra of the amides dissolved at 0.2 M concentration in D_2O at 35 °C. The data from NMR spectrum analysis agreed well with data obtained by titration[16] of the aziridine moieties. With two exceptions, most of these compounds were hydrolyzed rapidly, with half-lives of 10–80 min. However, the aziridine rings of **9** and **13**, which each contain an extra amino group separated from the phosphoryl by the amide nitrogen and two or three methylene groups, showed a comparative high resistance to hydrolysis. The half-life of **13** at 0.2 M concentration in D_2O was about 3.2 h, while the half-life of **9** under the same conditions was an amazing 167 h in D_2O. In a pH 7.0 buffer at 1.0 M concentration, the half-life of **9** was 132 h, indicating that the effect is not one

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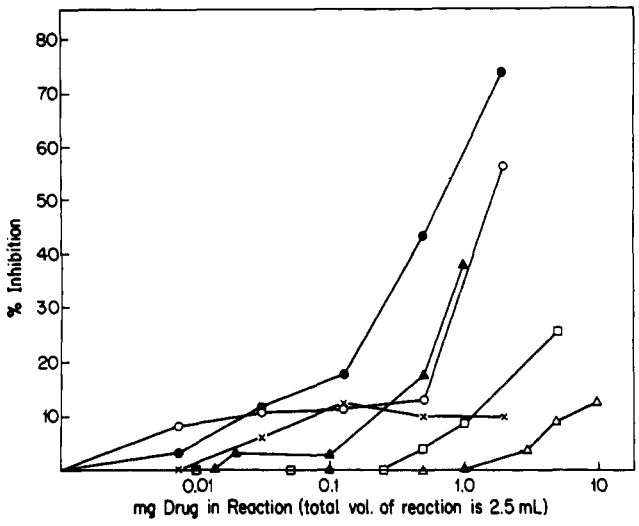

Figure 5. In vitro inhibition of electric eel cholinesterase by phosphoraziridines: **2** (○); **4** (×); **6** (●); **7** (□); **9** (▲); **10** (△).

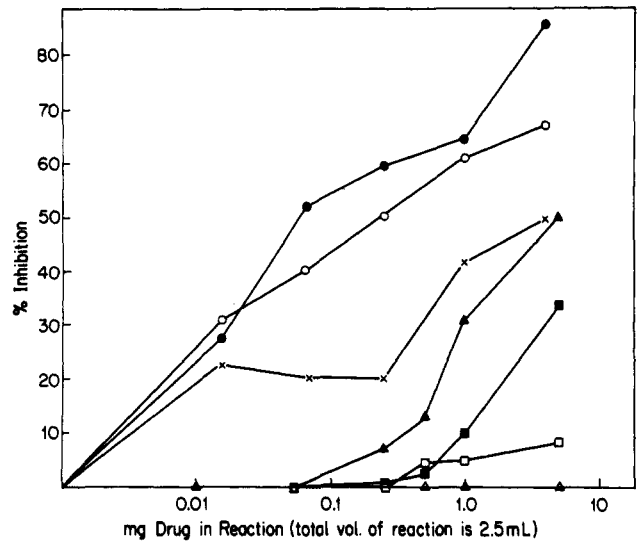

Figure 6. In vitro inhibition of bovine erythrocyte cholinesterase by phosphoraziridines: **2** (○); **3** (■); **4** (×); **6** (●); **7** (□); **9** (▲); **10** (△).

of simple increase in the general basicity of the medium because of the extra amine moiety. Apparently, the side chain amino group acts intramolecularly as a protection against the opening of the aziridine rings, probably by preventing the protonation of the aziridine nitrogen through offering a more basic site in close proximity to it.

Cholinesterase Inhibition. An in vitro assay of these compounds relative abilities to inhibit the activity of horse serum cholinesterase according to the procedure described by Lalka and Bardos[13] demonstrated a broad spectrum of inhibitory potencies for this series of phosphinic amides (Figure 4), from the total noninhibition caused by **3** to the very potent activities of **6** and **8**. An interesting alternating variation in cholinesterase inhibitory behavior with increasing chain length of the substituent amino function was noted for the *N*-methyl (**4**), *N*-ethyl (**6**), *N*-propyl (**7**), and *N*-butyl (**8**) derivatives.

