Synthesis of Potential Dual-Acting Radiation Sensitizer Antineoplastic Agents: 2,2-Dimethylphosphoraziridines Containing 2-Nitroimidazoles or Other Electron-Affinic Moieties

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In view of the in vivo demonstrated radiation-potentiating activities of several previously studied 2,2-dimethylphosphoraziridines, six new compounds incorporating the bis(2,2-dimethyl-1-aziridinyl)phosphinyl moiety, together with an electron-affinic group such as 2-nitroimidazole or nitrobenzyl, have been synthesized and tested (1) in vitro for ability to increase the effect of X-irradiation under hypoxic conditions on V-79 Chinese hamster lung fibroblast cells, (2) in vivo for antitumor activity in the absence of radiation against P388 leukemia in mice, and (3) in a preliminary experiment with compound **10** only, in combination with whole-body γ-radiation, using the P388 leukemia mouse model for in vivo radiation-potentiating activity. The chemical-alkylating activities and hydrolytic behavior of these compounds, as well as their antitumor activities without radiation, were found to be comparable to those of other 2,2-dimethylphosphoraziridines, while their in vitro radiosensitizing activities were at low concentrations generally comparable to that of misonidazole, with compound **8** showing superior activity. At higher concentrations, only compound **10** was sufficiently soluble and nontoxic to the cells for evaluation in this assay. Thus, the bis(2,2-dimethyl-1-aziridinyl)phosphinyl moiety does not seem to have contributed to the hypoxic radiosensitizing activities (only to the cytotoxicities) of the electron-affinic moieties in this in vitro assay. In comparison, the prototype 2,2-dimethylphosphoraziridine, ethyl [bis(2,2-dimethyl-1-aziridinyl)phosphinyl]carbamate (AB-132), showed at nontoxic doses no radiosensitizing activity in this assay, and at cytotoxic doses increased the cell-killing effect of each given dose of X-radiation additively under both hypoxic and oxic conditions. Conversely, only the 2,2-dimethylphosphoraziridine moiety appeared to participate in the moderate "therapeutic radiation-potentiating" activity indicated by compound **10** in the in vivo experiment using the P388 leukemia model (on day 1), as the misonidazole standard was inactive in this nonhypoxic system. Clearly, the mechanism of the in vivo observed radiation-potentiating effect of AB-132 and other 2,2-dimethylphosphoraziridines is different from that of the hypoxic radiosensitizers, but the possible synergism between the two biologically active moieties of the new compounds could not be demonstrated with the experimental models so far employed.

Previous studies demonstrated that phosphoraziridines containing 2,2-dimethyl-substituted aziridine moieties show some dramatic differences in their chemical as well as pharmacologic properties as compared to the corresponding unsubstituted or differently substituted aziridine derivatives.[1-3] While the latter compounds reacted more slowly, by S_N2 mechanisms,[4] and their pharmacologic properties, including bone marrow toxicity, resembled those of typical alkylating agents,[5-7] the 2,2-dimethylphosphoraziridines were found to undergo very fast hydrolysis and alkylation reactions by S_N1 mechanisms (via a *tertiary* carbonium ion intermediate[1,8,9]) and in the pharmacologic studies produced very little if any hematologic side effects.[3,10] This was clearly apparent during the clinical trials of the prototype 2,2-dimethylphosphoraziridines, ethyl [bis(2,2-dimethyl-1-aziridinyl)phosphinyl]carbamate (AB-132) and ethyl bis(2,2-dimethyl-1-aziridinyl)phosphinate (AB-163), for both of which central nervous system (CNS) toxicity rather than bone marrow toxicity was the dose-limiting side effect.[3,10,11] Much of the CNS toxicity appeared to be related to the potent cholinesterase inhibitory activity of these drugs,[12] which has been proposed to result from the formation of hydrolytic intermediates with phosphorylating rather than alkylating activities.[8,12]

Most important from the therapeutic point of view was the observation, first made serendipitously during the early clinical trials of AB-132 against bronchogenic carcinoma and carcinoma of the esophagus, that this drug significantly potentiated the therapeutic effects of X-irradiation.[3,13-16] Subsequent animal experiments, using various experimental tumor systems in mice,[17-20] confirmed the in vivo radiation-potentiating effects of AB-132 and of several other 2,2-dimethylphosphoraziridines including AB-163. More recent clinical pilot studies with AB-163 in conjunction with X-irradiation also indicated the potential benefit of such combinations.[11] However, attempts to

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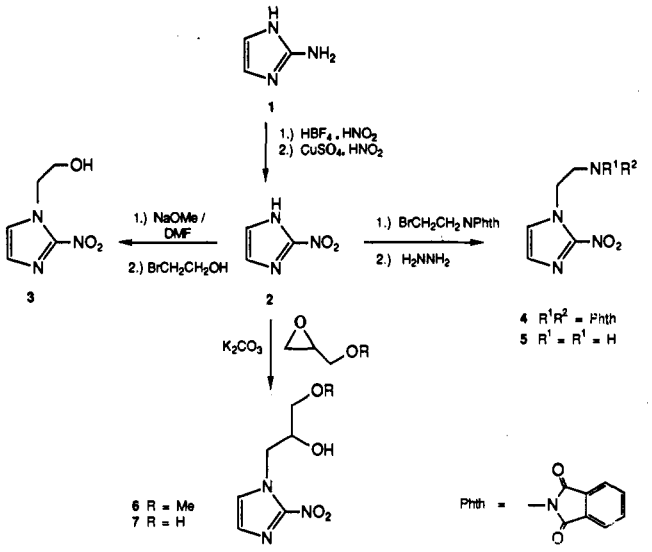
Scheme I



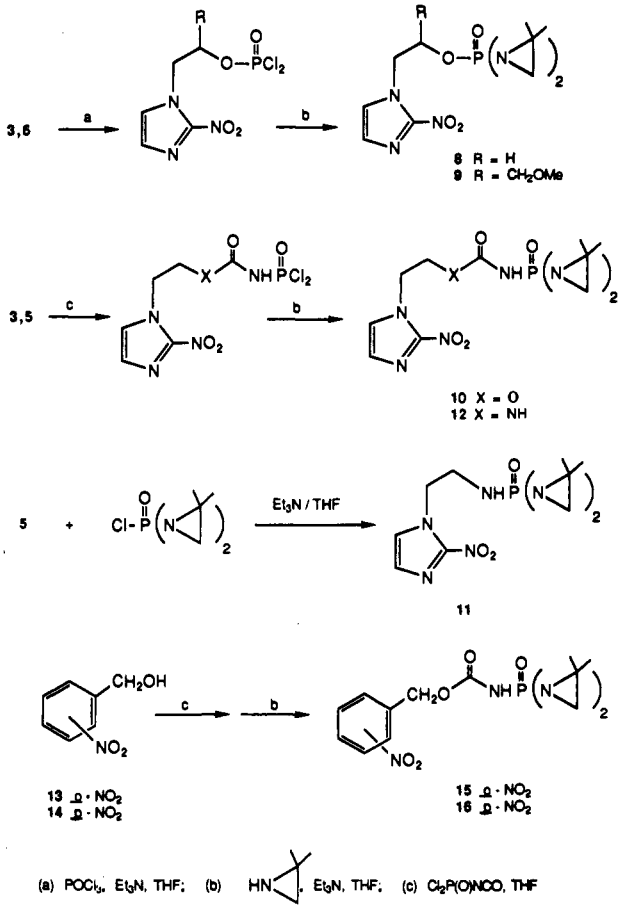
demonstrate in vitro the radiation-potentiating effects of these compounds in a V-79 (fibroblast) cell culture assay[21,22] gave results consistent with additive cytotoxicity[21] (see also below), while some indication of a radiosensitizing effect was obtained under oxic as well as hypoxic conditions in EMT-6 tumor cells.[21]

