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COMPARATIVE CHEMICAL AND BIOLOGICAL STUDIES OF FOUR PROTOTYPE PHOSPHORAZIRIDINE ANTINEOPLASTIC AGENTS

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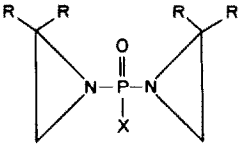
Abstract—The chemical alkylating activities of four prototype phosphoraziridine antineoplastic agents were compared with their biological effects on V-79 Chinese hamster lung fibroblasts. It was found that the chemical reactivity patterns correlate well with all of the biological parameters examined in this study, i.e. cytotoxicity, DNA synthesis, and production of alkali labile strand breaks. Specifically, the 2,2-dimethylaziridine derivatives (AB-132 and AB-163) showed higher initial activities reaching a plateau after a short reaction time in all of the systems used in this study while the unsubstituted aziridine derivatives (AB-100 and D-63) reacted more slowly but continued to exert their action in a linear fashion to produce greater overall effects. These findings are consistent with the conclusion that the difference between the time-dependent biological activities of these drugs closely follows the different chemical mechanisms of their alkylating reactions (S_N1 vs S_N2). The more rapid action and subsequent hydrolytic inactivation of the 2,2-dimethylphosphoraziridines as effective alkylators could be the basis of their lower hemopoietic toxicity compared to conventional alkylating agents including their own C-unsubstituted aziridine analogs. The much more rapid action of the 2,2-dimethylphosphoraziridines on DNA inside the cell may have some bearing on their radiation potentiating activity, but this aspect and the cholinesterase inhibitory activity of these agents (which may depend on phosphorylation) were not investigated in the present study.

The term "phosphoraziridine" has been conveniently employed when referring to the class of alkylating agents which possess aziridine rings attached to a phosphinyl group (phosphine oxide or sulfide moiety) via phosphorus-nitrogen bonds [1, 2]. Classical examples of phosphoraziridines include TEPA§ and thio-TEPA which have long been used in clinical cancer chemotherapy. Unlike the nitrogen mustards which may undergo uncatalyzed cyclization reactions to form ethyleniminium (aziridinium) ion intermediates [3, 4] that have the capability to react with nucleophiles, the phosphoraziridines, instead, require protonation of the aziridine ring nitrogens to form aziridinium ions; the latter, too, react with nucleophiles at one of the two ring carbon atoms. This mechanism has been suggested to impart a degree of selectivity to the phosphoraziridines because protonation would be favored in the more acidic microenvironment found in tumor cells relative to normal cells [5].

Early attempts in our laboratory to increase the therapeutic efficacy of the phosphoraziridines resulted in the synthesis of a series of bis(1-aziridinyl)-phosphinyl carbamates ("dual-antagonists") such as AB-100 and AB-103 (Fig. 1) which were quite active in a number of experimental animal tumor systems [6, 7] as well as in clinical trials [8–10] but did not seem to exhibit significant therapeutic advantages over conventional alkylating agents. Attempts to decrease the dose-limiting hemopoietic toxicity of these agents by adding substituents at the aziridine ring carbon atoms have led, in particular,



AB-100	R = H	X = $NHCO_2C_2H_5$
AB-103	R = H	X = $NHCO_2CH_2C_6H_5$
D-63	R = H	X = OC_2H_5
AB-132	R = CH_3	X = $NHCO_2C_2H_5$
AB-163	R = CH_3	X = OC_2H_5
AB-182	R = CH_3	X = $ONHCO_2C_2H_5$

Fig. 1. Chemical structures of some prototype ring-C-unsubstituted (AB-100, AB-103, D-63) and 2,2-dimethyl (AB-132, AB-163, AB-182) phosphoraziridines.

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§ Abbreviations: TEPA, triethylenephosphoramide; AB-132, ethyl bis(2,2-dimethylaziridinyl)phosphinyl carbamate; AB-163, ethyl bis(2,2-dimethylaziridinyl)-phosphinyl ester; AB-100, ethyl bis(1-aziridinyl)phosphinyl carbamate; D-63, ethyl bis(1-aziridinyl)phosphinyl ester; NBP, 4-(*p*-nitrobenzyl)pyridine; KOH, potassium hydroxide; TPAH, tetrapropyl ammonium hydroxide; PBS, phosphate-buffered saline; and TCA, trichloroacetic acid.

