Nitrobenzyl Phosphorodiamidates as Potential Hypoxia-Selective Alkylating Agents

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A series of novel nitrobenzyltetrakis(chloroethyl)phosphorodiamidates has been prepared, and its cytotoxicity has been evaluated against HT-29 cells under aerobic and hypoxic conditions and against murine bone marrow progenitor cells under aerobic conditions. All compounds were selectively toxic to HT-29 cells under hypoxic conditions, and the selectivity ratios varied from 1.6 to >90. Analogs lacking either the nitro group or the tetrakis(chloroethyl) moiety were not cytotoxic, confirming that the presence of both nitro and incipient alkylating groups are essential for activity. Surprisingly, some analogs were far more toxic to bone marrow progenitors than to HT-29 cells under aerobic conditions, suggesting that other activation mechanisms must exist in these hematopoietic cells. Cytotoxicity increased with increasing depth in the HT-29 spheroid model, consistent with the preferential hypoxic toxicity of these compounds. Alkaline elution experiments showed a greater number of DNA interstrand cross-links under hypoxic compared to aerobic conditions. The extent of cross-linking in hypoxic cells was essentially identical to that produced by an equitoxic dose of melphalan, suggesting that the cytotoxicity of these compounds results from phosphorodiamidate release and alkylation of DNA.

Introduction

A substantial body of evidence has accumulated supporting the existence of significant hypoxic fractions in animal tumors and in human tumor xenografts transplanted in immune deficient rodents.[1] While less direct evidence exists establishing the presence of hypoxic cells in human tumors *in situ*, an increasingly impressive body of evidence supports the conclusion that certain types of human tumors do indeed contain populations of cells which at any given time exist in a hypoxic environment.[2] It has been suggested that tumor hypoxia could be exploited to provide a therapeutic advantage through the design of hypoxia-selective cytotoxic agents (HSAs) that are activated to cytotoxic species by reduction at reduced oxygen tensions, a mechanism commonly referred to as bioreductive activation.[3,4]

While tumor hypoxia may provide an exploitable drug-activating environment, the rationale for developing agents that target hypoxic cells extends beyond considerations of selectivity. In addition to being relatively refractory to radiation therapy, hypoxic tumor cells have also been shown to be resistant to a number of drugs commonly used in conventional cytotoxic chemotherapy.[5] Hypoxic cells may be resistant to chemotherapy because of their nonproliferative status, their location beyond the effective diffusion distance of some agents, or their presence in regions of low oxygen tension (higher concentrations of which may be required for the uptake or activation of certain drugs). Hypoxic cells have also been reported to be more susceptible to certain mutagenic damage,[4] an important consideration since they may be exposed to sublethal, yet mutagenic, concentrations of chemotherapeutic agents. Hypoxia has also been implicated in the emergence of drug-resistant and metastatic phenotypes. Rice et al. have reported that transient hypoxia can result in amplification of the dihydrofolate reductase and P-170 glycoprotein genes with increased resistance to methotrexate and adriamycin, respectively.[6] Similarly, Young et al. reported that hypoxia resulted in the emergence of clones with markedly enhanced metastatic potential.[7] The data clearly indicate that hypoxic cells can negatively impact effective tumor therapy, identifying them as important targets for novel compounds that can eradicate cells located in protective hypoxic environments.

Several classes of HSAs have been developed and evaluated in preclinical as well as clinical settings. Perhaps the best example of a bioreductive compound with demonstrated clinical activity is the antitumor quinone Mitomycin C, developed by Sartorelli and colleagues.[3] Other classes of bioreductively-activated compounds, particularly analogs of nitroimidazoles, nitroacridines, and benzotriazene *N*-oxides, have also been developed and tested as potential hypoxic cytotoxic agents.[8] In addition to these agents, which are metabolized to toxic intermediates under hypoxic conditions, nitroheterocyclic analogues containing latent alkylating moieties which can be activated subsequent to reduction of the nitro group to more electron-donating species have also been reported.[9] We recently described a novel mechanism-based strategy for the design of hypoxia-activated alkylating compounds based on intramolecular base-catalyzed β-elimination of phosphoramide mustard (PDA) subsequent to reduction of a nitroquinoline.[10] We report here the synthesis and evaluation of a series of nitrophenyl phosphorodiamidate mustards to further explore the hypothesis that intramolecular catalysis can be utilized for the selective delivery of toxic alkylating species to hypoxic tumor cells.

Results and Discussion

Chemistry. The mechanistic hypothesis underlying the proposed hypoxic activation of these compounds is shown in Scheme 1. Because the rate-limiting step for nitro group reduction is addition of the first electron, reduction is expected to proceed to the corresponding

