Aryl Bis(diazeniumdiolates): Potent Inducers of *S*-Glutathionylation of Cellular Proteins and Their in Vitro Antiproliferative Activities

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A number of bis(diazeniumdiolates) that we designed to release up to 4 mol of nitric oxide (NO) and that are structural analogues of the NO prodrug and anticancer lead compound O^2-{2,4-dinitro-5-[4-(*N*-methylamino)benzoyloxy]phenyl} 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2- diolate (PABA/NO) were synthesized and studied. A majority of these compounds yielded higher levels of NO, were better inhibitors of proliferation of a number of cancer cell lines, and more rapidly induced substantially increased levels of *S*-glutathionylation of cellular proteins in comparison with PABA/NO. In most cases, the antiproliferative activity and extents of *S*-glutathionylation correlated well with levels of intracellular NO release. We report bis(diazeniumdiolates) to be a class of *S*-glutathionylating agents with potent antiproliferative and *S*-glutathionylating activity.

Introduction

Nitric oxide (NO[a]) is intimately involved in mediating a plethora of biological processes.[1−6] Numerous studies indicate that nitric oxide is also a promising therapeutic agent for the treatment of several physiological conditions and disease states, including cancer, culminating in the pursuit of nitric oxide prodrugs as potential drug candidates.[7−11] The wide-ranging physiological involvement and applicability of NO offer several opportunities for drug discovery but also present enormous challenges, most notably among these the delivery of nitric oxide selectively to the desired physiological site in order to minimize any potential side effects.

Diazeniumdiolate anions are prodrugs of nitric oxide that are widely used as reliable sources of NO in a number of chemical and biological studies.[12−16] Our laboratory has developed a number of promising diazeniumdiolate-based cancer drug candidates[17−22] such as O^2-{2,4-dinitro-5-[4-(*N*-methylamino)benzoyloxy]phenyl} 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate, PABA/NO, which has shown high potency as an antiproliferative agent.[23−25] PABA/NO was shown to have anticancer activity comparable to that of cisplatin in human ovarian cancer xenograft studies in a rodent model.[24] PABA/NO and analogous members of the arylated diazeniumdiolate class of prodrugs were conceived as substrates for glutathione *S*-transferase (GST),[26−28] a phase II detoxification enzyme that is frequently overexpressed by cancer cells. Attack by glutathione (GSH) on the 2,4-dinitroaryl ring results in the release of a diazeniumdiolate anion, which dissociates to form NO (Scheme 1).[23] This process was shown to be catalyzed by GST and thus presents one plausible mechanism for selective generation of potentially cytotoxic nitric oxide in tumors.

Addition of glutathione to the cysteine residues in proteins (*S*-glutathionylation) is a post-translational modification that alters protein structure and function.[29−34] A variety of chemical pathways involving thiol oxidation or nitrosation have been proposed to account mechanistically for protein *S*-glutathionylation.[35] Glutathionylation can serve as a protective mechanism against cysteine oxidation in proteins and is recognized as an important component of cellular redox signaling. Altered *S*-glutathionylation levels are observed in several pathological conditions such as melanoma, diabetes mellitus, HIV infection, and ischemia.[30] *S*-Glutathionylation of several proteins has been reported; notable among these are PTP1B, caspase-3, HIV protease, p38, ERK, and JNK. In addition to activation of stress kinases such as p38 and JNK, PABA/NO (10 μM) also induced rapid (<5 min) *S*-glutathionylation of ATP synthase, β-lactate dehydrogenase, protein disulfide isomerase, actin, and glucosidase-II.[24,25]