to the synthesis of a series of agents with distinctly different chemical and pharmacological properties, that is, the 2,2-dimethylphosphoraziridines [11–13] represented by AB-132, AB-163 and AB-182 (Fig. 1). These compounds show little or no hemopoietic toxicity [14–16] but were found to be potent inhibitors of cholinesterases [14, 17, 18]. The dose-limiting toxicity of these agents appeared to be due primarily to their effect on the CNS which, in turn, could be attributed, at least in part, to their anticholinesterase activities. Of greatest interest and possible therapeutic importance was the discovery that this class of compounds was capable of potentiating the antitumor effects of X-irradiation, an observation first made in the clinical trials of AB-132 [19] and then confirmed in animal experiments for this drug as well as AB-163 and AB-182 in several laboratories using P-388 [20, 21] and Friend virus-infected [22] leukemias in mice. More recent clinical pilot studies using AB-163 in conjunction with X-irradiation [16] further indicated the possible utility of 2,2-dimethylphosphoraziridines as radiation potentiating antitumor agents. It is this effect, in addition to their low hemopoietic toxicities, that has stimulated continuing interest in studies of the mechanism of action of phosphoraziridines to assist in the design of more effective congeners.

Several of our previous studies dealt with the distinctly different chemical alkylation mechanism of the 2,2-dimethylphosphoraziridines (S_N1 vs S_N2) as compared to those of their C-unsubstituted or differently substituted analogs [1, 2, 23]. However, the present paper provides the first comparative time-dependent study of the chemical and biological effects of the four prototype phosphoraziridines: the bis-(2,2-dimethyl)phosphinyl ethyl carbamate and ethyl ester, AB-132 and AB-163, and the corresponding ring-C-unsubstituted analogs, AB-100 and D-63, respectively, under carefully controlled conditions.

METHODS

Chemicals

AB-100, AB-132 and AB-163 previously synthesized by Bardos and coworkers [6, 11, 12] were recrystallized or redistilled, respectively, before use. D-63 (ethyl bis[1-aziridinyl]phosphinate) was synthesized according to our general procedure [12]. The purity of each compound was confirmed by chromatography and nuclear magnetic resonance spectroscopy. Tetrapropyl ammonium hydroxide (TPAH, 25% solution in water, v/v), trichloroacetic acid (TCA) and 4-(*p*-nitrobenzyl)pyridine (NBP) were purchased from the Eastman Kodak Co., Rochester, NY. NBP was recrystallized from ethanol before use. *N*-Phenylpiperazine was purchased from the Aldrich Chemical Co., Milwaukee, WI, and used as supplied. [^{14}C]Thymidine (54 mCi/mmol) and [^{3}H]thymidine (6.7 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Sarkosyl NL-30 was obtained from the Ciba-Geigy Corp., Greensboro, NC. Proteinase K was purchased from the Sigma Chemical Co., St. Louis, MO. All solvents used were spectral grade.

Chemical alkylating activity

Standard procedure. The method of Bardos *et al.* [24] was used in the following manner. For each measurement, 1.0 mL of 5% NBP in absolute ethanol was combined with 1.0 mL of 50 mM hydrogen potassium phthalate buffer (pH 4.2) and 1.0 mL of the phosphoraziridine (0.2 μmol) in absolute ethanol, in 75 mm screw cap test tubes (non-disposable) on ice. The reaction was initiated by placing the tightly capped, well shaken tubes in an 80 degrees Celsius (°) water bath and terminated by cooling to 4°. At various times triplicate tubes containing the same alkylating agent and one blank containing all of the components except the alkylating agent were taken out and cooled, and the color of the alkylated product was developed by adding 1.0 mL of chilled absolute ethanol and 0.6 mL of 0.1 N KOH in 80% ethanol/water. The absorbance of the alkylated product at 600 nm was calculated as the difference between the absorbance of the tubes containing the alkylating agent minus the absorbance of the blank at each corresponding time point. Under these conditions, the absorbance in the blanks did not change as a function of time.

Anhydrous procedure. This procedure has been described previously by Lalka and Bardos [25]. For each measurement, a 1.0 mL solution of 5% (w/v) NBP and 5% glacial acetic acid was combined with 1.0 mL acetophenone containing 0.2 μmol of the phosphoraziridine or, for the blank, acetophenone alone, in 75 mm screw top test tubes on ice. The reaction was initiated by placing the tightly capped, vigorously shaken tubes in an 80° water bath and terminated upon cooling to 4°. The color of the alkylated product was developed upon the addition of 1.0 mL of a 50% (v/v) solution of *N*-phenylpiperazine/acetophenone, and the absorbance was read at 575 nm. The specific absorbance of the alkylated product was calculated as the difference between the absorbance of each tube containing the alkylating agent and its corresponding blank at each time point.