A second set of in vitro assays of the cholinesterase inhibitory activities of a selected few of the compounds, using both electric eel cholinesterase and bovine erythrocyte cholinesterase, was then conducted (Figures 5 and 6). The tertiary amine bearing compound **9** was a fairly effective cholinesterase blocker. Compound **6** again exhibited powerful cholinesterase inhibitory behavior, greater

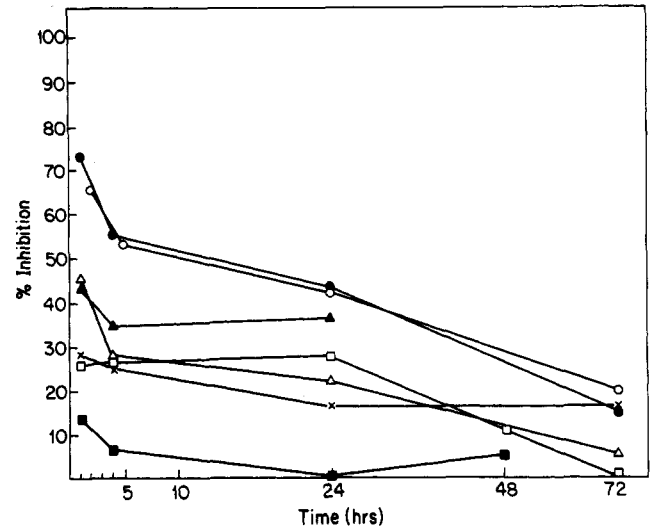

Figure 7. In vivo inhibition of whole blood cholinesterase in Balb/cCr mice by phosphoraziridines: **2** (○); **3** (■); **4** (×); **6** (●); **7** (□); **9** (▲); **10** (△).

than that of AB-163 (**2**), while **4** showed relatively minor inhibitory effects toward electric eel cholinesterase and moderate inhibition of bovine erythrocyte cholinesterase. Compound **7** had little potency in either assay, exhibiting a maximum of 9% inhibition of bovine erythrocyte cholinesterase at 2 mg/mL concentration and 26% inhibition of electric eel cholinesterase at the same concentration; the threshold concentrations of **7** for any activity to be observed were a relatively high 0.2 mg/mL in both assays. Comparison of **4**, **6**, and **7** in these assays further reinforces the observation of the alternating relationship of cholinesterase inhibitory potency to chain length in these *N*-(*n*-alkyl) derivatives. Compound **3**, which had been totally inactive toward horse serum cholinesterase, did show some minor inhibition of the bovine erythrocyte cholinesterase, with 0.2 mg/mL as a threshold concentration for any significant activity, and 34% inhibition at the maximum concentration tested, 2.0 mg/mL. The least active inhibitor of cholesterases in either of these two assays was compound **10**, which proved to be totally inert toward bovine erythrocyte cholinesterase at all concentrations up to 2.0 mg/mL and affected electric eel cholinesterase activity by only 13% at a maximum dosage of 4.0 mg/mL (threshold concentration of 1.0 mg/mL in this experiment).

An in vivo study of the drugs abilities to inactivate the whole blood cholinesterase of Balb/cCr mice showed results generally paralleling those obtained in the several in vitro studies. As shown in Figure 7, **6** again proved to be a potent blocker of cholinesterase, while **4** and **7** showed relatively minor inhibitory activities. Compound **3** was again confirmed as having very little inhibitory effect. Disappointingly, compound **10**, which had shown encouragingly low inhibition of electric eel and bovine erythrocyte cholinesterases in vitro, did exhibit significant inhibitory activity in this in vivo assay.

In Vivo Testing against Murine Tumors. The entire series of bis(2,2-dimethyl-1-aziridinyl)phosphinic amides was evaluated against P388 leukemia implanted ip. For each tested compound, doses of 256, 128, 64, and 32 mg/kg (and for compounds **3–5**, additional doses of 16 and 8 mg/kg) were administered ip to six mice per dose level. The measure of the drugs effectiveness at each dosage level is expressed as % T/C, a ratio of the average life span of the treated mice to that of a control group of 10 tumor-implanted, untreated mice. The maximum therapeutic effect obtained for each compound, within the dose range

Table I. Effect of Bis(2,2-dimethylaziridinyl)phosphinic Amides on P388 Leukemia

compd	opt dose,[a] mg/kg	max % T/C (cures/total)[b]
1 (AB-132)[c]	400	244
2 (AB-163)[c]	80	272 (2/6)
3	256[d]	206
4	128	239 (1/6)
5	128	272
6	256[d]	288
7	256[d]	363 (2/6)
8	256[d]	238
9	64	231
10	256[d]	225 (1/6)
11	256[d]	218
12	256	247
13	128	224
14	256[d]	106
15	256	169

[a] Dose at which therapeutic effects are maximized without evidence of excessive toxicity, administered ip on day 1 following ip implant of 10^6 P388 cells in six mice per dosage tested. [b] % T/C is the ratio of the average lifespan of treated mice to that of a control group of 10 tumor-implanted untreated mice. "Cures" were tumor-free mice surviving to day 30 or beyond. [c] Prototype 2,2-dimethylphosphoraziridines[6-12] used as standard compounds for comparison. [d] Highest dose level evaluated.