Recently, we reported the synthesis and study of a number of PABA/NO structural analogues.[36] Abundant NO was released from most of these prodrugs when treated with GSH. Furthermore, it was observed that analogues that released higher amounts of NO had superior antiproliferative activity while analogues that formed negligible amounts of NO under these conditions were inactive against human leukemia HL-60 cells, suggesting that nitric oxide is a component of the cytotoxicity of such prodrugs. Hence, it was envisaged that any further increase of the levels of nitric oxide may result in prodrugs that have superior cytotoxic effects in comparison with PABA/NO. Furthermore, nitrosative stress caused by NO and its redox forms is also proposed to mediate protein *S*-glutathionylation by PABA/NO (Scheme 1), suggesting that increased nitric oxide levels in comparison with PABA/NO should also lead to higher extents of *S*-glutathionylation.[24,25]

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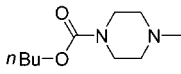[§] These authors contributed equally to this work.
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[a] Abbreviations: NO, nitric oxide; PABA/NO, O^2-{2,4-dinitro-5-[4-(*N*-methylamino)benzoyloxy]phenyl} 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate; GST, glutathione *S*-transferase; GSH, glutathione; DFM-FM diacetate, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DMSO, dimethyl sulfoxide; DEA/NO, sodium 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate; DMA/NO, sodium 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate; PROLI/NO, disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate; HBSS, Hanks's balanced salt solution; TBS-T, Tris-buffered saline−Tween-20; PBS, phosphate buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
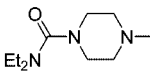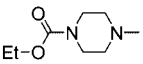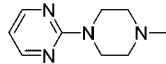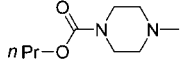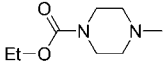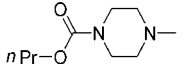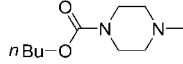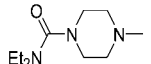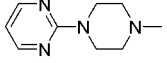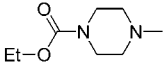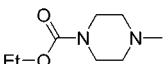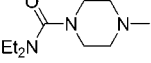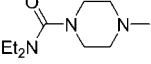
Scheme 1. Mechanism of Nitric Oxide Release from PABA/NO, an O^2-Arylated Diazeniumdiolate That Is Designed To Be Activated by Reaction with Glutathione[23,a]

[a] ○−SH is a representative protein that can be *S*-glutathionylated, and ○−S-SG is a representative *S*-glutathionylated protein.

Scheme 2. Design of Bis(diazeniumdiolate) Prodrugs That Release Elevated Amounts of NO

One strategy to increase the amount of NO released is to attach an additional diazeniumdiolate group to the aromatic ring; attack by two GSHs would result in the formation of up to 4 mol of NO per mole of prodrug (Scheme 2). On the basis of this design, we report the synthesis, glutathione-mediated nitric oxide release, *S*-glutathionylating ability, and in vitro antiproliferative activity of a library of nitric oxide prodrugs possessing two diazeniumdiolate groups.

Results

Synthesis. Earlier, we reported the synthesis of PABA/NO analogues from the sequential addition of a diazeniumdiolate ion and then 4-aminobenzoic acid derivatives to 1,5-difluoro-2,4-dinitrobenzene.[23] Using a similar strategy, we prepared the fluorides **3a**, **3b**, and **3c** (Scheme 3). Treatment of **3a** with a number of diazeniumdiolate anions (**2b−k**, Table 1) afforded **4a−j** in excellent yields (Table 2). By use of a similar procedure, compounds **5a**, **5c**, and **5d** were prepared from **3b**, again in excellent yields (Table 2). Compound **5b** was synthesized from **3c** in high yield (Table 2).

Finally, the diazeniumdiolate salt **2k** was used to prepare compound **6** and a prodrug form of 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO), **7** (Scheme 4).

We assume that compound **4g** is racemic, as the supplier did not comment on its stereochemistry. Compounds **6**, **5d**, **4i**, and **7** are assumed to be optically active and to have the configuration of the amino acid proline from which they were all ultimately prepared. The NMR spectra of **6** and **7** were those of single compounds, with no evidence of a second diastereomer that would be anticipated if significant racemization had occurred.