Cytotoxicity

Asynchronous V-79 Chinese hamster lung fibroblasts (obtained from Dr. F. W. Hetzel, Henry Ford Hospital, Detroit, MI) were plated at 3×10^2 cells per 100 mm^2 cell culture dish (Corning Glass Works, Corning, NY) 24 hr before exposure for 1 or 24 hr to increasing concentrations of each agent in a final volume of 5.0 mL. Seven days after plating, the resultant colonies in each dish were rinsed twice with phosphate-buffered saline (PBS, pH 7.4) and immediately fixed in methanol for 1 hr before staining with a 10% solution of methylene blue in PBS. Percent cell survival was calculated as the difference between the average number of untreated (control) colonies minus treated colonies divided by the average number of control colonies. The results are expressed as percent survival at a given concentration of phosphoraziridine.

[^{3}H]*Thymidine incorporation*

Asynchronous V-79 cells were plated at 1×10^5 cells per well in twenty-four-well Corning

cell culture dishes in 1.0 mL complete growth medium 24 hr before treatment with increasing concentrations of each agent. At various times thereafter the cells were washed free of drug and pulse-labeled for 1.0 hr with 1.0 μCi/mL [^{3}H]thymidine or re-fed and pulse-labeled 24 hr later. For 24-hr experiments, cell counts were obtained in order to normalize the data. After pulsing, the cells were rinsed twice with ice-cold PBS and incubated for 15 min at 4° with 1.0 mL of 10% trichloroacetic acid (TCA). After removing the TCA solution, the cells were lysed with 0.1 N NaOH and an aliquot of the lysate was removed, neutralized and counted. The results are expressed as percent [^{3}H] incorporation per 10^6 cells.

Alkaline elution analysis

Asynchronous V-79 cells plated at 1×10^6 cells per 100 mm^2 Corning cell culture dish in a total volume of 5.0 mL were labeled for 24 hr with 0.01 μCi/mL [^{14}C]thymidine followed by 12-hr incubation in the absence of the isotope. The cells were then treated with either drugs, X-irradiation (Cs-137 source, 4.25 Gy/min), or both, after which the DNA from the cells was analyzed for single-strand breaks according to the method of Kohn *et al.* [26]. Briefly, 1×10^6 cells in 10 mL of PBS from each treatment regimen were deposited by gravity onto separate 0.80 μm pore size polycarbonate filters (Nucleopore Corp., Pleasanton, CA) and lysed at 4° with 5 mL of lysis buffer containing 20 mM EDTA, 0.1% Sarkosyl NL-30, 4 M NaCl and 1.0 mg/mL proteinase K, pH 10.1. This was followed by washing each filter with 10 mL of 20 mM EDTA, pH 10.1. The DNA from each filter was eluted overnight employing a Gilson Rabbit eight channel peristaltic pump (flow rate = 0.04 mL/min) with 20 mM EDTA and 0.1% Sarkosyl NL-30 buffered to pH 12.4 with TPAH. Results are expressed as the fraction of DNA remaining on the filter versus time (volume of eluate collected at the constant flow rate). Initial experiments included the use of tritium-labeled cells treated with 1.5 Gy as a standard for each treatment regimen; however, no difference in the alkaline elution profile between experiments run in the presence or the absence of the standard was observed.

Interstrand cross-linking

Interstrand DNA cross-links were measured in a fashion similar to that described by Ewig and Kohn [27]. V-79 cells labeled with [^{14}C]thymidine as described above were exposed to 0.5 mM AB-163 or D-63 at 37° in complete growth medium 1 hr before treatment with 15 Gy of X-irradiation at 4°. After irradiation, the cells were immediately lysed on polycarbonate filters for 30 min with ice-cold lysis buffer containing 1 mg/mL proteinase K (to remove DNA-protein cross-links) and then subjected to alkaline elution analysis together with controls treated with X-irradiation or drugs alone.