tested, is summarized in Table I; these data were obtained from several experiments so that strict comparisons of effectiveness between compounds cannot be made due to the lack of concomitant testing. All of the tested compounds except **14** caused at least a 40% increase in lifespan (i.e., T/C ≥ 140%) at a dose of 32 mg/kg (data not shown), and the maximum effects were often obtained at 256 mg/kg, the highest dose level to be evaluated. The inactive derivative **14**, as well as the modestly active **15**, were water insoluble, but so were derivatives **5** and **8**, which showed substantial activity. A few of the new derivatives, **4**, **7**, and **10**, caused mice to be cured of P388. Most of the active derivatives had optimal doses (i.e., doses causing maximal therapeutic effect without unacceptable levels of toxicity) in the 128–256 mg/kg range and caused sufficient weight loss at 256 mg/kg as to make unlikely the prospect that higher doses, although not evaluated, would have been tolerated. In this assay the two clinically tested prototype 2,2-dimethylphosphoraziridines AB-132 and AB-163 were used as the standards.

Eleven of the new amides were subsequently tested against ip-implanted B16 melanoma (Table II) at several dose levels between 10 and 160 mg/kg for each compound, and 10 mice per dose level were tested. Also described in the tabular summary are the concomitant test results obtained for compound **2** and the clinically established alkylating agents cyclophosphamide and chlorambucil, which were included in selected experiments for purpose of comparison.

Compound **3** displayed consistently good activity in the ip B16 tumor model. The average maximum % T/C value based on six experiments was 214%. On two occasions a few mice were cured of B16 following treatment with **3**. Compound **9** was also quite active in this tumor model and produced maximum effects similar to those of compound **3** both times these compounds were tested in parallel. Compounds **6–8**, **10**, **12**, and **13** also showed meaningful but relatively less activities against ip B16, while **11** caused only borderline activity which could be described as inconsequential.

Compound **2** was also fairly active vs. ip B16 but inferior to **3** when both were tested concomitantly. Cyclophosphamide showed modest to good activity in this tumor

Table II. Effect of Selected Phosphoraziridines on B16 Melanoma

compd	test	opt dose,[a] mg/kg/inj	max % T/C (cures/total)[b]
2 (AB-163)	366	100[c]	188
3	366	100	310 (3/10)
	414	100	181
	455	40	214 (2/10)
	462	120[c]	191
	478	120	180
	510	80	210
4	436	60	120
	462	120[c]	153
5	436	60	118
	462	120[c]	153
6	455	120[c]	148
7	455	80	162
	462	120	144
8	455	120[c]	157
9	455	80[c]	210 (2/10)
	478	80	172
10	455	80	160
11	510	160[c]	132
12	510	160[c]	160
13	510	80	170
cyclophosphamide	436	100	143
	462	70	144
	478	120	180
chlorambucil	462	24[c]	121
	478	18	132
	510	36[c]	125

[a] Administered ip on days 1, 5, and 9 following ip implant of 0.5 mL of 10% w/v tumor breis. Optimal dose is that dosage which gives the maximal therapeutic effect without unacceptable toxicity (or the arbitrarily chosen maximum dosage, if indicated). [b] T/C is a ratio of average life span of the 10 treated mice to that of the 10 untreated control mice. "Cures" were tumor-free mice surviving to day 60. [c] Highest dose level evaluated.

Table III. Effect of Three New Phosphoraziridines on L1210 Leukemia

compd	opt dose,[a] mg/kg	max % T/C
3	60[b]	164
4	180[c]	207
5	180[c]	207

[a] Administered ip on day 1 following ip implant of 10^6 L1210 cells, in six mice per dosage tested. [b] At a higher dose of 180 mg/kg, compound **3** caused a T/C of 357% including two of six mice cured. But another two mice in the same group died prior to the first death of the untreated leukemic control mice due to undetermined cause; therefore, this experiment was excluded. [c] Highest dose level evaluated.

model whereas chlorambucil was only minimally active on two occasions and inactive in a third experiment.

Three of the phosphoramides were tested in the same experiment against ip-implanted L1210 leukemia (Table III), at several dose levels between 10 and 360 mg/kg for each compound with six mice per dose level tested. Compounds **4** and **5** achieved identical maximum increases in lifespan as reflected by a T/C value of 207%.

Compound **3** was included in this study but yielded a T/C only 164% at the (unexpectedly low) dose of 60 mg/kg. A higher dose of **3**, 180 mg/kg, caused two of six cures but also killed the same proportion of mice by day 5 post-implant and so this dose level as judged, in this study, to be too toxic for evaluation.