Scheme 3. Synthesis of Bis(diazeniumdiolate) Prodrugs

Glutathione-Activated Nitric Oxide Release. The amount of nitric oxide released from these compounds upon treatment with glutathione was determined (Table 3). PABA/NO released 1.4 mol of nitric oxide per mole under these conditions. A majority of these bis(diazeniumdiolate) prodrugs released at least twice as much NO as PABA/NO. Excellent yields (≥90%) of nitric oxide from several compounds such as **4b**, **4f**, **4h**, and **4j** were observed.

Antiproliferative Activity. Since PABA/NO has been shown to exhibit cytostatic activity against the leukemia HL-60 cell line,[23] this line was used as an initial screen for the antiproliferative activity of bis(diazeniumdiolates). All the PABA/NO analogues prepared in this work, except **7**, were found to possess excellent antiproliferative activity, with IC$_{50}$ values ranging from 0.9 to 4.0 μM (mean 1.7 μM) and in most cases better than that of PABA/NO (Table 3).

Three analogues, **4a**, **4i**, and **5b**, were then further tested for their antiproliferative activity against a number of other human cancer cell lines. Leukemia U-937, non-small-cell lung cancer H441 and A549, colon cancer HCT-116, HCT-15, and HT-29, ovarian cancer OVCAR-3 and OVCAR-5, and prostate cancer PC-3 cell lines were used for this study. Again, the compounds

Table 1. Diazeniumdiolate Salts (R_2N-$N_2O_2^-Na^+$) Used in the Synthesis of Aryl Bis(diazeniumdiolates)

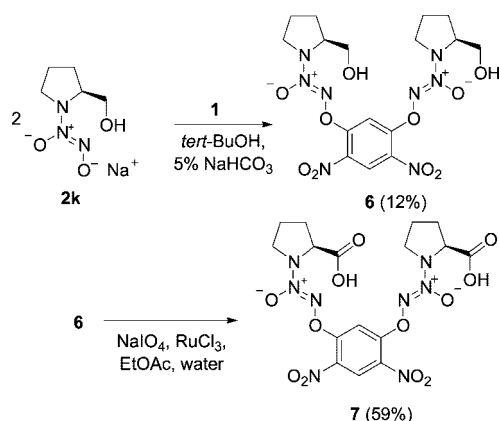| R_2N	compd	R_2N	compd	R_2N	compd
Me₂N—	2a	nBu-O-C(O)-N(piperazine)N—	2e	(2-Me-piperidine)N—	2i
Et₂N—	2b	Et₂N-C(O)-N(piperazine)N—	2f	(pyrrolidine)N—	2j
Et-O-C(O)-N(piperazine)N—	2c	(pyrimidin-2-yl)-N(piperazine)N—	2g	(2-CH₂OH-pyrrolidine)N—	2k
nPr-O-C(O)-N(piperazine)N—	2d	(piperidine)N—	2h		

Table 2. Bis(diazeniumdioalate) Prodrugs **4a−5d**

compd	R_2N substituent	R'_2N substituent	precursors	yield (%)
4a	Me₂N—	Et-O-C(O)-N(piperazine)N—	3a, 2c	84
4b	Me₂N—	nPr-O-C(O)-N(piperazine)N—	3a, 2d	95
4c	Me₂N—	nBu-O-C(O)-N(piperazine)N—	3a, 2e	93
4d	Me₂N—	Et₂N-C(O)-N(piperazine)N—	3a, 2f	94
4e	Me₂N—	(pyrimidin-2-yl)-N(piperazine)N—	3a, 2g	85
4f	Me₂N—	(piperidine)N—	3a, 2h	90
4g	Me₂N—	(2-Me-piperidine)N—	3a, 2i	81
4h	Me₂N—	(pyrrolidine)N—	3a, 2j	79
4i	Me₂N—	(2-CH₂OH-pyrrolidine)N—	3a, 2k	72
4j	Et₂N—	Me₂N—	3a, 2b	91
5a	Et₂N—	Et-O-C(O)-N(piperazine)N—	3b, 2c	77
5b	Et-O-C(O)-N(piperazine)N—	Et₂N-C(O)-N(piperazine)N—	3c, 2f	81
5c	Et₂N—	Et₂N-C(O)-N(piperazine)N—	3b, 2f	90
5d	Et₂N—	(2-CH₂OH-pyrrolidine)N—	3b, 2k	87

4a, **4i**, and **5b** were found to be superior inhibitors of cell proliferation and generally showed greater antiproliferative activity in comparison with that of PABA/NO (Table 4), with the leukemia cell line appearing to be the most sensitive.