RESULTS

NBP was originally employed by Epstein *et al.* [28] and others [29, 30] as an analytical reagent for the colorimetric determination of the amounts of a given alkylating agent present in blood and tissues. Subsequently, we showed that the *rates* of reaction of various nitrogen mustards with NBP as a model nucleophile could be used for the comparison of the chemical alkylating activities [24]. In the present study we employed this method for the determination of the comparative alkylating activities of the prototype 2,2-dimethyl and ring-C-unsubstituted phosphoraziridines. As seen in Fig. 2, both 2,2-dimethylaziridine derivatives (AB-132 and AB-163) showed high initial rates of alkylation which, however, began to decline after 10 min and approached zero rate of reaction (plateau) during the first hour of incubation, both the initial rate and overall extent of alkylation being somewhat higher for the ester AB-163 than for the carbamate AB-132. In contrast, the ring-C-unsubstituted analogs (AB-100 and D-63) showed substantially slower initial rates of alkylation which remained unaltered (linear) through the time course studied, resulting after 2 hr in a greater overall extent of alkylation as compared to that obtained with the 2,2-dimethylaziridine analogs. In addition, the carbamate AB-100 showed markedly higher alkylating activity than the ester, D-63.

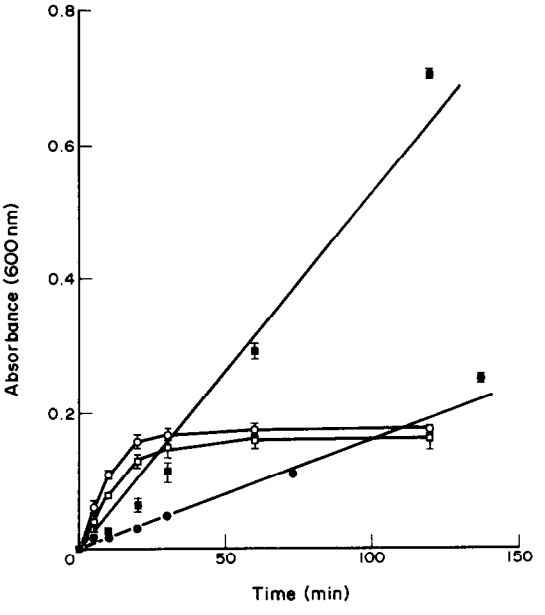

Fig. 2. Comparative chemical alkylating activities of AB-100 (■), D-63 (●), AB-132 (□) and AB-163 (○) under standard (aqueous) assay conditions. Equivalent concentrations (0.2 μmol) of each agent were reacted with NBP (final concentration = 1.67%) at 80°. At the times indicated, the amount of alkylated product was determined colorimetrically at 600 nm after the addition of 0.6 mL of 0.1 N KOH. Each point is the mean ± SEM of triplicate measurements of two separate experiments.

To determine the significance of the observed differences in NBP reactivities between the four prototype phosphoraziridines with respect to their mechanism of action, it was necessary to investigate the effects of small variations in the reaction

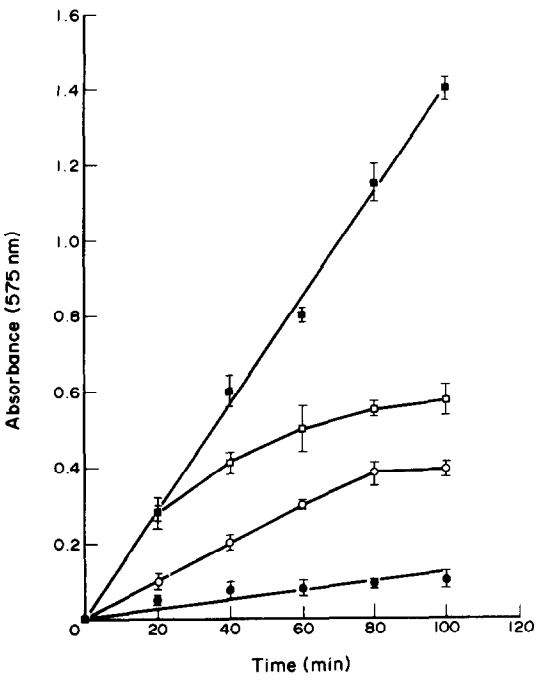

Fig. 3. Comparative chemical alkylating activities of AB-100 (■), D-63 (●), AB-132 (□) and AB-163 (○) under anhydrous assay conditions. Equivalent concentrations (0.2 μmol) of each agent were reacted with NBP (final concentration = 2.5%) at 80°. At the times indicated, the amount of alkylated product was determined colorimetrically at 545 nm after the addition of 0.5 mL of *N*-phenylpiperazine. Each point is the mean ± SEM of triplicate measurements of two separate experiments.