Although the mechanism of the in vivo observed radiation-potentiating activity of 2,2-dimethylphosphoraziridines has not been established as yet, and several alternative hypotheses are still under consideration,[3,8,9] the above-mentioned in vitro results seem to exclude the possibility that the mode of action of these compounds would be similar to that of misonidazole[23] or other hypoxic radiosensitizers.[24-26] Therefore, it appeared of interest to combine in a single molecule the bis(2,2-dimethyl-1-aziridinyl)phosphinyl function with an "electron affinic" nitroimidazole or nitrobenzene moiety,[24,25] in hope that the two differently acting groups will act synergistically in potentiating the antitumor effect of X-radiation.

Chemistry

The starting point for the synthesis of the nitroimidazole phosphoraziridines described here is 2-nitroimidazole (**2**) or azomycin (Scheme I), which was synthesized from 2-aminoimidazolium sulfate (**1**)[27] by the method of Agrawal.[28] N-Alkylation of **2** with bromoethanol to yield **3** was found to proceed in higher yield with the sodium salt[30] instead of the silver salt.[29] However, a much smaller scale ($^1/_{25}$)

Scheme II



was employed than reported in the literature,[30] and it was found to be necessary to avoid excess base in the reaction by using the red color of the sodium salt in the presence of NaOMe as an indicator.[31] Misonidazole (**6**) and the desmethyl analogue **7** were obtained by the reaction of **2** with methyl glycidyl ether and glycidol, respectively.[32]

The method described in the patent literature[33] for the synthesis of **5** involves refluxing of **2** in ethylenimine (aziridine) and proceeds in very low yield. An alternative route was developed in which the sodium salt of **2** is reacted under anhydrous conditions with (bromoethyl)-phthalimide to afford **4**, which can then be readily deprotected by hydrazinolysis. Although the product precipitates from the reaction as the free amine rather than as the phthalhydrazide salt, removal of residual phthalhydrazide by acid/base treatment, chromatography, or recrystallization proved to be difficult due to the chemical and thermal lability of the product. A reversible solvent adduct also seemed to be formed when crystallization from ethanol was attempted. Isolation of **5** was finally achieved by extraction of the product into a large volume of ethyl acetate.

The syntheses of phosphoraziridine derivatives of the nitroimidazoles and nitrobenzyl alcohols were based on methods reported previously by Bardos and co-workers for

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Table I. Activity against P-388 Leukemia in Mice

compd	dose/kg ip[a] mg	dose/kg ip[a] μmol	MST,[b] %T/C	AWC,[c] g, day 6
8	256	746	228	−2.8
	128	373	189	−1.3
	64	186	150	−1.1
	32	93	111	−0.9
	16	47	122	+0.1
	8	23	106	+0.2
9	256	661	200	−1.5
	128	330	150	−0.3
	64	165	133	+0.2
	32	83	122	+0.4
	16	41	100	+0.8
	8	21	106	+1.2
10	256	663	169	−1.0
	128	331	156	−1.1
	64	166	144	−0.5
	32	83	125	+0.8
	16	41	125	+0.6
	8	21	113	+1.6
12	256	664	122	−0.4
	128	332	122	−0.3
	64	166	111	+0.3
	32	83	100	+1.3
	16	42	111	−0.2
	8	21	111	+0.2
15	256	670	153	−1.5
	128	335	141	−1.1
	64	167	129	−1.1
	32	84	118	−0.4
	16	42	106	0
	8	21	106	+0.6
16	256	670	119	+0.6
	128	335	113	+2.0
	64	167	113	+1.4
	32	84	106	+2.1
	16	42	113	+1.2
	8	21	113	+1.4
AB-132	400	1453	244	−1.3
	200	727	231	−0.2
	100	363	181	−0.4
	50	182	163	−0.7
AB-163	200	861	433 (1 cure)	−5.2
	100	430	289	−2.6

[a] Compounds were administered ip, in a single injection, at the dose indicated, 1 day following the ip implant of 10^6 P388 leukemia cells. [b] Percent T/C (MST) refers to the median survival time of drug-treated mice divided by the median survival time of control mice, × 100, and expressed as a percentage. [c] AWC refers to average weight change, expressed in grams, determined between day 0 and day 6 of the experiment.

alkyl esters[34] and carbamates.[35] Thus, **3** and **6** were converted to the corresponding phosphorodichloridates, which were then treated in situ with 2,2-dimethylaziridine and Et_3N (Scheme II). After removal of traces of starting material and decomposition products by thin-layer chromatography in the presence of Et_3N, the desired phosphinates, **8** and **9**, were obtained. Reaction of **3** with phosphorisocyanatidic dichloride[36] and then 2,2-dimethylaziridine yielded carbamate **10**. Since phosphorylation of **7** with $POCl_3$ was likely to yield a cyclic phosphodiester, reaction was attempted with bis(2,2-dimethyl-1-aziridinyl)phosphinic chloride,[37] prepared by the reaction of 2 equiv of 2,2-dimethylaziridine with $POCl_3$. Neither hydroxy group was phosphorylated: however, both groups appeared to participate in a reaction with phosphorisocyanatic dichloride to yield a polymer instead of the desired carbamate. Reaction of the amine **5** with bis(2,2-dimethyl-1-aziridinyl)phosphinic chloride did yield the desired phosphinic amide **11**, based on the NMR spectrum of the product. However, subsequent chromatography to remove small amounts of starting material resulted in extensive decomposition. The corresponding phosphinylurea **12** could be prepared from **5** by using the isocyanate procedure, and it crystallized readily.