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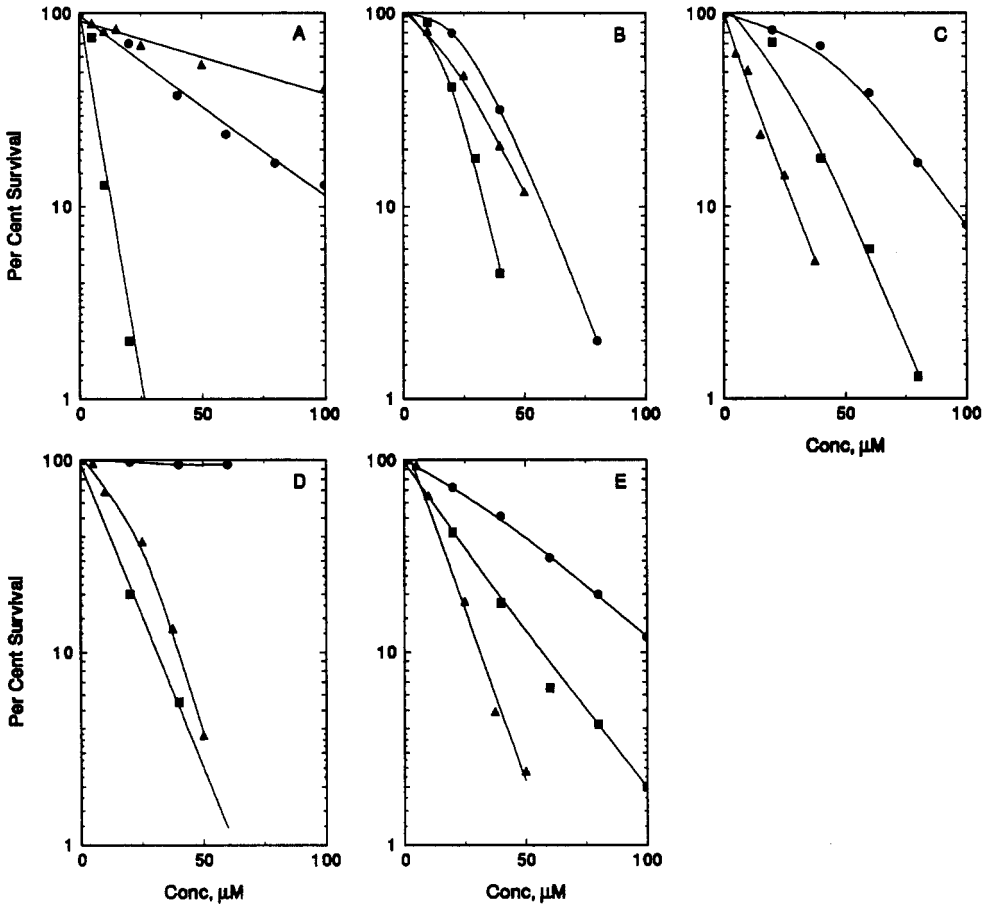
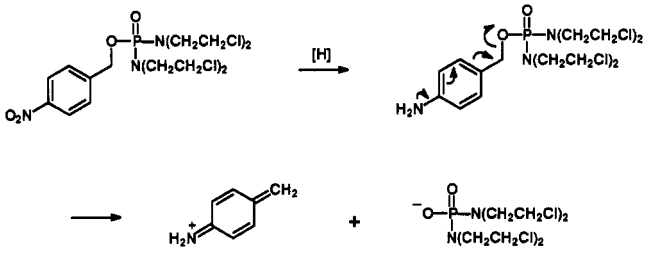
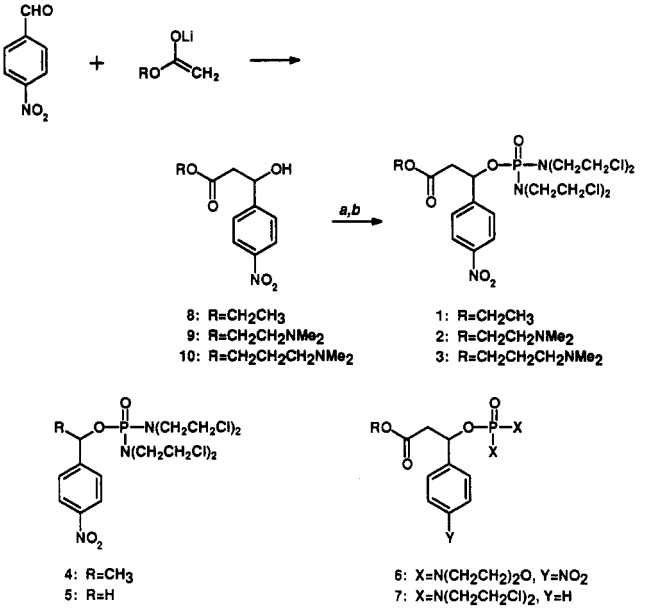


Figure 1. Clonogenic survival of HT-29 cells under aerobic (●) or hypoxic (■) conditions (4-h exposure) and of murine bone marrow granulocyte/macrophage progenitors (▲) (2-h exposure). Panel A, compound 1; panel B, compound 2; panel C, compound 3; panel D, compound 4; panel E, compound 5.

Scheme 1



Scheme 2[a]



[a] (a) $((ClCH_2CH_2)_2N)_2PCl$, Et_3N; (b) *t*-BuOOH.

aniline derivative. Lone-pair-assisted expulsion of the phosphorodiamidate serves to activate the alkylating moiety. The phosphorodiamidates **1–3**, **6**, and **7** were prepared as outlined in Scheme 2. 4-Nitrobenzaldehyde (benzaldehyde in the case of **7**) was reacted with the lithium enolate of the corresponding esters to generate β-hydroxy esters **8–10**. The benzylic alcohols were then reacted with *N,N,N′,N′*-tetrakis(chloroethyl)chlorophosphorous diamide in the presence of triethylamine followed by oxidation with *tert*-butyl hydroperoxide to produce the phosphorodiamidates **1–5**. Analog **6** was prepared by reaction of **8** with dimorpholinophosphinic chloride followed by *tert*-butyl hydroperoxide oxidation.