conditions on the relative activities of these compounds. Therefore, we conducted a series of experiments in which we determined the alkylating activities of all four compounds at different pH values (between 3.5 and 5.5) and concentrations (between 25 and 250 mM) of the buffer hydrogen–potassium phthalate. As expected, the alkylating activities of all four compounds increased with the lowering of the pH, but their *relative* alkylating activities remained the same. As to the buffer concentration, higher than 50 mM inhibited the colored product formation. The optimal pH value of the buffer was found to be 4.0 to 4.2 and the optimal buffer concentration, 50 mM, resulting in a final pH of 4.8 after the addition of 5% NBP. These were, therefore, the standard conditions of the reaction medium employed in the experiments shown in Fig. 2 which gave highly reproducible results.

To gain a better understanding of the mechanism of the reaction of these agents with NBP, we compared their reaction rates also under the anhydrous conditions used previously in our pharmacokinetic studies for the quantitation of the 2,2-dimethylaziridines extracted from serum [25]. As shown in Fig. 3, alkylation by the ring-C-unsubstituted phosphoraziridines remained linear with time, while the 2,2-dimethylaziridines again showed a plateau effect but without exhibiting the higher initial rates of reaction relative to the former. In contrast to the results obtained in our standard (aqueous) NBP alkylation experiments (see above), AB-132 was in this system significantly more reactive than AB-163, and the difference between the two unsubstituted phosphoraziridines, AB-100 and D-63, was even more pronounced than in the aqueous system.

The comparative cytotoxicities of these agents were determined by measuring their effects on the clonicity of V-79 Chinese hamster lung fibroblasts following 1- and 24-hr exposure, respectively, to increasing concentrations of each drug (Table 1). The data presented for both 50 and 90% inhibition clearly show that the 2,2-dimethylphosphoraziridines exerted their maximal cytotoxicities after 1 hr of exposure, and continued exposure to the drug had no further effect. The ethyl ester AB-163 was more cytotoxic than the carbamate AB-132. In contrast, the ring-C-unsubstituted phosphoraziridines required more time to manifest their cytotoxicities which, after 24 hr of incubation, were much higher than those of the corresponding 2,2-dimethyl-substituted analogs. Moreover, the carbamate AB-100 was markedly more cytotoxic than the ester D-63.

The effect of each agent on the incorporation of [^{3}H]thymidine into the DNA of V-79 cells also revealed a striking difference between the ring-C-unsubstituted and 2,2-dimethyl-substituted phosphoraziridines (Table 2). When the cells were pulse-labeled after 1-hr exposure to the drugs, neither of the unsubstituted compounds had any effect on [^{3}H]thymidine incorporation while both 2,2-dimethyl-substituted analogs inhibited this response in a concentration-dependent fashion. When the pulse-labeling was conducted 24 hr after exposure to the alkylating agents, the inhibition of [^{3}H]thymidine incorporation was comparable for all four agents and at the higher concentration (1.0 mM) approached 100%. Thus, the longer time period required for the inhibition of [^{3}H]thymidine incorporation by the unsubstituted phosphoraziridines was apparently not due to slower uptake of the drug but rather to their slower intracellular action.

Since the patterns of cytotoxic activities of these prototype phosphoraziridines appear to be in good agreement with those of their chemical alkylating activities, this would indicate that the cytotoxicity of these drugs is closely related to the alkylation of DNA. Therefore, we decided to investigate this relationship more directly by determining the ability of these compounds to produce alkali labile lesions, or strand breaks in cellular DNA. The technique of alkaline elution analysis [26] was employed to determine the strand breaks caused in the DNA of V-79 cells exposed to these drugs for 1 and 24 hr, respectively, followed by alkali treatment. The results given in Fig. 4 for AB-163 and D-63 indicate that after 1 hr of incubation with the cells, the 2,2-dimethyl-substituted phosphoraziridine, AB-163, produced more DNA strand breaks than the corresponding ring-C-unsubstituted derivative D-63. The reverse was true after 24-hr treatments with these drugs. In this case, D-63 produced more alkali labile strand breaks than did AB-163 which again

Table 1. Concentrations of prototype ring-C-unsubstituted and 2,2-dimethyl-phosphoraziridines required for 50 and 90% inhibition of the clonal growth of V-79 Chinese hamster lung fibroblasts