With similar methods, both nitrobenzyl alcohols **13** and **14** were transformed into nitrobenzyl carboxamidophosphoraziridines **15** and **16**. Attempts to prepare the corresponding ester of **16** were thwarted by the conversion of **14** to nitrobenzyl chloride by $POCl_3$ and by its lack of reactivity with bis(2,2-dimethyl-1-aziridinyl)phosphinyl chloride.

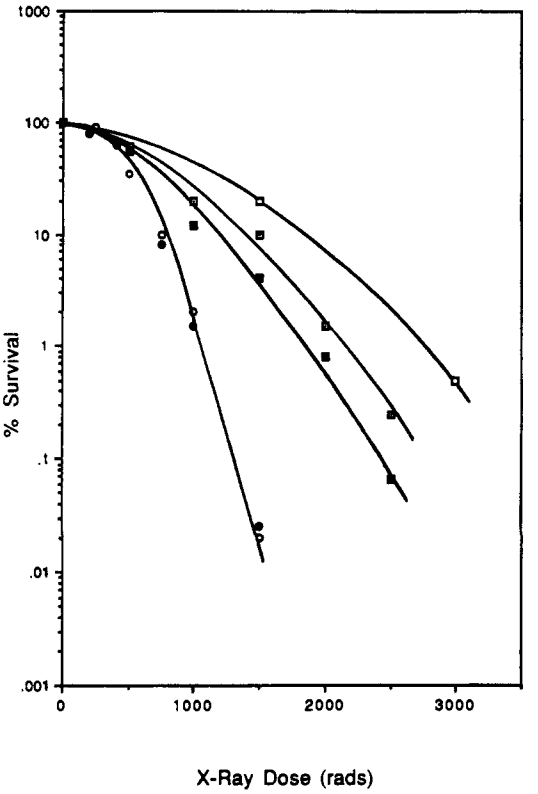

Figure 1. Survival curves for V-79 cells irradiated under oxic and hypoxic conditions following treatment with **10**. Drug/atmosphere combinations are as follows: no drug/N_2 (□), 0.5 mM **10**/N_2 (⊡), 1.0 mM **10**/N_2 (■), no drug/air (○), 1.0 mM **10**/air (●).

Antitumor Activities without Irradiation

The four nitroimidazole phosphoraziridines (**8–10** and **12**) and the *o*- and *p*-nitrobenzyl phosphoraziridines (**15** and **16**) were screened for in vivo antitumor activity (without irradiation) against P-388 leukemia in CDF_1 mice as described previously.[37] The results are given in Table I. The two nitroimidazole esters (**8** and **9**) were the most active compounds in this series, the former being about as active on a molar basis as the prototype phosphoraziridine ethyl carbamate AB-132 (although much lower than the ethyl ester AB-163).[37] Nitroimidazole carbamate **10** and *o*-nitrobenzyl carbamate **15** also showed significant albeit moderate antitumor activities, while poorly soluble *p*-nitrobenzyl carbamate **16** and nitroimidazole ureido derivative (**12**) were essentially inactive in this system.

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Table II. Sensitizer Enhancement Ratios ($SER_{1\%}$)[a]

concn, mM	8	9, 369209[d]	10, 369206[d]	12, 369210[d]	15, 369208[d]	16	miso
0.1	1.24			1.12	1.13	1.10	1.10
0.3	tox						
0.5	tox	tox	1.26	1.20	1.26	1.19	
1.0			1.40	1.36	tox	tox	1.45
1.5			1.50	(insol)			1.55
NCI ratio[b] $C_{1.6}^{drug}/C_{1.6}^{miso}$		(tox)[c]	0.9[c]	(insol)[c]	(insol)[c]		(1.0)

[a] $SER_{1\%} = R_a/R_p$, with R_a = radiation dose at 1% survival in absence of drug, and R_p = radiation dose at 1% survival in presence of drug. [b] Ratio of $SER_{1\%}$ values at 1.6 mM concentration of the drug vs. misonidazole. [c] Results obtained from the Radiotherapy Development Branch, Division of Cancer Treatment, National Cancer Institute, N.I.H. [d] NSC no.

Table III. Effect of Drug Treatment in Combination with γ-Radiation on P-388 Leukemia in Mice

treatment[a]	dose,[b] mg/kg; radiation, R	MST[c] %T/C (no. of cures[d])	AWC,[e] g, day 8
control	none	100	7.75
radiation (RT)	0; 200	115	4.67
RT	0; 400	145	5.00
RT	0; 600	170	4.58
10	250; 0	190	1.67
10	375; 0	180	2.00
10, RT	250; 200	190	1.92
10, RT	250; 400	200	1.17
10, RT	250; 600	230	0.67
AB-163	120; 0	400 (3)[d]	0.83
AB-163, RT	120; 200	250 (3)[d]	0.33
AB-163, RT	120; 400	160 (4)[d]	-0.93
AB-163, RT	120; 600	80 (2)[d]	-3.58
misonidazole	1000; 0	97	
misonidazole, RT	1000; 200	103	
misonidazole, RT	1000; 400	155	5.17
misonidazole, RT	1000; 600	150	5.50

[a] Six mice in each group. [b] Radiation was given 1 h after a single ip drug injection. [c] %T/C calculated from median life span as described (Table I); survivors are not included in %T/C calculations. [d] Number of surviving animals (out of six) on day 60. [e] Average weight change.

In Vitro Radiation-Sensitizing Activities

All six compounds were tested for their ability to potentiate the cytotoxic effect of X-irradiation under hypoxic conditions in a cell culture assay employing Chinese hamster lung fibroblast line V-79 cells.[22] The survival curve for one of the compounds (**10**), which was essentially identical with that of misonidazole (**6**), is shown in Figure 1. The radiation-sensitizing activities of the compounds were compared by use of their "sensitizer-enhancement ratio", $(SER)_{1\%}$ values, which were determined from the corresponding survival curves at various concentrations, as previously described.[22] These data are given in Table II. At the lowest (0.1 mM) concentration tested nitroimidazole ester derivative **8** appeared to have the greatest radiosensitizing activity (greater than that of misonidazole), but the hypoxic cytotoxicity of this compound (observed even at 0.3 mM) prevented its further evaluation. All the other nitroimidazole and nitrobenzyl derivatives showed at the lower concentrations similar or slightly less radiosensitizing activities than misonidazole, which was used in our assay as the positive control. However, only nitroimidazole carbamate **10** could be fully evaluated up to the 1.6 mM concentration used by the National Cancer Institute for comparison with their misonidazole standard; this compound was closely similar to misonidazole throughout the concentration range studied. All the other compounds were either cytotoxic or insoluble at or about 1.0 mM concentration. The prototype 2,2-dimethylphosphoraziridine AB-132 showed no radiosensitizing activity in this assay (SER = 1.0) and, at cytotoxic doses, increased the cell-killing effect of each given dose of X-radiation additively with the same constant increment (at a given dose of the drug) under both hypoxic and oxic conditions (Figure 2).