A few compounds were selected for antitumor evaluation against additional murine tumor models. The results of these studies are summarized in Table IV. For a compound to be considered active vs. sc B16 it must achieve a T/C of ≥140%, and as such none of the phosphoraziridines[17] tested were active. Cyclophosphamide, how-

Table IV. Advanced Antitumor Testing of Selected Phosphoraziridines

tumor implant site[a]	compd	expt	opt dose, mg/kg (inj)	treatment schedule; route	max % T/C (cures/total)[b]
B16 sc	2 (AB-163)	366A	100	1, 5, 9; ip	119
	3	366A	50	1, 5, 9; ip	123
		416	40	1, 5, 9; iv	110
	4	436	10	1, 5, 9; iv	126
	5	436	10	1, 5, 9; iv	108
	cyclophosphamide	436	100	1, 5, 9; iv	205
C26 ip	2 (AB-163)	42	120	5, 8; ip	144
	3	51	120	1, 4; ip	190 (3/8)
		61	80	1, 4; ip	221 (2/8)
	4	51	60	1, 4; ip	166
	5	51	80	1, 4; ip	127
	9	61	50	1, 4; ip	170
	cyclophosphamide	51	125	1, 4; ip	134
C26 sc	2 (AB-163)	42	120	5, 8; ip	130
	3	51	50	1, 4; ip	127
	4	51	25	1,4; ip	151
	5	51	60	1, 4; ip	125
	cyclophosphamide	51	125	1, 4; ip	227 (1/8)
M109 ip	2 (AB-163)	130	150	1, 4; ip	146
	3	130	150	1, 4; ip	157
M109 sc	2 (AB-163)	130	100	1, 4; ip	104
	3	130	70	1, 4; ip	99
	3	130	70	1, 4; iv	104

[a] Tumor implant levels are described in the materials and methods section. [b] "Cures" were tumor-free mice surviving to day 60 or beyond.

ever, included in one of the sc B16 experiments, produced a maximum T/C of 205%.

In contrast to the sc B16 results, several phosphoraziridines tested against ip colon 26 carcinoma were active (T/C ≥ 125%) to varying degrees. Compound **3** was the most active of those evaluated. In two experiments **3** cured a few mice of ip C26 while achieving T/C values of ≥190%. Compounds **4** and **9** showed moderate activity in this model, T/C values of 166% and 170%, respectively. The level of activity of compounds **2–5** vs. sc C26 was less than that observed against ip C26. In contrast, cyclophosphamide was only minimally active vs. ip C26 but showed very good activity against sc C26.

Finally, compounds **2** and **3** were tested against the M109 lung carcinoma. While both compounds displayed modest activity vs. ip M109, neither was active against sc M109.

Conclusions. Several compounds in this series of bis(2,2-dimethyl-1-aziridinyl)phosphinic amides show promise as antineoplastic drugs. Compound **9**, and to a lesser extent **13**, are of interest because of their greater stability in aqueous media, which would facilitate their use for parenteral injection and, in addition, extend the presence of effective drug concentration in the tissues. Each shows greater antitumor effectiveness (at equal dosages) than its structural analogues in the series (considering **9** to be structurally analogous to **8** and **10**, and **13** analogous to **11** and **12**). However, both **9** and **13** show significant toxicities at the higher doses tested; thus, the optimal doses, which avoid unacceptable toxicity, are lower than the doses considered optimal for most members of the series and yield % T/C values at their optimal dose levels that are approximately the same as for the other compounds of this series at their respective (higher) optimal dose levels. Moreover, the cholinesterase inhibitory activity of **9** does not augur well for its use in cancer chemotherapy.

Considering the simple *n*-alkylamine derivatives **4**, **6**, **7**, and **8**, it may be noted that the maximum % T/C against P388 leukemia increases with increasing *n*-alkyl chain length up to the *n*-propylamine **7** and then decreases again at **8**; this is undoubtedly because, while **7** is reasonably water soluble, **8** is sparingly soluble in water. Insolubility might also explain the low activities seen for **14** and **15**, both water insoluble. Compound **7** showed a remarkably high effectiveness against P388, although its activity in inhibiting B16 melanoma was only approximately the same as for the other members of the series that were tested against this tumor system. An interesting alternating relationship between chain length and both the rate of hydrolysis and cholinesterase inhibition appears to exist for this subgroup of *n*-alkylamine derivatives; both the methyl (**4**) and the propyl (**7**) derivatives were comparatively more stable in water than the rapidly hydrolyzed ethyl- (**6**) and butyl- (**8**) substituted analogues, and concomitantly **6** and **8** were much more effective inhibitors of cholinesterases than **4** and **7**. The duality of this relationship is not surprising considering that cholinesterase inhibition by bis(2,2-dimethyl-1-aziridinyl)phosphinic compounds has been found to be caused not so much by the actual phosphoraziridine alkylating agents as by their hydrolytic intermediates.[13,14] An explanation for the variability in hydrolytic stabilities for this series of compounds has not yet been determined, however. Because of its relatively better hydrolytic stability and lower cholinesterase inhibitory activity, especially toward the acetyl cholinesterases, coupled with its comparatively good antineoplastic effectiveness as demonstrated by the limited testing done so far, **7** shows some promise and appears to merit further study.