Cytotoxicity. Compounds **1–5** reduced cell survival under both aerobic and hypoxic conditions (Figure 1a–e; Table 1). As expected, each compound produced greater cell kill when cells were treated under hypoxic conditions. The hypoxic/aerobic differentials (determined from the ratio of IC_{90} values calculated from the log-linear portion of the aerobic and hypoxic survival curves) are 8.1, 1.6, and 1.9 for compounds **1**, **2**, and **3**, respectively. The dimethylamino groups were introduced into the ester moiety in order to enhance the aqueous solubility of these compounds. However, it is not apparent why this modification should reduce selectively the hypoxic toxicity of the ester 3–4-fold. There was initially some concern that the presence of the (dimethylamino)ethyl group in **2** might

Table 1. Cytotoxicity of Nitrobenzyl Phosphorodiamidates 1–5

compd	LC_{90}, μM[a] aerobic	hypoxic	G/M	aerobic/hypoxic ratio
1	106 ± 6	13 ± 3	268 ± 8	8.1
2	55 ± 4	34 ± 3	55 ± 3	1.6
3	95 ± 3	50 ± 2	29 ± 2	1.9
4	>2000	22 ± 1	39 ± 4	>90
5	109 ± 2	55 ± 4	32 ± 2	2.0

[a] Concentration that reduces the clonogenic survival by 1 log (90%). Values are determined by linear regression of the data points on the log linear part of the dose–response curves and are reported ±SE.

enhance toxicity via intramolecular catalysis of the β-elimination reaction, so the (dimethylamino)propyl ester 3 (which should not undergo intramolecular β-elimination) was also prepared. The aerobic toxicity of 1 and 3 are comparable and somewhat lower than 2, suggesting that, if intramolecular β-elimination is occurring for 2, its contribution is modest. The effect of the ester group on cytotoxicity was assessed by preparing compounds 4 and 5, in which the acetate ester groups are replaced by methyl and hydrogen, respectively. Compound 4 was the most selective in this series, exhibiting essentially no cytotoxicity to HT-29 cells under aerobic conditions. Remarkably, the unsubstituted benzyl compound 5 was much less selective than 4, resulting from increased aerobic toxicity and decreased hypoxic toxicity compared to 4. The basis for these differences is not known. Finally, additional support for the proposed mechanism of cytotoxicity was obtained via the synthesis and evaluation of two analogs of 1. For compound 6, the alkylating moieties were replaced by a morpholine ring, and for compound 7, the nitro group was replaced by hydrogen. Analogs 6 and 7 showed essentially no cytotoxicity to HT-29 cells under aerobic or hypoxic conditions at concentrations up to 300 μM, confirming that the nitro group and the electrophilic substituent on the phosphoramidate are essential for cytotoxic activity.

In an attempt to assess the toxicity of these compounds to normal tissue, the cytotoxicity of compounds 1–5 to murine bone marrow granulocyte/macrophage progenitors (GM-CFC) was evaluated in an *ex vivo* assay. The GM-CFC toxicity of ester 1 following a 2-h exposure was less than half the aerobic toxicity to HT-29 cells following a 4-h exposure (triangles, Figure 1; Table 1). In marked contrast, however, compounds 2–5 were highly toxic to GM progenitors, having toxicity comparable to their hypoxic toxicity to HT-29 cells. The desnitro analog 7 was essentially nontoxic in the GM-CFC assay (>70% survival at 50 μM). Presumably compounds 2–5 are undergoing reductive activation in bone marrow progenitor cells, but neither the greater selectivity of 1 nor the mechanism of the process is understood.

Cytotoxicity in HT-29 Spheroids. In order to provide further support for the preferential hypoxic toxicity of these compounds, we sought to evaluate their cytotoxic potential in multicell tumor spheroids. The Hoescht 33342 flow cytometric technique described by Durand[11] was utilized to compare the toxicity of 1 in cell populations isolated from increasing depths of drug-treated spheroids. HT-29 spheroids were incubated with 1 for either 4 or 16 h. In each case, cell survival decreased progressively from the outermost (aerobic) to innermost (hypoxic) populations (Figure 2). The fraction of cells from the outermost population surviving 4-h drug treatment was comparable to that obtained in similar experiments with aerobic single

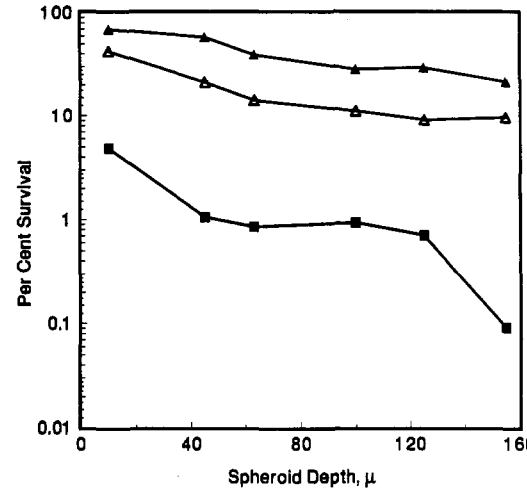


Figure 2. Survival of HT-29 cells isolated from spheroids treated with 1: (▲) 25 μM, 4-h treatment; (△) 50 μM, 4-h treatment; (■) 25 μm, 16-h treatment. Details are described in the text.