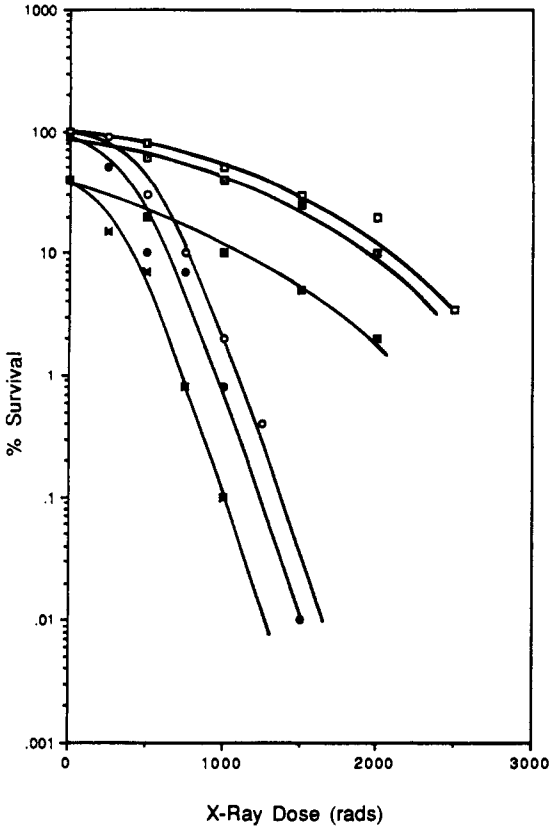


Figure 2. Survival curves for V-79 cells irradiated under oxic and hypoxic conditions following treatment with AB-132. Drug/atmosphere combinations are as follows: no drug/N_2 (□), 0.3 mM AB-132/N_2 (⊡), 0.5 mM AB-132/N_2 (■), no drug/air (○), 0.3 mM AB-132/air (●), 0.5 mM AB-132/air (*).

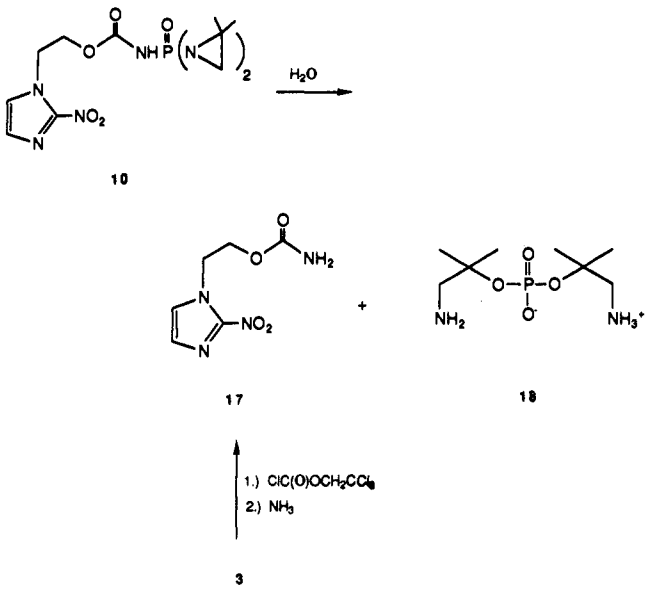
In Vivo Combination of Radiation with Chemotherapy

Only compound **10** was tested in vivo in combination with whole-body γ-radiation in a preliminary experiment conducted at the Medical College of Virginia. Essentially, Wodinsky's P-388 leukemia combination radiotherapy–chemotherapy model[38] was employed with $B6D2F_1$, instead of CDF_1, mice according to the modification of Wampler et al.[20] The results are given in Table III.

The phosphoraziridine standard used in this experiment was AB-163, which had previously shown significant radiation-potentiating activity in this system, with apparent "therapeutic synergism" at optimal dose levels of the drug and radiation.[20] However, in the present experiment, the 120 mg/kg dose of AB-163 selected for the positive control

(38) Wodinsky, I.; Swiniarski, J.; Kensler, C. J.; Venditti, J. M. *Cancer Chemother. Rep., Part 2* **1974**, *4*, 73.

Scheme III



10 17 18 3

was apparently somewhat higher than optimal for the combination effect, as it produced maximal survival time (with three out of six mice "cured") by itself, and additional treatment with radiation progressively decreased survival with increasing radiation dose, indicating toxic deaths, yet in the 400 R group there were four of six survivors. In this experiment, AB-163 did not provide an entirely satisfactory positive control, because only one dose level of AB-163 was used. Misonidazole, on the other hand, included as the electron-affinic sensitizer control, was inactive in this system, as could be expected on the basis of the literature,[39,40] under the test conditions employed, since P-388 leukemia on day 1 does not have a large hypoxic fraction.[40] γ-Radiation alone showed in this system only moderate antitumor activity, consistent with previous reports,[20,38] which reached its maximum at 400 R. In comparison, the test compound **10** at 250 mg/kg showed marked antitumor activity by itself, which did not increase further upon raising the dose to 375 mg/kg. With the 250 mg/kg dose, γ-radiation treatment 1 h after injection of the drug (on day 1), caused small increases of the median survival time up to a T/C value of 230% (at 600 R). This corresponds only to an addition of the separate effects of the drug and the radiation, but since it could not be attained with either modality by itself (see Table III), a moderate degree of "therapeutic synergism"[41] between **10** and γ-radiation, i.e., "therapeutic radiation potentiation" (as defined by Goldin et al.[42]), appears to be demonstrated.

Studies of the Mechanisms of Hydrolysis and Alkylation

In order to determine whether the presence of the 2-nitroimidazole moiety alters the mode of aqueous hydrolysis of the phosphoraziridine, the hydrolysis of **10** was followed by ^{1}H NMR at 37 °C in D_2O. The first aziridine ring was found to open within 1 h (40% in 10 min), while the second aziridine required 7 h to completely hydrolyze. In a parallel study with AB-132, the first ring opened at least twice as slowly. After incubation of **10** for 24 h, a precipitate formed that was identified as carbamate **17** by comparison with an authentic sample prepared from **3** (Scheme III). This is consistent with the formation of ethylurethane late in the hydrolysis of AB-132. In addition, the spectra of the final water-soluble product from **10** were found to be identical with those of diester **18** which is formed from AB-132 as the final hydrolysis product.[8,43] Thus, the breakdown of **10** appears to occur through the same pathway previously proposed for AB-132,[8] although the presence of the nitro group seems to accelerate the first aziridine ring opening step.