Protein *S*-Glutathionylation. All but one of the bis(diazeniumdiolate) prodrugs induced rapid protein *S*-glutathionylation in human leukemia HL-60 cells (Figure 1). Treatment with compound **7** did not result in protein *S*-glutathionylation. The
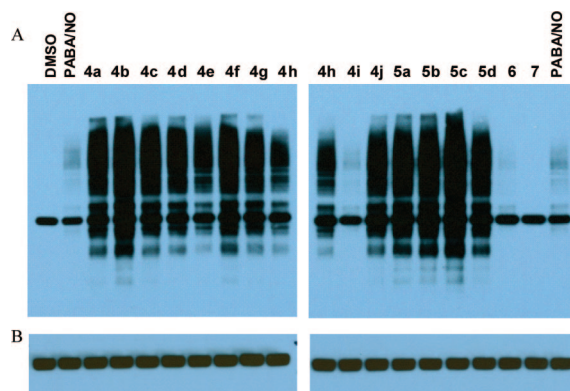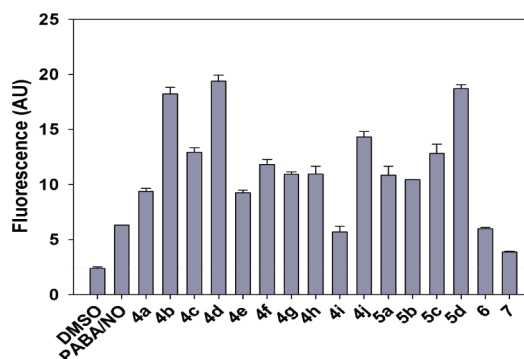
Scheme 4. Synthesis of Bis(diazeniumdiolate) Prodrug Forms **6** and **7**

Figure 1. (A) Equal numbers of HL-60 cells were treated with 5 μM PABA/NO, with the bis(diazeniumdiolate) prodrugs, or with the vehicle DMSO. Ten micrograms of cellular protein lysates was separated by SDS−PAGE under nonreducing conditions and immunoblotted using antiglutathione antibodies. (B) The immunoblots shown in (A) were stripped and reprobed with β-actin antibodies as a loading control. The band visible in the DMSO control in (A) is β-actin, which has been shown to be spontaneously glutathionylated at physiological conditions.[37,38]

Table 3. Glutathione-Activated Nitric Oxide Release from Bis(diazeniumdiolate) Prodrugs and PABA/NO and Their in Vitro Antiproliferative Activity in the HL-60 Human Leukemia Cell Line

compd	moles of NO released	IC$_{50}$ (μM)^a	TGI (μM)^b
PABA/NO	1.4	3.9	>10
4a	2.7	1.2	2.0
4b	3.7	1.6	3.9
4c	3.1	0.9	2.7
4d	3.5	1.9	4.9
4e	3.5	1.2	5.0
4f	3.7	1.7	4.9
4g	3.0	1.5	4.9
4h	3.6	2.3	4.9
4i	3.0	1.0	2.0
4j	3.8	1.0	2.3
5a	3.1	2.1	5.0
5b	2.9	0.9	3.0
5c	3.0	2.9	>10
5d	3.0	1.8	4.0
6	2.5	4.0	>10
7	3.5	9.6	>10

^{a} IC$_{50}$ = concentration required to inhibit cell proliferation by 50%. ^{b} TGI = concentration providing total growth inhibition.