The parent compound **3** appears to be the most promising member of the series. It combines a very low (in some instances, nonexistent) cholinesterase inhibitory activity with significant effectiveness against all of the ip-implanted neoplasms tested. The hydrolytic stability of **3** is somewhat better than that of **7**, making it more conveniently utilizable as a drug that should be injectable as an aqueous solution (**3** is highly water soluble). Further evaluation of **3** is planned.

The bis(2,2-dimethyl-1-aziridinyl)phosphinic amides have proved to be interesting antineoplastic agents, showing moderate-to-good activities in most cases against several transplanted tumors in mice but exhibiting a wide range of variability in some of their properties relevant to

(17) This term is used loosely, to cover any compound containing a phosphorus atom linked to aziridine moieties.

their pharmaceutical usefulness such as hydrolytic stability and cholinesterase inhibition. The results presented here show that the anticholinesterase and antineoplastic activities of this series of agents are independent properties and can be separated from each other. It remains to be seen whether or not there is any relationship between the anticholinesterase activity and radiation potentiating effect of the 2,2-dimethylphosphoraziridines[15] and whether or not the remarkably low hematologic toxicity of such agents as AB-163 is dependent on their rapid hydrolyzability. The variability of these properties seen in the case of the amide series permits us to investigate the possible existence of such interrelationships that may provide a basis for the design of more useful chemotherapeutic agents.

Experimental Section

Melting points were determined on a MelTemp apparatus and are uncorrected. NMR spectra were obtained on a Varian T-60 spectrometer, and values are reported in parts per million (δ) from Me_4Si. Analyses were performed by Atlantic Microlabs, Atlanta, GA. Thin-layer chromatography was performed with Analtech silica gel GHLF plates.

2,2-Dimethylaziridine was supplied by Polyscience, Inc. Most of the alkylamines used were obtained from the Aldrich Chemical Co., and those with boiling points sufficiently above ambient temperature were distilled before use. The tetrahydrofuran used as the solvent in most of the preparations and purifications of the phosphoraziridines was distilled from CaH and stored over metallic sodium to ensure its dryness. Because of the susceptibility of the 2,2-dimethylaziridine moiety to hydrolysis, all of the syntheses were performed under a dry N_2 atmosphere, and stringent precautions to exclude moisture were taken during handling of these phosphoraziridines.

General Synthetic Procedures. To a well-stirred solution of 1.0 equiv of $POCl_3$ and a 5–10% excess over 3 equiv of triethylamine in tetrahydrofuran (150 mL for a 0.025-mol scale reaction), chilled to −40 °C under N_2, is added very slowly, via a dropping funnel, a solution of 2.0 equiv of 2,2-dimethylaziridine in THF (20–30 mL for a 0.025-mol scale reaction), over a period of 1–2 h. The resulting reaction mixture is permitted to warm gradually to ambient temperature over a period of at least 1 h. At this point, the mixture might be vacuum filtered directly into a second reaction vessel to remove the precipitated triethylamine hydrochloride or the slurry may be used in the next stage without filtration. This THF solution of bis(2,2-dimethyl-1-aziridinyl)-phosphinic chloride and triethylamine is then combined with the appropriate primary or secondary amine by one of the following procedures:

Method A. The filtered bis(aziridinyl)phosphinic chloride solution is chilled at −30 to 0 °C, and a gaseous amine (NH_3, CH_3NH_2, $CH_3CH_2NH_2$) is bubbled through the well-stirred solution for 1–1.5 h. The flask is then stored, unstirred, at 4 °C overnight; it is then permitted to warm to ambient temperature and rapidly vacuum filtered to remove amine hydrochloride salts. The filtrate is then concentrated at reduced pressures and purified by the appropriate technique.