Little difference in the initial rate and general pattern of the reaction with the model nucleophile *p*-nitrobenzylpyridine (NBP)[44,45] was observed between **10** and AB-132,[9] indicating that the presence of the electron-affinic moiety did not affect the alkylating reactions of the bis-(2,2-dimethyl-1-aziridinyl)phosphinyl function.

Discussion

It may be a "truism" not always verified by experimental evidence, but it is generally assumed that two drugs acting at the same site in a similar manner may even antagonize each other or, at best, will have only an additive effect. Synergistic action is believed to depend on the drugs acting at different sites of the metabolic or pharmacologic "network" and by different mechanisms, but each targeting the same biological event. The new compounds reported in the present paper were designed to incorporate the essential structural features of two differently acting radiation-potentiating agents, i.e., those of a misonidazole-type hypoxic radiosensitizer and those of a 2,2-dimethylphosphoraziridine-type alkylating/phosphorylating agent with demonstrated in vivo radiation-potentiating activity.

The chemical and biological properties of these new compounds, described above, clearly show that the two active moieties incorporated within the same molecules do not interfere with (or modify) each other's actions: first, these "hybrid" compounds show a similar range of in vivo antitumor activities (without radiation) as do other 2,2-dimethylphosphoraziridines,[9,22,37,46] and (as shown for carbamate **10**) they also have the same characteristic patterns of hydrolysis and alkylation; second, these compounds demonstrate in vitro hypoxic radiosensitizing activities similar to those of other 2-nitroimidazole and nitrobenzyl derivatives. It may not be surprising that the phosphoraziridine moiety does not seem to have contributed to the in vitro hypoxic radiosensitizing activity (only, perhaps, to the cytotoxicity) of the electron-affinic portion of the molecule, since the prototype AB-132 itself showed no such activity in this assay. In fact, the present paper includes the first demonstration that the 2,2-dimethylphosphoraziridines do not act as hypoxic radiosensitizers (see Figure 2). However, it is interesting to note that in the case of RSU1069, a highly effective radiosensitizer introduced by Adams and co-workers,[47] the attachment of

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a monofunctional aziridine (ethylenimine) to the side chain of a 2-nitroimidazole derivative similar to misonidazole significantly increased the enhancement ratio (SER > 2.0 compared to 1.4 for the same concentration of misonidazole).[47] Since ethylenimine per se is not known to have any radiosensitizing activity, it may possibly act by covalently binding the electron-affinic moiety to the receptor (DNA) via alkylation. The bis(2,2-dimethyl-1-aziridinyl)phosphinyl moiety of our compounds, a less potent and shorter lived alkylating function compared to ethylenimine,[12] does not seem to do this.

The reason for the variation of cytotoxicities among the various compounds is unclear. It should be noted that the nitroaromatic compounds themselves may display hypoxic cytotoxicity due to their metabolic reduction to products binding to DNA and proteins.[48,49] This, however, is a delayed effect.[26] On the other hand, the cytotoxic action of 2,2-dimethylphosphoraziridines manifests itself during the first hour of exposure (due to the unusually fast DNA interstrand cross-linking effect of these agents)[9] and then levels off, resulting in lower overall (24-h) cytotoxicity and generally much lower in vivo toxicity as compared to other alkylating agents.[9] Therefore, the in vitro "cytotoxicity" data should not be taken too strictly as a measure of drug toxicity. For example, 8 was much more "cytotoxic" than 10 (Table II), but the two compounds showed little difference in their in vivo toxicities (Table I). Therefore, 8 would appear to be the choice candidate for radiation-potentiation studies in animals on the basis of its superior radiosensitizing activity at 0.1 mM, despite its cytotoxicity at the higher concentrations.

Several possible mechanisms have previously been proposed to explain the radiation-potentiating effects of the 2,2-dimethylphosphoraziridines.[3,8,9] Two of these, involving the inhibition of repair of the DNA single-strand breaks via phosphorylation of the free 3'-hydroxyl groups[8] and the extraordinarily rapid cross-linking of the DNA strands via S_N1-type alkylation,[9] are based on the unique chemical properties of these agents. These have been observed in vitro with isolated DNA or in appropriate cell culture systems. Other mechanisms, including the possibility that there is some indirect pharmacologic relationship between the effects of the drug and the X-ray,[3] may require in vivo systems for their manifestations. However, regardless of its mechanism, in view of the fact that the radiation-potentiating effect of the 2,2-dimethylphosphoraziridines has been so far demonstrated only in a few in vivo model systems (in addition to clinical cancers in man, see the Introduction), it is desirable that the new compounds should be evaluated in the same or other in vivo test systems that are sensitive to this effect.

One such system is Wodinsky's P388 leukemia combination radiotherapy–chemotherapy model[38] which had been used by both Wodinsky et al.[19] and Wampler et al.[20] to demonstrate the therapeutic radiation-potentiating activities[42] of two prototype 2,2-dimethylphosphoraziridines. Only compound 10 of the present series was tested against this model in a preliminary experiment, and the results indicated that 10 was a moderately active radiation-potentiating antitumor agent (see Table III and explanation in text, above). However, this activity of 10 appears to have been due solely to its phosphoraziridine moiety, as the 2-nitroimidazole moiety could not have made much contribution in light of the inactivity of the misonidazole control included in this experiment. It has been reported that under the test conditions employed, misonidazole is ineffective because P388 leukemia on day 1 does not have a large hypoxic fraction.[40] For this reason, the P388 leukemia model, at least in its original form, cannot be used for the demonstration of possible synergism between the two radiosensitizing moieties of these compounds. Using it in its advanced form (e.g. on day 6) would probably increase the activity of the electron-affinic moiety[40] but may substantially decrease the activity of the 2,2-dimethylphosphoraziridine component.[19]

Compound 10 was also tested by the Radiation Research Program of the National Cancer Institute, using an in vivo–in vitro assay with EMT6/SF tumors in BALB/c mice and a growth-delay assay with the squamous cell carcinoma SCCVII in C3H mice; in these tests, compound 10 was a less active radiosensitizer than misonidazole. However, these assays were designed for the comparison of various electron-affinic hypoxic radiosensitizers, and their sensitivities to the antitumor effect of 2,2-dimethylphosphoraziridines, with or without radiation, are unknown (as the results with 10 indicate, probably quite low); therefore, lacking any comparative data for a standard agent (e.g. AB-132), we cannot regard these results as conclusive for the evaluation of our new series of compounds as potential "dual-acting radiation sensitizers".

In the present paper, we reported the synthesis of a new series of compounds incorporating the structural features as well as the biological activities of both the 2,2-dimethylphosphoraziridine-type antitumor agents and the nitroimidazole-type hypoxic radiosensitizers. The biological studies reported here indicate that these compounds are "properly constructed" in that their two differently acting moieties do not interfere with each other and are operational by themselves. To evaluate their possible "synergistic" radiation-potentiating effects and their potential usefulness in cancer treatment would require further studies using different in vivo models as well as a variety of dose schedules of drug and radiation.