	Inhibitory concentration (mM)			
	1-hr Exposure		24-hr Exposure	
Compound	IC_{50}	IC_{90}	IC_{50}	IC_{90}
AB-100	0.30 ± 0.200	0.75 ± 0.210	0.01 ± 0.003	0.03 ± 0.021
D-63	0.75 ± 0.070	>1.0	0.02 ± 0.007	0.05 ± 0.019
AB-132	0.40 ± 0.140	0.90 ± 0.140	0.43 ± 0.110	0.98 ± 0.002
AB-163	0.11 ± 0.010	0.29 ± 0.010	0.12 ± 0.021	0.25 ± 0.070

Cells were exposed to increasing concentrations (0.01 to 1.0 mM) of each agent for 1 or 24 hr. Seven days after treatment the resultant colonies were fixed in methanol and stained with methylene blue. The IC_{50} and IC_{90} values (means ± SEM) were obtained from two separate survival curves generated over the concentration range indicated, each point measured in triplicate.

Table 2. Inhibition of [^{3}H]thymidine incorporation into the DNA of V-79 Chinese hamster lung fibroblasts by prototype ring-C-unsubstituted and 2,2-dimethyl phosphoraziridines as a function of each compound's concentration and contact time

Compound	Concentration (mM)	Time (hr)	% Inhibition
AB-100	0.1	1	0
	1.0	1	0
	0.1	24*	24.0 ± 1.7
	1.0	24	84.2 ± 7.1
D-63	0.1	1	3.0 ± 4.2
	1.0	1	0
	0.1	24	18.1 ± 12.6
	1.0	24	75.4 ± 8.3
AB-132	0.1	1	20.2 ± 7.3
	1.0	1	76.6 ± 6.2
	0.1	24	18.4 ± 4.4
	1.0	24	92.2 ± 1.3
AB-163	0.1	1	28.3 ± 2.5
	1.0	1	67.8 ± 3.2
	0.1	24	22.7 ± 15.2
	1.0	24	82.4 ± 15.1

Cells were exposed to increasing concentrations (0.01 to 1.0 mM) of each agent for 1 or 24 hr before pulsing with 1.0 μCi [^{3}H]thymidine for 1 hr. The amount of radioactivity incorporated into acid-precipitable material was determined by liquid scintillation counting. Percent inhibition values (mean ± SEM) at a given concentration relative to untreated controls were obtained from two concentration/response curves generated over the concentration range indicated, each point measured in triplicate. Control cells incorporated 0.17 pmol[^{3}H]thymidine/10^6 cells/hr.

* Corrected for viable cells.

did not appear to cause significant additional damage after the first hour of exposure.

However, the data in Fig. 4 may significantly underestimate the actual number of strand breaks because the formation of alkali stable interstrand cross-links by these drugs would be expected to counteract the effects of strand breaks on these measurements. To estimate the effect of interstrand cross-links on the alkaline elution rate of each agent, the cells were incubated with 0.5 mM AB-163 and D-63, respectively, for 1 hr and subsequently irradiated with a standard dose (15 Gy) of X-ray at 4°, according to the method of Ewig and Kohn [27].

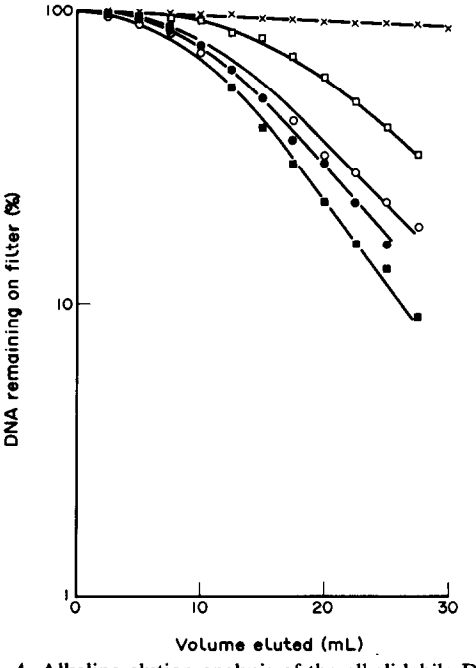

Fig. 4. Alkaline elution analysis of the alkali-labile DNA lesions (strand breaks) produced in V-79 Chinese hamster lung fibroblasts following exposure to 1.0 mM AB-163 for 1 (○) and 24 (●) hr, or to 1.0 mM D-63 for 1 (□) or 24 (■) hr respectively; untreated control (×). Cells labeled with [^{14}C]thymidine were lysed at pH 10.1 in the presence of proteinase K on polycarbonate filters. DNA from the lysed cells was eluted from the filter with 0.1% Sarkosyl NL-30 and 20 mM EDTA buffered to pH 12.4 with TPAH at a flow rate of 0.04 mL/min, and 2.5-mL fractions were collected. The radioactivity in each fraction was determined by liquid scintillation counting. Three separate experiments produced nearly identical results.