Method B. The unfiltered bis(aziridinyl)phosphinic chloride solution is chilled at −30 to 0 °C, and to it is added a solution of an alkylamine, dialkylamine, or cyclic amine (1.05–1.2 equiv) in 20 mL of THF at a moderate rate from a dropping funnel. The mixture is then stirred overnight at 4 °C, for those preparations involving low-boiling amines, or at room temperature when the amine has a boiling point above 50 °C. Filtration of the mixture to remove amine hydrochlorides is followed by concentration at reduced pressures and purification by the appropriate method.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic Amide (3).** The general synthetic procedure, using method A, was followed by using these modifications: The filtered bis(2,2-dimethyl-1-aziridinyl)phosphinic chloride solution was diluted to twice its original volume, a dry ice/acetone cold finger condenser was used to condense escaping NH_3 back into the flask during the addition phase, and in the filtration of the final reaction mixture the collected solids were washed copiously with hot THF until no more solids were observed to dissolve. The concentrated filtrate was recrystallized from THF to give **3** in 53.9% yield; mp 169–177 °C with sublimation noted at 150 °C. Anal. ($C_8H_{18}N_3OP$) C, H, N.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic *N*-Methylamide (4).** The general procedure, method A, was used. The concentrated filtrate was passed through a shallow bed of silica gel with 2% triethylamine/10% methanol/88% CH_2Cl_2 as eluent, reconcentrated, and distilled at 96–104 °C (0.10–0.14 mmHg) to give **4** in 83.3% yield as an oil that solidified on standing, to a waxy solid, not analytically pure. A pure sample was obtained by a fractional sublimation at 35–38 °C under vacuum. The initial sublimate, an oil, was removed from the sublimer collection surface before the remaining crystalline material was sublimed. Four sequential such sublimations gave pure **4**, mp 66–67.5 °C. Anal. ($C_9H_{20}N_3OP$) C, H, N.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic *N,N*-Dimethylamide (5).** The general procedure, method B, was used. Distillation at 57.5 °C (0.15 mmHg) gave colorless liquid **5** in 75.6% yield. Anal. ($C_{10}H_{22}N_3OP$) C, H, N.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic *N*-Ethylamide (6).** The general procedure, method A, was used. Bulb-to-bulb distillation at 0.5 mmHg gave **6** as a white, waxy solid that was not analytically pure. This was applied to a silica gel column and eluted rapidly with THF, using positive air pressure to force a rapid flow. Extended exposure of the phosphoraziridines to silica gel results in decomposition. The eluate was concentrated to a solid and sublimed under vacuum at 39 °C to give white, crystalline, hygroscopic **6** in 74.5% yield; mp 52–60 °C. Anal. ($C_{10}H_{22}N_3OP \cdot {}^1/_4H_2O$) C, H, N.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic *N*-Propylamide (7).** The general procedure, method B, was used. Distillation of the crude product at 99 °C (0.25 mmHg) gave **7** in 68.7% yield as an oil that solidified on standing. Sublimation at 39 °C under vacuum gave a crystalline sample of **7**, mp 43–46 °C. Anal. ($C_{11}H_{24}N_3OP$) C, H, N.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic *N*-Butylamide (8).** The general procedure, method B, was followed. The crude product was vacuum distilled bulb-to-bulb to give a 73.3% yield of **8** as a waxy, slightly yellow semisolid, not analytically pure. A sample of this material was purified by three sequential fractional sublimations (as described in the preparation of **4**) with a 54.3% recovery of **8** as white crystals, mp 55–56.5 °C. Anal. ($C_{12}H_{26}N_3OP$) C, H, N.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic *N*-(3-(Dimethylamino)-1-propyl)amide (9).** Method B of the general procedure was employed. Distillation of the crude product at 119 °C (0.45 mmHg) gave **9** in 40.4% yield, as a slightly yellowed oil that solidified after refrigeration. Anal. ($C_{13}H_{29}N_4OP$) C, H, N.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic *N*-(3-Methoxy-1-propyl)amide (10).** Method B of the general procedure was used. Vacuum distillation at 129–140 °C (0.42–0.45 mmHg) gave somewhat yellowed **10** as an oil in a 64.3% yield. Bulb-to-bulb redistillation in vacuo gave nearly colorless **10** in 58.5% yield, pure by TLC and NMR evidence. Anal. ($C_{12}H_{26}N_3O_2P \cdot {}^1/_4H_2O$) C, H, N.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic Piperidide (11).** The general procedure, method B, was used. Vacuum distillation caused some decomposition, giving impure **11** in 51.6% yield. Column chromatography on silica gel using 50:50 acetone/CH_2Cl_2 with 1% added triethylamine as eluent, with application of positive air pressure to force a rapid flow rate and subsequent bulb-to-bulb distillation under vacuum, gave **11** as a slightly yellow oil, pure by TLC analysis, in 37.2% yield.

***P,P*-(2,2-Dimethyl-1-aziridinyl)phosphinic Morpholide (12).** Method B of the general procedure was followed. Bulb-to-bulb vacuum distillation gave a 72.8% yield of **12** as a colorless oil. TLC showed some base line impurities in this material. The material was filtered through a 5-cm bed of silica gel with THF as eluent, under pressure. Redistillation of the eluate, bulb-to-bulb under vacuum, gave a 94% recovery of **12** as an oil pure by TLC. Anal. ($C_{12}H_{24}N_3O_2P \cdot {}^1/_2H_2O$) C, H, N.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic *N'*-Methylpiperazide (13).** The general procedure, method B, was employed. The crude product was rapidly chromatographed under pressure on a 4-cm bed of silica gel with THF eluent to remove nonmobile impurities detected by TLC (10% methanol/CH_2Cl_2).