Experimental Section

Nuclear magnetic resonance spectra were obtained on a Varian T-60 or an FT-80 spectrometer. Chemical shifts are reported in ppm downfield from TMS; either TMS or DSS at 0 ppm was the internal shift standard. Coupling constants are first-order approximations. Resonance assignments use the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; br, broad. Infrared spectra were recorded on a Perkin-Elmer 197 spectrophotometer or a Nicolet 7199 Fourier Transform instrument. Melting points were determined in an open capillary tube in a Mel-Temp apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was performed on Analtech silica gel GF Uniplates. Preparative TLC was conducted on plates coated with Machery Nagel silica gel P/UV_{254}. Elemental analyses were performed by Atlantic Microlabs, Inc., Altanta, GA.

2,2-Dimethylaziridine was purchased from Bodman Chemicals, Aston, PA. (AB-132 was prepared in our laboratory.[35]) All reactions involving organophosphorus compounds were conducted under an inert atmosphere with anhydrous solvents, and products filtered under a flow of N_2 gas. Solvents were removed on a rotary evaporator at room temperature. The phosphoraziridines were protected from exposure to moisture and heat and were not allowed to stand for prolonged periods of time at room temperature.

2-Nitroimidazole (2). The method of Agrawal[28] was modified slightly in that the solution of the diazonium salt was stirred only very briefly (2 min) after addition of $NaNO_2$ to 1 (88.5 mmol) was completed. It was then treated immediately with $CuSO_4$ followed by $NaNO_2$ to avoid extensive decomposition of the diazonium salt.

2-(2-Nitro-1-imidazolyl)ethanol (3). This preparation was conducted as reported by Beaman,[30] with the following modification to avoid excess base, which was found to lower the yield of 3. Methanolic NaOMe (3 N) was added dropwise to a sus-

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pension of **2** (4.00 g, 35.4 mmol) in dry DMF (40 mL) until a red solution was obtained. Further **2** was added until the color just returned to yellow. The methanol was distilled off by heating the bath to 152 °C, and then the reaction with 2-bromoethanol and subsequent workup were performed as reported. Recrystallization (EtOH/Norit) of the crude product provided 1.92 g (35%) of **3** as yellow-green needles: mp 114–117 °C (lit.[30] mp 112–114 °C); ^{1}H NMR (DMSO-d_6) δ 3.76 (m, 2 H, CH_2O), 4.50 (t, 2 H, CH_2N, J = 5 Hz), 5.02 (t, 0.3 H, OH, J = 5 Hz), 7.20 and 7.63 (2 s, 2 H, CH=CH).

1-(2-Phthalimidoethyl)-2-nitroimidazole (4). The sodium salt of **2** (2.26 g, 20.0 mmol) in DMF (20 mL) was generated with NaOMe/MeOH as described for **3**, and the methanol was distilled off. After the addition of *N*-(2-bromoethyl)phthalimide (4.83 g, 19.0 mmol), the reaction was stirred at 110 °C for 5 h and then allowed to stand at room temperature overnight. The mixture was evaporated, and the resulting powder was washed with half-saturated Na_2CO_3 (4 × 70 mL) and H_2O (2 × 75 mL) and dried in vacuo (in presence of P_2O_5). Recrystallization (EtOAc) provided 4.52 g (79% based on **2**) of **4** as yellow crystals: mp 203–214 °C; ^{1}H NMR (DMSO-d_6) δ 4.08 (m, 2 H, CH_2N_{Phth}), 4.67 (m, 2 H, CH_2N_{Imid}), 7.03 and 7.58 (2 d, 2 H, CH=CH, J = 1 Hz), 7.80 (s 4 H, arom); IR (KBr) 1490, 1355 (NO_2) cm^{-1}. Anal. ($C_{13}N_{10}N_4O_4$) C, H, N.

1-(2-Aminoethyl)-2-nitroimidazole (5). Hydrazine hydrate (1.0 g, 20 mmol) was added to a suspension of **4** (3.87 g, 13.5 mmol) in absolute EtOH (160 mL), and the mixture was stirred at reflux for 4.5 h and then allowed to sit overnight at room temperature. The reaction was filtered and the precipitate washed with EtOH (50 mL). The combined filtrates were evaporated and then twice treated with EtOH (50 mL) and evaporated to remove residual hydrazine. The residue was then twice digested with THF (50 mL), filtered, and evaporated (without warming). The resulting powder was dissolved in EtOAc (1 L) at room temperature and filtered again. Evaporation and coevaporation from THF yielded 1.21 g (57%) of **5** as a pale tan powder. An analytical sample was prepared by rapid recrystallization from hot EtOAc, decanting from a resin upon cooling: mp 83–91 °C (lit.[33] mp 91–92 °C); ^{1}H NMR (DMSO-d_6) δ 2.23 (br s, 2 H, NH_2), 2.90 (t, 2 H, CH_2NH_2, J = 6 Hz), 4.37 (t, 2 H, CH_2N_{Imid}), 7.12 and 7.59 (2 d, 2 H, CH=CH, J = 1 Hz). Anal. ($C_5H_8N_4O_2$) C, H, N.

2-(2-Nitro-1-imidazolyl)ethyl Bis(2,2-dimethyl-1-aziridinyl)phosphinate (8). A solution of **3** (0.471 g, 3.0 mmol) and Et_3N (0.30 g, 3.0 mmol) in dry THF (17 mL) was added dropwise over 1 h to a stirred solution of $POCl_3$ (1.07 g, 7.0 mmol) in THF (5 mL) at −5 °C. The suspension was stirred for another 75 min at 0 °C and then allowed to reach room temperature and filtered under a N_2 flow. The residual powder was washed with THF (20 mL), and the filtrate and washings were evaporated then dissolved in THF and reevaporated to an oil, which was then dried in vacuo for 30 min. The crude phosphorodichloridate was dissolved in THF (10 mL) and added dropwise over 15 min to a stirred solution of 2,2-dimethylaziridine (0.441 g, 0.56 mL, 6.2 mmol) and Et_3N (0.708 g, 7.0 mmol) in THF (10 mL) at −5 °C. The suspension was stirred for another 19 h at 4 °C, allowed to reach room temperature, and filtered. The precipitate was washed with THF (15 mL), and the filtrates were evaporated to an oil, which was subsequently chromatographed by preparative TLC (THF/3% Et_3N). The product was extracted with 1,2-dimethoxyethane, the extracts were evaporated, and residual solvent was removed by multiple evaporations from 1,2-dichloroethane and CH_2Cl_2. Drying in vacuo (40 °C) yielded 0.95 g (92%) of **8** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.38 (s, 12 H, Me), 2.10 (d, 4 H, azir CH_2, $J_{P,H}$ = 14 Hz), 4.59 (m, 4 H, CH_2CH_2), 7.17 and 7.30 (2 d, 2 H, CH=CH, J = 1.5 Hz); IR (neat) 1490 and 1360 (NO_2), 1240 (P=O) cm^{-1}. Anal. ($C_{13}H_{22}N_5O_4P{\cdot}0.5H_2O$) C, H, N.