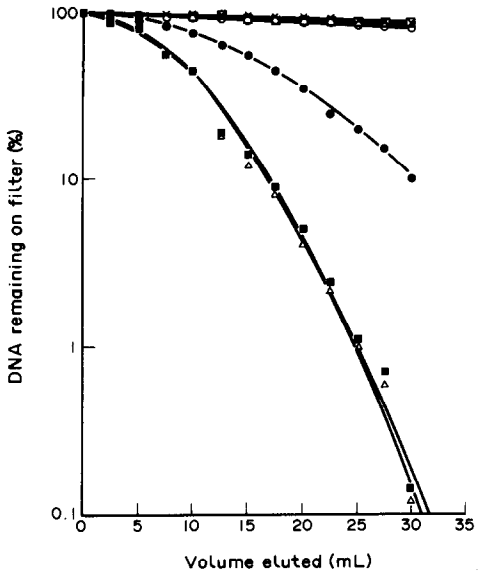

Fig. 5. Determination of DNA interstrand cross-links produced in V-79 Chinese hamster lung fibroblasts by 1-hr exposure to 0.5 mM AB-163 or D-63 respectively. Cells labeled with [^{14}C]thymidine were lysed at pH 10.1 in the presence of proteinase K on polycarbonate filters immediately following treatment with 15 Gy X-ray alone (△), D-63 alone (□), AB-163 alone (○), D-63 followed by 15 Gy X-ray (■), and AB-163 followed by 15 Gy X-ray (●); the untreated control is shown by (×). The DNA from the lysed cells was eluted from the filter with 0.1% Sarkosyl NL-30 and 20 mM EDTA buffered to pH 12.1 with TPAH at a flow rate of 0.04 mL/min, and 2.5-mL fractions were collected. The radioactivity in each fraction was determined by liquid scintillation counting. Cross-links are expressed by the difference of the percent DNA retained on the filter when the irradiation was preceded by treatment with the agent as compared to treatment with irradiation alone. Three separate experiments produced nearly identical results.

Using this method, the inhibition of the increase in the elution rate produced by the standard X-ray dose is a measure of the cross-linking effect of each drug on the DNA strands. As seen in Fig. 5, AB-163 caused substantial cross-linking during 1 hr of incubation* while the cross-linking effect of D-63 did not manifest itself during this time period. Considering the overlapping opposite effects of DNA strand breaks [26] and interstrand cross-links [27] on the alkaline elution rate profile [32], it is clear that the differences between the direct effects of these two compounds on DNA are actually even larger than those apparent in Figs. 4 and 5. At any rate, both the strand breaking and interstrand cross-linking effects of the 2,2-dimethyl-substituted and unsubstituted phosphoraziridines on the DNA inside the cells parallel their time-dependent relative cytotoxicities and thymidine incorporation rates, as well as the characteristic patterns of their reaction rates with NBP in the standard (aqueous) assay for chemical alkylating activity.

* The very rapid formation of alkali stable interstrand cross-links in the DNA of CCRF/CEM human leukemia cells upon exposure of the cells to AB-163 was observed, using the Rydberg procedure [31], by Dr. P. Kanter of Roswell Park Memorial Institute (personal communication, cited with permission).

DISCUSSION

The phosphoraziridines as a class of compounds are, as evident from their structure, alkylating agents requiring protonation to exert their action. What makes the 2,2-dimethyl-substituted phosphoraziridines different from other alkylating agents, including their own ring-C-unsubstituted analogs, are (1) their unusually low hemopoietic toxicities, (2) their cholinesterase inhibitory activities, and (3) their *in vivo* observed radiation potentiating activities. In the present study, we have attempted to find some explanation for the different behavior of these agents by comparing the rate patterns of some of their chemical and biological activities to those of their ring-C-unsubstituted analogs.