Figure 2. Intracellular levels of NO measured as DAF-NO derivative fluorescence intensities in arbitrary units (AU). Error bars represent standard deviations of three independent experiments carried out with the final concentration of the compound being 5 μM.

levels of *S*-glutathionylation for two compounds (**4i** and **6**) were comparable to those of PABA/NO. The 13 remaining compounds caused massive glutathionylation of intracellular proteins, much more intense than the levels resulting from PABA/NO.

Intracellular Nitric Oxide Release. The intracellular level of nitric oxide after bis(diazeniumdiolate) treatment was quantified by measuring the extent of reaction with the nitric oxide-sensitive fluorophore, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate). HL-60 cells were preloaded with DAF-FM diacetate, followed by treatment with the bis(diazeniumdiolate) prodrugs or the solvent (DMSO) control or PABA/NO. Most bis(diazeniumdiolate) analogues of

PABA/NO were found to produce significantly higher levels of fluorescence, indicating greater amounts of intracellular nitric oxide than that produced by PABA/NO (Figure 2). However, analogues **4i**, **6**, and **7** formed intracellular levels of NO comparable to or lower than those of PABA/NO. The amounts of NO released intracellularly did not correlate significantly with those obtained upon reaction with glutathione in simple phosphate buffer ($P = 0.08$). Lack of consistency between the buffered GSH solution and intracellular NO release measurements may reflect differences in cell permeability of individual compounds.

Table 4. Antiproliferative Activity of **4a**, **4i**, **5b**, and PABA/NO against Selected Cancer Cell Lines

cell line	**4a**		**4i**		**5b**		PABA/NO	
	IC$_{50}$ (μM)	TGI (μM)	IC$_{50}$ (μM)	TGI (μM)	IC$_{50}$ (μM)	TGI (μM)	IC$_{50}$ (μM)	TGI (μM)
U-937	1.3	3.0	0.8	1.2	0.9	3.0	2.7	5.5
H441	5.0	7.0	2.1	4.5	1.7	4.0	4.9	>10
A549	9.1	>30	5.1	6.0	3.9	10	>10	>10
HCT-116	2.4	4.0	1.6	3.6	1.5	4.0	5.5	>10
HCT-15	2.5	4.0	1.7	3.3	1.6	4.5	5.7	>10
HT-29	4.5	10	2.4	4.5	2.5	6.5	7.0	>10
OVCAR-3	1.3	2.3	1.7	3.8	1.4	4.0	5.0	9.5
OVCAR-5	3.3	9.0	2.5	4.0	1.9	5.5	7.8	>10
PC-3	5.0	7.0	3.3	7.0	3.3	8.0	>10	>10
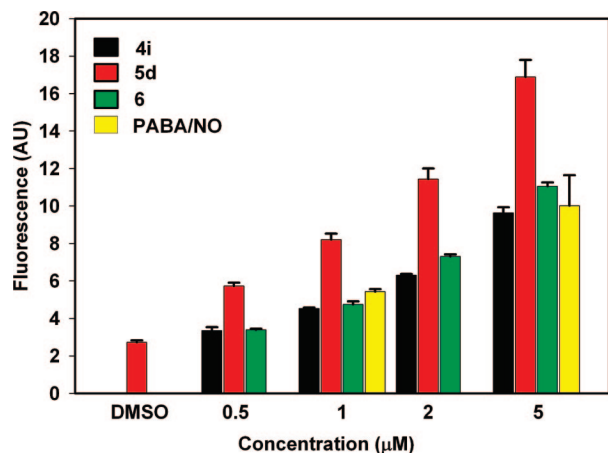
Figure 3. Intracellular levels of NO measured as DAF-NO derivative fluorescence intensities in arbitrary units (AU). Error bars represent standard deviations of three independent experiments.