1-(2-Nitro-1-imidazolyl)-3-methoxy-2-propyl Bis(2,2-dimethyl-1-aziridinyl)phosphinate (9). A solution of misonidazole (**6**)[32] (0.716 g, 3.56 mmol) and Et_3N (0.51 g, 5.0 mmol) in THF (25 mL) was added dropwise to a stirred solution of $POCl_3$ (3.45 g, 22.5 mmol) in THF (15 mL) at 0 °C over 90 min. After the reaction was stirred for another 3 h, the crude phosphorodichloridate was isolated and immediately reacted with 2,2-dimethylaziridine and Et_3N (9 mmol each) as described for the synthesis of **8**. The resulting oil was purified by preparative TLC using absolute EtOH/1,2-dichloroethane (1:4) for extraction of the product. Crystallization from ether yielded 0.70 g (51%) of **9** as pale yellow crystals. An analytical sample was obtained by recrystallization (ether/petroleum ether): mp 68–74 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (m, 12 H, Me), 2.03 (m, 4 H, azir CH_2), 3.38 (s, 3 H, MeO), 3.63 (d, 2 H, CH_2O, J = 4 Hz), 4.77 (m, 2 H, CH_2N), 4.90 (m, 1 H, CH), 7.17 and 7.23 (2 d, 2 H, CH=CH, J = 1 Hz); IR ($CHCl_3$) 1490 and 1360 (NO_2), 1240 (P=O) cm^{-1}. Anal. ($C_{15}H_{26}N_5O_5P$) C, H, N.

2-(2-Nitro-1-imidazolyl)ethyl [Bis(2,2-dimethyl-1-aziridinyl)phosphinyl]carbamate (10). A solution of **3** (0.943 g, 6.0 mmol) in THF (40 mL) was added dropwise to phosphorisocyanatidic dichloride[36] (0.975 g, 0.60 mL, 6.1 mmol), in THF (20 mL) over 2 h at 0 °C, and then the bath was removed and stirring continued for 30 min. Since some **3** still remained, additional isocyanate (0.03 mL) was added, and stirring was continued for another 30 min. The solution of the carbamoyl phosphorodichloridate was immediately transferred to a dropping funnel and added dropwise over 1 h to a stirred solution of 2,2-dimethylaziridine (0.85 g, 12 mmol) and Et_3N (1.22 g, 12 mmol) in THF (40 mL) at −5 °C. After stirring for 19 h at 4 °C, additional aziridine and Et_3N (0.6 mmol each) were introduced, and the reaction was stirred for 4 h at 4 °C to reach completion. The suspension was filtered and the precipitate washed with cold THF (40 mL). Evaporation of the filtrates and crystallization from THF/hexanes yielded 0.99 g (43%) of **10** as a pale yellow powder: mp 106 °C (dec); ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 12 H, Me), 2.25 (d, 4 H, azir CH_2, J = 14 Hz), 4.50 and 4.73 (2 m, 4 H, CH_2CH_2), 7.10 and 7.40 (2 d, 2 H, CH=CH, J = 1 Hz), 7.58 (br s, 1 H, NH); IR (KBr) 3230 (NH), 1725 (C=O), 1470 and 1360 (NO_2), 1220 (P=O) cm^{-1}. Anal. ($C_{14}H_{23}N_6O_5P$) C, H, N.

***N*-[2-(2-Nitro-1-imidazolyl)ethyl]bis(2,2-dimethyl-1-aziridinyl)phosphinic Amide (11).** To a solution of $POCl_3$ (0.23 g, 1.5 mmol) in THF (10 mL) at −30 °C was added Et_3N (0.30 g, 3.0 mmol) in THF (7 mL) over 20 min, followed by 2,2-dimethylaziridine (0.213 g, 3.0 mmol) in THF (7 mL) over 45 min. To this solution of bis(2,2-dimethyl-1-aziridinyl)phosphinic chloride was then added **5** (0.234 g, 1.5 mmol) and Et_3N (1.5 mmol) in THF (15 mL) over 45 min. After the suspension had stirred for 20 h at 4 °C, it was filtered at room temperature and the precipitate washed with THF (93% of theoretical amount of $Et_3N{\cdot}HCl$ was recovered). Evaporation of the filtrate yielded a yellow oil (0.53 g) which was the desired product based on the NMR spectrum, but which contained a small amount of **5** and polymer based on TLC: ^{1}H NMR ($CDCl_3$) δ 1.40 (m, 12 H, CH_3), 2.03 (d, 4 H, azir CH_2), 2.7–3.8 (br, m, 3 H, CH_2NHP), 4.55 (m, 2 H, CH_2N_{Imid}), 7.10 and 7.30 (2 d, 2 H, CH=CH, J = 1 Hz). Attempted chromatography using preparative plates (5:2 1,2-dichloroethane/methanol) resulted in partial decomposition.

***N*-[2-(2-Nitro-1-imidazolyl)ethyl]-*N'*-[bis(2,2-dimethyl-1-aziridinyl)phosphinyl]urea (12).** A solution of **5** (0.234 g, 1.5 mmol) in THF (10 mL) was added dropwise over 2 h to phosphorisocyanatidic dichloride[36] (0.246 g, 1.54 mmol) in THF (10 mL) at −20 to −30 °C. The resulting solution of ureidophosphorodichloridate was then allowed to reach room temperature, transferred to a dropping funnel, and added to a stirred solution of 2,2-dimethylaziridine (0.220 g, 3.1 mmol) and Et_3N (0.32 g, 3.1 mmol) in THF (10 mL) at −5 °C over 30 min. The reaction was stirred for 19 h at 4 °C, allowed to reach room temperature, and filtered, and the precipitate washed with THF (20 mL). The residual powder was then extracted with THF (60 mL) and hot THF (100 mL), these extracts were evaporated, and the resulting powder was dried in vacuo to afford 0.28 g (48%) of **12**. An analytical sample was obtained by recrystallization from CH_2Cl_2/ether/petroleum ether: mp 162 °C dec; 80-MHz ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 12 H, Me), 2.20 and 2.16 (2 d, 4 H, azir CH_2), 3.64 (pseudo-q, 2 H, CH_2NH, J = 7 Hz), 4.58 (t, 2 H, CH_2N_{Imid}, J = 7 Hz), 5.44 (br d, 1 H, NH), 7.11 and 7.36 (2 d, 2 H, CH=CH, J = 1 Hz), 7.71 (br s, 1 H, NHP=O); IR (KBr) 3287 (NH), 1667 (C=O), 1483 and 1366 (NO_2), 1282 (P=O) cm^{-1}. Anal. ($C_{14}H_{24}N_7O_4P{\cdot}0.5H_2O$) C, H, N.