Overall, the results reported here demonstrate for the first time that the 2,2-dimethylaziridines are more rapidly and initially more effectively acting alkylating agents than their unsubstituted analogs, both in their chemical reactions with a model nucleophile and in their biological activities (cytotoxicity, inhibition of DNA biosynthesis) involving reactions with DNA inside the cells. However, they reach the limit of their effects after a short time and become essentially inactive thereafter in all of the chemical and biological systems studied. This clear difference in their activity patterns as compared to the ring-C-unsubstituted derivatives, which act more slowly but continue to act over a longer time period, could possibly be sufficient to explain the remarkably low bone marrow toxicities of the 2,2-dimethylaziridines relative to their significant antitumor activities, without having to invoke a different type of mechanism than alkylation.

Aside from the structural alterations of single bases (which may cause mutation), the typical macromolecular changes induced by alkylation of DNA with bifunctional alkylating agents (causing cytotoxicity) include: (a) single-strand breaks, (b) cross-linking of DNA strands, and (c) formation of cross-links between DNA and protein. In the present studies, the DNA–protein cross-links were eliminated by the routine use of proteinase K in the alkaline elution assays [32], and the two other macromolecular parameters were determined separately (but not independently) from each other. Due to the nature of their overlapping opposite effects on the experimental measurements, the results obtained for both the strand breaks and cross-links should be considered as minimal values; however, even so they demonstrate the dramatically faster action of the 2,2-dimethylphosphoraziridine, AB-163, as compared to its ring-C-unsubstituted analog, D-63, directly on the DNA inside the cells.

In addition, this study provides further insight into the chemical mechanism of alkylation of the prototype 2,2-dimethylaziridines and their ring-C-unsubstituted analogs. Using the model nucleophile NBP, the significantly higher *initial* rate of alkylation shown in the aqueous assay by the 2,2-

dimethylphosphoraziridines (which was not noted previously) is presumably due to the ability of these agents to react by either S_N1 or S_N2 mechanisms [1]. However, these agents rapidly lose their reactivities as a consequence of competing hydrolysis or other side reactions involving the formation of non-alkylating intermediates [2, 17]. The ring-C-unsubstituted phosphoraziridines show an essentially linear rate of alkylation consistent with their homogeneous S_N2 mechanism and the absence of competing side reactions. In this assay, the phosphinyl carbamate AB-100 is a much more effective alkylator than the corresponding phosphinic ester derivative (D-63). The difference between the reactivities of the carbamate and the ester becomes even more striking when the reaction is conducted under anhydrous conditions in which case AB-132 also becomes markedly more active than AB-163. This indicates that the role of the tautomerizable proton of the carbamate group of AB-100 (or AB-132) makes a significant contribution to the rate of reaction of the aziridine ring with the nucleophile, presumably due to internal protonation of the aziridine ring nitrogens. The results obtained under the standard aqueous conditions are, of course, more relevant to the biological activities, as shown by their surprisingly good correspondence with the data obtained in the cell culture systems.

With respect to the cholinesterase inhibitory activity, our previous studies have provided evidence that this effect is not due to alkylation but rather to the inhibitory effect of a non-alkylating hydrolytic intermediate that may act via phosphorylation [2, 17]. We have subsequently shown that this effect is not general to all 2,2-dimethylphosphoraziridines but is strongly dependent on the nature of the third substituent on the phosphorus atom [18]. The present study does not provide any additional information concerning this interesting albeit undesirable side-effect.

Regarding the radiation potentiating activity of the 2,2-dimethylphosphoraziridines, the question is still open whether this effect is in any way related to the different mechanism and rate pattern of alkylation of these compounds as compared to conventional alkylating agents or whether it is not due to alkylation at all but to the phosphorylating action of the hydrolytic intermediates, as we proposed previously [2]. Moreover, the *in vivo* observed radiation potentiating effect could be due to pharmacologically synergistic actions of the drug and X-ray at different sites in the organism [14]. This question is under continuing investigation.

In view of the unique and therapeutically useful properties of the 2,2-dimethylphosphoraziridines, a large number of such compounds containing a variety of non-alkylating moieties attached to the bis(2,2-dimethylaziridinyl)phosphinyl group have been synthesized recently, including amides [18], quinones [33], nucleosides [34, 35], nitroimidazoles [36], acridines and steroids, most of which are still under evaluation. The results of the present study of the prototype phosphoraziridines have been found useful in the comparative characterization of these new series of derivatives and, more generally, in our efforts to design novel types of alkylating agents [37] having similarly low hemopoietic toxicities but enhanced antineoplastic and/or radiation potentiating activities.

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