cells (compare Figures 2 and 1A). In contrast, cell kill at a depth of 150 μm was less than expected on the basis of the cell studies, perhaps as a consequence of higher oxygen tension at this depth in the spheroid compared to the hypoxic cell suspensions, or due to poor drug penetration. When the exposure interval was increased to 16 h, cell survival in each fraction was further reduced. This longer treatment period resulted in an even greater hypoxic/aerobic differential, producing nearly a 2 log difference in cell kill between the outer and inner fractions. These results are consistent with the proposed activation scheme for this class of compounds and clearly demonstrate the potential of this strategy for preferentially targeting this difficult population.

DNA Cross-Link Formation. If bioreductive activation and alkylation according to Scheme 1 were responsible for the selective hypoxic toxicity of these compounds, it should be possible to detect enhanced cross-linked formation in the DNA of cells exposed under hypoxic conditions. HT-29 cells were exposed to compound 1 (15 μM, 4 h) under aerobic and hypoxic conditions and prepared for alkaline elution 16 h after drug treatment. This time interval was selected to allow adequate time for completion of interstrand cross-link formation. Immediately before lysis on the elution filters, control and drug-treated cells were exposed to 4.0 Gy γ-irradiation to induce DNA strand breaks. As shown in Figure 3, DNA from control cells eluted from the filter at a constant rate over the collection period. In contrast, the elution rate of irradiated DNA from drug-treated cells was greatly reduced, providing clear evidence for interstrand cross-link formation. Cross-links were detected in DNA of cells exposed to 1 under aerobic conditions, consistent with observed aerobic toxicity. However, like cell kill, the magnitude of cross-link formation was increased in DNA from cells treated under hypoxic conditions. Interestingly, cross-link formation in hypoxic cells was comparable to that detected in HT-29 cells exposed to an equitoxic dose of the alkylating agent L-phenylalanine mustard, further supporting a correlation between cross-link formation and cytotoxicity of 1 under hypoxic conditions.

In conclusion, we have demonstrated the feasibility of combining nitrophenyl group reduction with phosphorodiamidate expulsion to provide selective toxicity to hypoxic cells. Penetration and selective cytotoxicity have

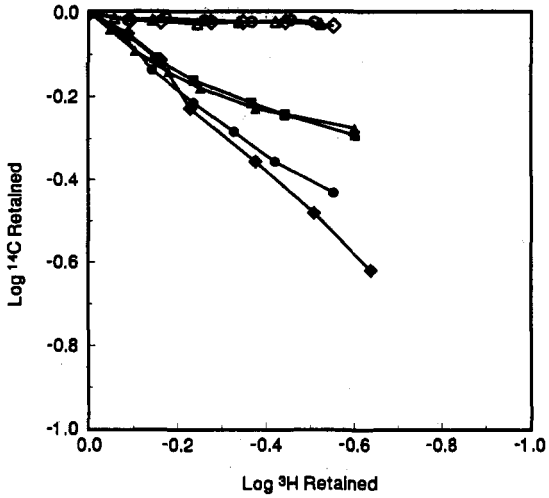


Figure 3. Alkaline elution of HT-29 cells. Cells were treated with 1 (15 μM, 4 h) or melphalan (15 μM, 1 h), allowed to recover (16 h), and then given 400 R (filled symbols) or 0 R (open symbols) before elution: (◇) control; (○) 1, aerobic; (△) 1, hypoxic; (●) 1, aerobic + 400 R; (▲) 1, hypoxic + 400 R; (■) melphalan + 400 R; (◆) 400 R.

been demonstrated in the spheroid model, and cytotoxicity also correlates with DNA interstrand cross-linking. However, selectivity with respect to aerobic bone marrow progenitor cells is observed with only one analog, suggesting that mechanisms other than reduction in a hypoxic environment can activate these prodrugs.

Experimental Section

General Introduction. NMR spectra were recorded on a Bruker WP-270SY spectrometer in $CDCl_3$. ^1H NMR spectra were measured at 270.19 MHz and are reported in the following format: chemical shift (number of protons, multiplicity, coupling constant(s) in hertz). ^{31}P NMR spectra were measured at 109.37 MHz with broadband ^1H decoupling and are reported as ppm from triphenylphosphine oxide in toluene-d_8 present as a coaxial reference. Melting points were measured on a Meltemp apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN, and Midwest Microlab, Indianapolis, IN. Mass spectral data were obtained from the Mass Spectrometry Facility at the School of Pharmacy, University of California, San Francisco, CA. Chromatographic separations were performed by flash chromatography on silica gel grade 60. All reactions were carried out under a nitrogen atmosphere unless otherwise specified or reagents containing water were used. Reagents and solvents were introduced via syringe where appropriate.