2-Nitrobenzyl [Bis(2,2-dimethyl-1-aziridinyl)phosphinyl]carbamate (15). A solution of 2-nitrobenzyl alcohol **13** (0.918 g, 6.0 mmol) in 15 mL of THF was added dropwise over 70 min to phosphorisocyanatidic dichloride[36] (0.61 mL, 0.99 g, 6.2 mmol) in 15 mL of THF at 0–2 °C. The reaction was allowed to warm to ambient temperature over 30 min, at which time TLC (1,2-

dimethoxyethane) indicated completion. This (2-nitrobenzyl)-carbamoyl phosphorodichloridate solution was then dropped slowly, over 40 min, into a solution of 2,2-dimethylaziridine (1.17 mL, 0.92 g, 13.0 mmol) and triethylamine (1.32 g, 13.0 mmol) in 20 mL of THF at −2–0 °C and the resulting slurry stirred at 4 °C for 19 h. The cold reaction mixture was filtered and the precipitate washed with 50 mL of cold THF. Evaporation of the filtrates and recrystallization (THF/hexanes) provided 1.66 g (72%) of **15** as white crystals: mp 123–135 °C; ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 12 H, CH_3), 2.26 (d, 4 H, azir CH_2, J = 14 Hz), 5.60 (s, 2 H, benzyl CH_2), 7.3–8.3 (m, 5 H, arom and NH); IR ($CHCl_3$) 3405 (NH), 1740 (C=O), 1525 and 1345 (NO_2), 1240 (P=O) cm^{-1}. Anal. ($C_{16}H_{23}N_4O_5P$) C, H, N.

4-Nitrobenzyl [Bis(2,2-dimethyl-1-aziridinyl)phosphinyl]carbamate (16). With a procedure analogous to that reported for **15** starting from 4-nitrobenzyl alcohol, **16** was obtained as white plates (55%) after recrystallization (THF/hexanes): mp 119–131 °C; ^{1}H NMR ($CDCl_3$) δ 1.42, 1.47 (2 s, 12 H, azir CH_3), 2.29 (d, 4 H, azir CH_2, J = 14 Hz), 5.28 (s, 3 H, benzylic CH_2), 7.60 (br d, 3 H, aryl (J = 8 Hz) and overlapping NH), 8.23 (d, 2 H, aryl, J = 8 Hz); IR ($CHCl_3$) 3410 (NH), 1740 (C=O), 1615 (NH), 1525 and 1350 (NO_2), 1240 (P=O) cm^{-1}. Anal. ($C_{16}H_{23}N_4O_5P$) C, H, N.

2-(2-Nitro-1-imidazoyl)ethyl Carbamate (17). This compound was prepared by the procedure reported in the patent literature:[30] mp 146–148.5 °C (lit.[30] mp 143.5–145 °C); FT-IR (KBr) 3330 and 3150 (NH_2), 1750 (C=O), 1625 (NH), 1490 and 1365 (NO_2) cm^{-1}.

Aqueous Hydrolysis of 10. A solution of **10** (29 mg, 0.075 mmol) in D_2O (0.5 mL) at 37 °C was monitored by ^{1}H NMR for 25 h, at which time a crystalline precipitate was observed. After incubation for another day, the mixture was cooled and filtered, and the crystals were washed with D_2O to yield 6 mg (40%) of white flakes that were identified as **17** on the basis of the following: mp 142–146 °C; TLC (1:1 CH_2Cl_2/EtOAc) identical migration with **17**; FT-IR (KBr) nearly identical with synthetic **17**.

The mother liquor was lyophilized to yield a white foam (10 mg) that was identical on the basis of FT-IR (KBr) and ^{1}H NMR (D_2O) spectra with **18**, a sample of which was obtained by hydrolysis of AB-132 in D_2O at 37 °C for 50 h, followed by ether extraction and lyophilization. ^{1}H NMR (D_2O) δ 1.27 and 1.41 (2 s, 12 H, CH_3), 2.96 (s, 2 H, CH_2NH_2), 3.08 (s, 2 H, $CH_2NH_3^+$) (High-field spectra were obtained on a GN-500 (General Electric) spectrometer at the National Institute of Environmental Health Sciences, Laboratory of Molecular Biophysics, Research Triangle Park, NC); IR (KBr) 3420–2640 (NH_3+, POH), 1620 and 1540 (NH), 1095 and 1070 (P=O, POC), 965 cm^{-1}.

Reaction with NBP. Reaction with NBP was conducted as previously described[45] in buffered (pH 4.0) aqueous EtOH at 80 °C.

Radiation-Sensitization Studies. Evaluation of the ability of the various compounds studied to potentiate the effects of X-irradiation on V-79 Chinese hamster lung fibroblast cells under hypoxic conditions was performed in our laboratory as described previously.[22] The cultured cells were transferred to culture dishes or flasks with fresh medium, and the samples to be evaluated under hypoxic conditions were ventilated for 4 h with humidified N_2 containing 5% CO_2 while the "oxic" samples were kept in a normal atmosphere. The cells were then treated with freshly prepared drug solution, incubated for 30 min, then X-irradiated. One hour past addition of the drug, the medium was replaced and the cells incubated for a short time, then detached, made into single-cell suspensions, and plated into cell culture dishes in appropriate cell concentrations. Incubation in a CO_2 incubator for ca. 1 week was followed by fixing, staining, and counting of the formed colonies.

In Vivo Biological Testing. (a) Without Radiation (Bristol Laboratories). Compounds were tested against P-388 murine leukemia cells in CDF_1 mice as described in a previous publication.[37] The mice (six per dose level of each drug, with 10 mice in leukemia control groups) were implanted ip with 10^6 P388 cells, then on the next day treated ip with the appropriate doses of the experimental drugs. The experiments were generally terminated 30 days postimplant, with weight changes being noted on day 6.

(b) **Combination of Drug with Radiation (Medical College of Virginia).** Compound **10** was tested in combination with radiation by using the Wodinsky Model.[38] P388 leukemia was maintained by weekly passage of 10^6 cells into DBA/2 mice. $B6D2F_1$ mice, six per group, were used for testing. Each received 10^6 cells on day 0 with treatment on day 1. Compound **10** was given by ip injection 1 h before radiation. Radiation was delivered with a ^{60}Co source by a previously described method.[20] Mice were observed until death or for 60 days. Weight changes were recorded on day 8.