The reconcentrated eluate was then vacuum distilled bulb-to-bulb to give **13** as a colorless oil in 67.4% yield. Anal. ($C_{13}H_{27}N_4OP \cdot {}^1/_2H_2O$) C, H; N: calcd, 18.97; found, 18.56.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic *N*-Cyclohexylamide (14).** A solution of cyclohexylamine (2.10 g, 0.021 mol) in 5 mL of dry diethyl ether was added over 20 min by dropping funnel to $POCl_3$ (1.53 g, 0.01 mol) in 20 mL of dry ether at 2 °C under N_2. The resulting slurry was stirred for 1 h at 0 °C and for 3 h at ambient temperature and then was filtered to remove the cyclohexylammonium chloride byproduct. The dichloridate solution was transferred to a dropping funnel and added over 30 min to a solution of 2,2-dimethylaziridine (1.49 g, 0.021 mol) and triethylamine (2.13 g, 0.021 mol) in 25 mL of ether at 0–2 °C under N_2. Stirring was continued at 4 °C overnight, and then the suspension was filtered and the filtrate concentrated at reduced pressure. Recrystallization (twice) from ethyl ether yielded 0.793 g (27.8% of **14** as rhombic, colorless crystals), mp 110–112 °C. Anal. ($C_{14}H_{28}N_3OP$) C, H, N.

***P,P*-Bis(2,2-dimethyl-1-aziridinyl)phosphinic Anilide (15).** A solution of $POCl_3$ (3.83 g, 0.025 mol) and triethylamine (11 mL, 0.079 mol) in 150 mL of THF was cooled to −40 °C and treated over a 1-h period with a solution of aniline (2.33 g, 0.025 mol) in 20 mL of THF. The slurry was allowed to warm gradually to ambient temperature over 1 h. The dropping funnel was recharged with 2,2-dimethylaziridine (3.56 g, 0.05 mol) in 20 mL of THF, and this solution was added to the reaction mixture at a moderate rate. Stirring at ambient temperature overnight was followed by filtration and concentration of the filtrate at reduced pressure. The resulting residue was agitated with dry diethyl ether and filtered to isolate **15** as a powdery solid in a 27.1% yield; mp 158–162 °C. Anal. ($C_{14}H_{22}N_3OP$) C, H, N.

Hydrolysis Rate Studies. A small (0.025–0.05 g) sample of a *P,P*-bis(2,2-dimethyl-1-aziridinyl)-*N*-alkylphosphinic amide, or of **2** used as a reference for comparison, was placed in a 5-mm NMR tube and dissolved in a sufficient volume of D_2O to achieve a 0.2 or 1.0 M concentration. NMR spectra were obtained at appropriate time intervals, with the samples being stored at 37 °C between spectra. The relative concentrations of intact aziridine moieties and hydrolysis intermediate moieties were calculated on the basis of integrations of the proton signals of the ring-opening dimethylaziridine moieties.

Cholinesterase Inhibition Studies. In Vitro Inhibition of Horse Serum Cholinesterase. The in vitro inactivation of horse serum cholinesterase by the *P,P*-bis(2,2-dimethyl-1-aziridinyl)-*N*-alkylphosphinic amides was measured by the method of Lalka and Bardos.[13] A 20-mL aliquot of a 5 unit/mL solution of the enzyme in 0.066 M NaH_2PO_4 buffer of pH 7.4 was combined with 1.0 mL of a freshly prepared 2.94×10^{-3} M solution of the phosphoraziridine in the same buffer. The solution was incubated at 37 °C with mild agitation, and at appropriately chosen time intervals 1.5-mL aliquots were withdrawn and cooled to 24 °C in a water bath. Each aliquot, and an accompanying matched control containing no phosphoraziridine, was combined with 1.5 mL of 5.6×10^{-5} M procaine hydrochloride in the same buffer, and the initial rate of decrease of absorbance at 300 nm was measured as described.[13]

In Vitro Inhibition of Bovine Erythrocyte Cholinesterase and Electric Eel Cholinesterase. Reagents. Purified cholinesterase Type XII from bovine erythrocytes and electric eel type V-S (Sigma) were reconstituted and diluted in 0.9% NaCl and stored at −20 °C. A 150 mg/mL stock solution of acetylcholine chloride (Sigma) was prepared in the same manner and stored at 4 °C. This preparation is stable for approximately 2 weeks. Stock buffer was prepared as follows: 0.02 M sodium barbital (4.124 g/L), 0.004 M KH_2PO_4 (0.545 g/L), 0.6 M KCl (4.73 g/L). As a stock buffer indicator solution, Bromthymol blue (dibromothymolsulfonephthalein, 100 mg) was dissolved in 2 mL of 2 N NaOH. One liter of stock buffer was added and adjusted to pH 8.0.