2-(Ethoxycarbonyl)-1-(4-nitrophenyl)ethyl *N,N,N',N'*-Tetrakis(2-chloroethyl)phosphorodiamidate (1). Phosphorus trichloride (1.00 mL, 11.4 mmol) was added to dichloromethane (100 mL). Bis(2-chloroethyl)amine hydrochloride (4.29 g, 24.0 mmol) was suspended in this solution. Triethylamine (16.7 mL, 120 mmol) was added via syringe over 5 min and the resulting mixture stirred for 10 min at room temperature. Hydroxy ester 8 (2.99 g, 12.5 mmol) was dissolved in dichloromethane (20 mL) and added. The reaction mixture was stirred for 10 min and then cooled to −20 °C. *tert*-Butyl hydroperoxide (4.0 mL, 3.0 M in 2,2,4-trimethylpentane, 12 mmol) was added and the bath was allowed to warm to room temperature. The reaction mixture was filtered over a short column of silica. The filtrate was added to dilute aqueous hydrochloric acid (100 mL) and extracted with dichloromethane (100 mL), followed by drying ($MgSO_4$) and concentration. The residue was chromatographed (5% acetone:dichloromethane) to give 1 (3.69 g, 52% based on 8) as an oil: R_f = 0.35 (5% acetone:dichloromethane); ^1H NMR δ 8.27 (2H, d, J = 8.6), 7.62 (2H, d, J = 8.6), 5.89 (1H, dd, J = 14.1, 6.9), 4.12 (2H, q, J = 7.0), 3.68 (4H, m), 3.47 (8H, m), 3.16 (5H, m), 2.76 (1H, m), 1.22 (3H, t, J = 6.9); ^{31}P NMR δ −8.53. Anal. ($C_{19}H_{28}Cl_4N_3O_6P$) C, H, N.

2-[[2-(Dimethylamino)ethoxy]carbonyl]-1-(4-nitrophenyl)ethyl *N,N,N',N'*-Tetrakis(2-chloroethyl)phosphorodiamidate Hydrochloride (2). Phosphorus trichloride (2.00 mL, 22.9 mmol) was dissolved in dichloromethane (100 mL). Bis(2-chloroethyl)amine hydrochloride (8.6 g, 48 mmol) was added and the stirred mixture for 15 min at room temperature. Triethylamine (17.6 mL, 127 mmol) was added dropwise over 10 min and the mixture stirred for an additional 15 min. Hydroxy ester 9 (4.9 g, 17 mmol), dissolved in a minimum of dichloromethane, was added and the mixture stirred 30 min. The reaction mixture was cooled to −20 °C and *tert*-butyl hydroperoxide (8.0 mL, 3.0 M in 2,2,4-trimethylpentane, 24 mmol) was added. The reaction mixture was stored overnight at −20 °C. Concentrated hydrochloric acid was added dropwise to make the reaction mixture acidic to moistened pH paper (pH 5–6). The solution was dried ($MgSO_4$) and filtered over a short column of silica. The filtrate was evaporated and the residue chromatographed (25% methanol: ethyl acetate) to give 2 (2.73 g, 25% based on 9) as a hydrate: R_f 0.42 (25% methanol:ethyl acetate); ^1H NMR ($CDCl_3$) δ 8.27 (2H, d), 7.80 (2H, d), 6.32 (1H, m), 4.61 (1H, m), 4.39 (1H, m), 3.68 (4H, m), 3.42 (8H, m), 3.15 (6H, m), 2.92 (6H, s); ^{31}P NMR ($CDCl_3$) δ −9.28. Anal. ($C_{21}H_{38}Cl_5N_4O_8P$) C, H, N.

2-[[3-(Dimethylamino)propoxy]carbonyl]-1-(4-nitrophenyl)ethyl *N,N,N',N'*-Tetrakis(2-chloroethyl)phosphorodiamidate Hydrochloride (3). Phosphorus trichloride (1.00 mL, 11.5 mmol) was dissolved in dichloromethane (100 mL). Bis(2-chloroethyl)amine hydrochloride (4.1 g, 23.0 mmol) was added and the mixture stirred for 15 min at room temperature. Triethylamine (8.0 mL, 58 mmol) was added dropwise over 10 min and the mixture stirred for an additional 15 min. Hydroxy ester 10 (1.0 g, 4.0 mmol) dissolved in a minimum of dichloromethane was added and the mixture stirred for 30 min at room temperature. The reaction mixture was cooled to −20 °C, and *tert*-butyl hydroperoxide (4.0 mL, 3.0 M in 2,2,4-trimethylpentane, 12 mmol) was added. The reaction mixture was stored overnight at −20 °C and filtered cold. The filtrate was washed with water (3 × 200 mL), dried, and evaporated. The residue was dissolved in methanol (5 mL) and diluted with water (45 mL). The mixture was loaded on a short column of C18. The column was washed with water (2 × 50 mL) and 50% methanol: water (50 mL) and eluted with methanol. The methanol was evaporated and the residue chromatographed (25% methanol: ethyl acetate containing 1% hydrochloric acid) to give 3 (0.49 g, 16% based on 10) as a hydrate: R_f 0.37 (25% methanol:ethyl acetate); ^1H NMR ($CDCl_3$) δ 8.29 (2H, d, J = 8.8), 7.68 (2H, d, J = 8.8), 5.92 (1H, dd, J = 14.1, 7.3), 4.21 (1H, m), 4.02 (1H, m), 3.70 (6H, m), 3.46 (6H, m), 3.19 (5H, m), 2.84 (7H, m), 2.25 (2H, m); ^{31}P NMR ($CDCl_3$) δ −8.73. Anal. ($C_{22}H_{40}N_4O_8PCl_5$) C, H, N.

1-(4-Nitrophenyl)ethyl *N,N,N',N'*-Tetrakis(2-chloroethyl)phosphorodiamidate (4). Phosphorus trichloride (2.00 mL, 22.9 mmol) and bis(2-chloroethyl)amine hydrochloride (8.19 g, 45.9 mmol) were added to dichloromethane (300 mL). Triethylamine (15.9 mL, 115 mmol) was added over 5 min via syringe. The mixture was stirred for 15 min at room temperature. 2-(*p*-Nitrophenyl)ethanol (1.28 g, 7.63 mmol) was added and stirred for 1 h. The reaction mixture was cooled to −20 °C, and *tert*-butyl hydroperoxide (8.4 mL, 3.0 M in 2,2,4-trimethylpentane, 25 mmol) was added with vigorous stirring. The reaction was stored overnight at −20 °C. The mixture was filtered and washed with hydrochloric acid (2 × 200 mL, 10% aqueous), sodium carbonate (1 × 200 mL, 10% aqueous), and water (2 × 200 mL). The organic phase was dried, filtered, evaporated, and chromatographed (5% acetone:dichloromethane) to give 4 (2.53 g, 67%) as an oil: R_f = 0.67 (10% acetone:dichloromethane); ^1H NMR ($CDCl_3$) δ 8.27 (2H, d, J = 7.8), 7.56 (2H, d, J = 7.8), 5.65 (1H, dd, J = 6.6, 6.6), 3.71 (4H, m), 3.49 (8H, m), 3.26 (4H, m), 1.70 (3H, d, J = 6.6); ^{31}P NMR ($CDCl_3$) δ −8.92; MS m/z 494 (MH^+).

4-Nitrobenzyl *N,N,N',N'*-Tetrakis(2-chloroethyl)phosphorodiamidate (5). Phosphorus trichloride (5.0 mL, 2.0 M in dichloromethane, 10 mmol) was added to dichloromethane (100 mL) and cooled to 0 °C. Bis(2-chloroethyl)amine hydrochloride (3.8 g, 21 mmol) was suspended in this solution. Triethylamine (8.5 mL, 61 mmol) was added dropwise via syringe and the resulting mixture stirred for 10 min. *p*-Nitrobenzyl alcohol was dissolved in 20 mL of dichloromethane and added all at once to

the reaction. The mixture was stirred for 10 min and cooled to −20 °C. *tert*-Butyl hydroperoxide solution (3.5 mL, 11 mmol) was added and the bath was allowed to warm to room temperature. Ethyl acetate (100 mL) was added and the mixture filtered. The filtrate was washed with hydrochloric acid (250 mL, 10% aqueous) and with water (250 mL), followed by drying ($MgSO_4$) and concentration. The residue was chromatographed (10% acetone: dichloromethane) to give **5** (2.98 g, 62%): R_f = 0.43 (10% acetone: dichloromethane); mp = 64 °C; ^{1}H NMR δ 8.26 (2H, d, J = 8.7), 7.56 (2H, d, J = 8.7), 5.17 (2H, d, J = 7.8), 3.64 (8H, m), 3.46 (8H, m); ^{31}P NMR δ −8.12. Anal. ($C_{15}H_{22}Cl_4N_3O_4P$) C, H, N.

2-(Ethoxycarbonyl)-1-(4-nitrophenyl)ethyl Dimorpholinophosphinate (6). Phosphorus trichloride (2.00 mL, 22.9 mmol) was dissolved in dichloromethane (200 mL) and cooled to 0 °C. A mixture of triethylamine (9.55 mL, 68.8 mmol) and morpholine (4.00 mL, 45.9 mmol) was added dropwise over 10 min and the mixture stirred for an additional 10 min at 0 °C. Hydroxy ester **8** (2.73 g, 11.5 mmol) was dissolved in a minimum of dichloromethane and added to the reaction. After 30 min the reaction mixture was cooled to −20 °C and *tert*-butyl hydroperoxide (8.4 mL, 3.0 M in 2,2,4-trimethylpentane, 25.2 mmol) was added. The reaction mixture was stored overnight at −20 °C. Ethyl acetate (200 mL) was added and the mixture filtered and evaporated. The residue was chromatographed (ethyl acetate) to give **6** (0.49 g, 9% based on **7**) as an oil: R_f = 0.16 (ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 8.27 (2H, d, J = 8.7), 7.62 (2H, d, J = 8.7), 5.82 (1H, dd, J = 14.1, 7.0), 4.10 (2H, m), 3.57 (6H, m), 3.46 (3H, m), 3.10 (5H, m), 2.85 (4H, m), 1.21 (2H, t); ^{31}P NMR ($CDCl_3$) δ −7.12. Anal. ($C_{19}H_{30}N_3O_9P$) C, H, N.