Assay. Buffer indicator solution (1.0 mL) and 1.2 mL of distilled H_2O were added to individual tubes. Drugs were diluted in 0.9% NaCl, and 0.1 mL was added. Positive controls received 0.1 mL of 0.9% NaCl. Following drug addition, 0.1 mL of cholinesterase (1–4 units/mL) was placed in the reaction mixture. Negative controls received cholinesterase which was heat inactivated at 60 °C for 10 min. After 30-min incubation at 37 °C, 0.1 mL of acetylcholine chloride was added to arrive at a final concentration of 0.033 M. After 45-min incubation at 37 °C, A_{620} was measured in a Beckman DU spectrophotometer. The net change in values was used to calculate the percent of inhibition.

In Vivo Inhibition of Cholinesterase in Mice. Intraperitoneal injections of drugs diluted in 7.5% sodium bicarbonate were administered to Balb/cCr mice. Control animals received 7.5% sodium bicarbonate. Whole blood (0.01 mL) was collected into heparinized capillary pipettes following retroorbital puncture at various time intervals post-injection. Whole blood was placed into tubes containing 5 mL of 0.002 M phosphate buffer, pH 7.8, containing bromthymol blue. Acetylcholine chloride was then added to arrive at a final concentration of 0.0165 M. To calculate the net change in absorbance, a set of control tubes received 0.1 mL of 0.9% NaCl in place of substrate. Samples were incubated for 45 min at 37 °C, and A_{620} was measured in a Beckman DU spectrophotometer.

In Vivo Testing against Murine Tumors. Mice. Balb/C, DBA/2, C57BL/6, (C57BL/6 × DBA/2)F_1 (BDF_1) and Balb/C × DBA/2)F_1 (CDF_1) mice of both sexes, 16–20 g, were used for the maintenance of tumors via in vivo transplantation and for chemotherapy experiments.

Murine Tumors. P388 and L1210 leukemias were maintained in ascitic form in DBA/2 mice. B16 melanoma (B16) was maintained as a sc growing tumor in C57BL/6 mice. Madison 109 lung carcinoma (M109) and colon 26 carcinoma (C26) were maintained sc in BALB/c mice.

Drug Preparations and Treatments. The phosphoraziridines were usually dissolved in water, or absolute ethanol followed by aqueous dilution, and administered to mice within 15 min of dissolution. In a few instances, compounds were dissolved in Me_2SO or ethanol and upon dilution in water had to be suspended with the aid of Tween 80. Cyclophosphamide was dissolved in saline, and chlorambucil was suspended in water and carboxymethylcellulose. Injection volumes were 0.5 mL for ip administration and 0.2–0.25 mL for iv administration. Treatment schedules varied according to the tumor test and are described in the tables.

Antitumor Testing. Experiments involving L1210 and P388 leukemias were initiated by implanting 10^6 cells ip into CDF_1 mice. Each drug-treated group for each dose level consisted of six mice, and leukemia control groups were composed of 10 mice.

In the P388 experiments, dose levels of 256, 128, 64, and 32 mg/kg, and for compounds **3–5**, 16 and 8 mg/kg, were evaluated. In the L1210 experiments, **3** was tested at 30, 60, 120, 180, 240, and 360 mg/kg dosages, and **4** and **5** were tested at 10, 20, 40, 80, 120, and 180 mg/kg dosages.

B16 experiments were begun by either implanting BDF_1 mice with tumor fragments sc or inoculating them ip with 0.5 mL of a 10% w/v tumor brei suspension. Both drug-treated and control groups consisted of 10 mice per dose level tested. In each experiment three or four dose levels within the range 10–160 mg/kg were evaluated.

M109 and C26 experiments were initiated by implanting CDF_1 mice with 0.5 mL ip or 0.1 mL sc of 2% (M109) or 1% (C26) tumor brei suspensions. Both drug-treated and control groups consisted of eight mice per dose level. For the C26 experiments dose levels of 25, 50, 80, and 120 mg/kg were evaluated.

L1210 and P388 experiments were usually terminated 30 days post-implant, occasionally as many as 60 days post-implant. All other experiments were terminated 60 days post-implant. Mice alive at the end of an experiment were autopsied and judged to be cured if no signs of disease were visible. Mice were observed daily, and antitumor activity was determined based upon (a) the proportion of mice cured and (b) the % T/C using the median survival time (MST) in the drug-treated (T) and untreated tumor control (C) groups. Mice dying prior to the first death among parallel untreated tumor control mice were presumed to have died from drug toxicity and were excluded from calculations of MST. No result of therapy is reported in which deaths attributed to drug toxicity exceeded 17% in the treated group.

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