2-(Ethoxycarbonyl)-1-phenethyl *N,N,N',N'*-Tetrakis(2-chloroethyl)phosphorodiamidate (7). A phosphorus trichloride solution (10.0 mL, 2.0 M in dichloromethane, 20 mmol) was added to dichloromethane (200 mL) and cooled to 0 °C. Bis-(2-chloroethyl)amine hydrochloride (7.5 g, 42 mmol) was added and stirred. Triethylamine (14.0 mL, 100 mmol) was added slowly via syringe and the resulting mixture stirred for 10 min. Ethyl 3-phenyl-3-hydroxypropionate (1.90 g, 10 mmol) was dissolved in 20 mL of dichloromethane and added all at once. The reaction mixture was stirred for 10 min and cooled to −20 °C. *tert*-Butyl hydroperoxide solution (3.5 mL, 3.0 M in 2,2,4-trimethylpentane, 11 mmol) was added and the bath warmed to room temperature. Ethyl acetate (100 mL) was added and the mixture filtered. The filtrate was washed with hydrochloric acid (250 mL, 3% aqueous) and with water (250 mL), followed by drying ($MgSO_4$) and concentration. The residue was chromatographed (5% acetone: dichloromethane) to give **7** as an oil (2.2 g, 42%): R_f = 0.42 (5% acetone:dichloromethane); ^{1}H NMR δ 7.40 (5H, s), 5.80 (1H, ddd, J = 16.2, 7.36, 2.94), 4.13 (2H, q, J = 7.36), 3.65 (5H, m), 3.39 (7H, m), 3.03 (5H, m), 2.81 (1H, dd, J = 16.18, 2.94); ^{31}P NMR δ −8.86. Anal. ($C_{19}H_{29}Cl_4N_2O_4P$) C, H, N.

Ethyl 3-Hydroxy-3-(4-nitrophenyl)propionate (8). Ethyl acetate (10.0 mL, 115 mmol) was dissolved in THF (100 mL) and cooled to −78 °C. Lithium bis(trimethylsilyl)amide (115 mL, 1.0 M in THF, 115 mmol) was added and stirred for 10 min at −78 °C. 4-Nitrobenzaldehyde (15.5 g, 115 mmol) was dissolved in THF (100 mL) and added all at once with vigorous stirring. After stirring for 5 min at −78 °C, hydrochloric acid (200 mL, 10% aqueous) was added and the reaction mixture was warmed to room temperature. The reaction mixture was brought to pH 3 with concentrated hydrochloric acid and extracted with ether (3 × 200 mL). The combined organic extracts were dried ($MgSO_4$) and evaporated to give **8** (20.7 g, 81%); mp = 43 °C; R_f = 0.76 (10% acetone:dichloromethane); ^{1}H NMR ($CDCl_3$) δ 8.19 (2H, d, J = 8.6), 7.57 (2H, d, J = 8.6), 5.25 (1H, m), 4.21 (2H, q, J = 7.1), 3.68 (1H, d, J = 2.7), 2.74 (2H, dd, 8.1, 4.5), 1.27 (3H, t, J = 7.1). Anal. ($C_{11}H_{13}NO_5$) C, H, N.

2-(*N,N*-Dimethylamino)ethyl 3-Hydroxy-3-(4-nitrophenyl)propionate (9). Lithium bis(trimethylsilyl)amide (100 mL, 1.0 M in THF, 100 mmol) was cooled to −78 °C. 2-(*N,N*-Dimethylamino)ethyl acetate (15.0 mL, 106 mmol) was added dropwise over about 10 min and stirred 10 min at −78 °C. 4-Nitrobenzaldehyde (15.2 g, 100 mmol) was dissolved in THF (60 mL) and added all at once. After stirring for 5 min, hydrochloric acid (125 mL, 10% aqueous) was added and the reaction mixture was allowed to warm to room temperature. Brine (100 mL) and ether (200 mL) were added. The mixture was acidified with concentrated hydrochloric acid, and the phases were separated. The aqueous phase was made basic with potassium carbonate and extracted with dichloromethane. The organic phase was dried, stirred with activated carbon, filtered, and evaporated to give **9** (17.59 g, 62%): mp = 77 °C; R_f = 0.28 (33% methanol:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 8.23 (2H, d, J = 8.6), 7.61 (2H, d, J = 8.6), 5.24 (1H, dd, J = 10.9, 5.1), 4.53 (1H, m), 4.26 (1H, m), 2.81 (1H, dd, J = 10.9, 2.1), 2.62 (3H, m), 2.33 (6H, s). Anal. ($C_{13}H_{18}N_2O_5$) C, H, N.

3-(*N,N*-Dimethylamino)propyl 3-Hydroxy-3-(4-nitrophenyl)propionate (10). Lithium bis(trimethylsilyl)amide (34 mL, 1.0 M in THF, 34 mmol) was cooled to −78 °C. 3-(*N,N*-Dimethylamino)propyl acetate (5.0 g, 34 mmol) was added dropwise over 5 min and stirred for 10 min at −78 °C. 4-Nitrobenzaldehyde (5.2 g, 34 mmol) was dissolved in a minimum of THF and added all at once. After stirring for 1 min at −78 °C, hydrochloric acid (100 mL, 10% aqueous) was added and the reaction mixture warmed to room temperature. The acidic reaction mixture was extracted with ethyl acetate (100 mL) and the aqueous phase was made basic with sodium carbonate. The resulting mixture was extracted with ethyl acetate (3 × 100 mL). The combined organic extracts were dried ($MgSO_4$) and evaporated to give **10** (6.6 g, 65%): mp = 80 °C; R_f = 0.14 (33% methanol:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 8.23 (2H, d, J = 8.6), 7.62 (2H, d, J = 8.6), 5.26 (1H, dd, J = 10.9, 5.1), 4.30 (2H, m), 2.72 (4H, m), 2.42 (6H, s), 1.98 (2H, m). Anal. ($C_{14}H_{20}N_2O_5$) C, H, N.

***In Vitro* Hypoxia-Selectivity Assay.** HT-29 human colon carcinoma cells were cultured in α-MEM supplemented with 10% FBS and antibiotics as previously described.[12] Cytotoxicity was assayed in duplicate under both aerobic and hypoxic conditions. For aerobic exposures, HT-29 cells in exponential growth were suspended in 10 mL of α-MEM at all cell density of 1–2 × 10^5 cells/mL and transferred to glass treatment vials. For hypoxic exposures HT-29 cells in α-MEM at a concentration of 1–2 × 10^7 cells/mL were incubated for 10 min at 37 °C in a Hamilton gas-tight syringe to deplete oxygen and then injected into 10 mL of α-MEM degassed for 3 h immediately prior to use with a 97% N_2/3% CO_2 gas mixture. The drug was dissolved in DMSO and diluted 100-fold with α-MEM to prepare a stock solution. Controls were treated with 1% DMSO in α-MEM. The treatment vials were incubated for 4 h while continually gassing them with 97% air/3% CO_2 or 97% N_2/3% CO_2 for aerobic and hypoxic exposures, respectively. Aerobic and hypoxic exposures were performed simultaneously for each compound assayed using the same drug stock solution. Following incubation, the cell suspensions were centrifuged, the drug-containing medium decanted, and the cells washed with drug-free medium. Appropriate dilutions from each treatment group were plated in 60-mm culture dishes and incubated for 14 days. The dishes were washed and stained and all colonies containing >50 cells counted. Cell survival was calculated as the ratio of the colonies formed in the drug-treated groups versus untreated controls. Duplicate values are generally within ±10%.

***Ex Vivo* GM-CFC Assay.** This assay was carried out essentially as described by Kohn and Sladek.[13] Briefly, bone marrow cells were flushed from the femurs and tibias of two BDF_1 mice using drug exposure medium (1 mL) and a 27-gauge needle. The cells were pooled, and the concentration of viable cells was adjusted to 2 × 10^6/mL. Cells were exposed to drug for 2 h at a cell density of 4 × 10^5 cells/mL (5 mL total volume). Immediately following drug exposure, cells were harvested by centrifugation (800*g*, 5 min). Cells were resuspended in drug free α-MEM, and the viable nucleated cells were counted. The GM-CFC assay was carried out by a modification of the method of Worton *et al.*[14] as described previously.[15] Control groups produced 225 ± 10 (SE) colonies/10^5 nucleated cells plated.

***In Vitro* Spheroid Studies.** HT-29 spheroids were initiated by seeding 5 × 10^4 cells into a 60-mm nonadherent culture dish and incubating for 5 days under an atmosphere containing 3% CO_2.[16] Following incubation the cell aggregates were transferred to spinner flasks in α-MEM supplemented with 10% FBS and 25 mM glucose. Spheroids of 600–800 μM diameter were manually selected for experiments with the aid of a calibrated microscope reticle. Spheroids were incubated with drug for 4 h followed by a 20-min exposure to a 2 mM solution of Hoescht

33342 dye. The spheroids were dissociated into single-cell suspensions in fresh medium. Fluorescence intensity is inversely proportional to the depth into the spheroid,[9] so the cells were sorted into fractions based on the fluorescence intensity using a Becton-Dickinson FACStar flow cytometer equipped with a 6-W argon-ion laser emitting 50 mW at a wavelength of 351–365 nm. The corresponding depth of each fraction from the surface of the spheroid was calculated according to Durand.[17] Each fraction was plated and clonogenic survival determined as described above.

DNA Cross-Linking Studies. DNA interstrand cross-linking was determined by the alkaline elution method.[18] HT-29 cells were prepared for drug treatment by incubation with [^{14}C]-thymidine (0.02 μCi/mL) for 48 h followed by a 24-h incubation in the absence of [^{14}C]thymidine. Reference cells were similarly prepared by incubation with [^{3}H]thymidine. [^{14}C]-Labelled cells were treated with drug for 2 h. Both cell groups were combined 16–20 h after drug treatment and irradiated at 0 °C (3.0 Gy, 250 kVp) at a dose rate of 1.78 Gy/min. The cells were then lysed on polycarbonate filters with a solution containing sodium dodecyl sulfate (1%) and EDTA (20 mM) at pH 10, treated 1 h with proteinase K (0.5 mg/mL), and eluted from the filter with 2% tetrapropylammonium hydroxide containing EDTA (20 mM) and 0.1% sodium dodecyl sulfate at pH 12.1. Fractions (6 mL) were collected hourly for 6 h.

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