Scheme 5. Proposed Mechanism of Nitric Oxide Release from Bis(diazeniumdiolate) Prodrugs
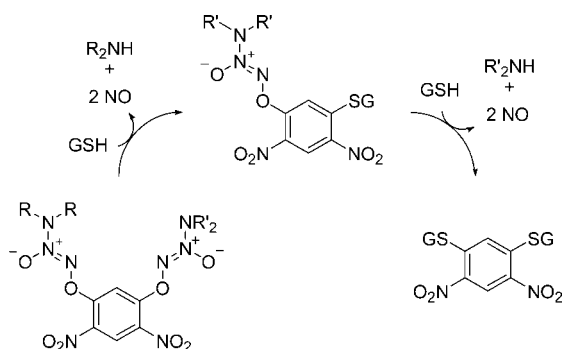
We were curious about the structure–property relationships among **4i**, **5d**, and **6**. All three bear a prolinol diazeniumdiolate substitutent at one position of the dinitrophenyl ring, the difference being that the other substituent is the diazeniumdiolate of dimethylamine (**4i**), diethylamine (**5d**), or a second prolinol (**6**). All three had low IC$_{50}$ values in the HL-60 cell screen (1.0, 1.8, and 4.0 μM, respectively, Table 3), but the diethylamine derivative was far more active than the other two as an inducer of *S*-glutathionylation (Figure 1) and intracellular release of active NO (Figure 2). While we were confident that these were reproducible observations, a referee's question led us to run an additional experiment. In this case, the three compounds were compared with PABA/NO at four different concentrations ranging from 0.5 to 5 μM. The results, summarized in Figure 3, once again showed that **5d** was substantially more active than the other three. The mechanistic origins of this difference remain to be determined.

Discussion

The results show that adding a second diazeniumdiolate group to the aromatic ring of previously reported arylated diazeniumdiolate prodrugs generally increased the amount of NO released (Scheme 5) on reaction with glutathione in neutral buffer solution relative to the mono(diazeniumdiolated) analogue PABA/NO, as expected, providing significantly higher antiproliferative activity than PABA/NO across a diverse panel of tumor cell lines.

Within the group of bis(diazeniumdiolates) reported here, however, no significant correlation between IC$_{50}$ values and

amounts of nitric oxide released upon treatment with GSH was derived. For example, the IC$_{50}$ value of **4j**, which released 3.8 mol of NO, was 1.0 μM while **4i** showed a similar inhibitory effect but released only 3.0 mol of NO under similar conditions. While the analogue **6** showed good antiproliferative activity against human leukemia HL-60 cells, the inhibitory activity of the PROLI/NO analogue **7** was diminished in comparison with all other prodrugs, although it formed 3.5 mol of NO when treated with GSH. This observation is consistent with our earlier finding that the 2,4-dinitrophenyl prodrug form of PROLI/NO did not significantly inhibit proliferation of HL-60 cells even at 50 μM.[39]

The total growth inhibition (TGI) index for several of these analogues was found to be less than 5 μM. Also, when one of the diazeniumdiolate groups was diazeniumdiolated dimethylamine **2a**, the size and nature of the substituent at the 5-position of the aromatic ring did not appear to play any significant role in the antiproliferative activity (IC$_{50}$ values of **4a**–**j** and **5a** ranged from 0.9 to 2.3 μM; TGIs were 2–5 μM).

Intracellular NO release correlated well with the antiproliferative activities and *S*-glutathionylating abilities, however. For example, from the intracellular NO release study with DAF-FM diacetate, compound **7**, which had the lowest levels of fluorescence among the analogues, showed both diminished antileukemic activity and lowered *S*-glutathionylation levels. All compounds with superior TGIs (≤ 5 μM), with the exception of **4i**, also formed greater intracellular levels of NO in comparison with PABA/NO (TGI > 10 μM). The intracellular NO release from **4i** was comparable with that of the prolinol analogue **6**, but both these analogues had diminished intracellular NO-releasing capacities in comparison with **5d**. This observation was consistent over the concentration range 0.5–5 μM (Figure 3). At this time, we are unable to provide a rationale to account for these observations. However, the levels of intracellular nitric oxide formed from the prolinol analogues **4i**, **5d**, and **6** correlated well with their *S*-glutathionylating abilities, suggesting that cell permeability is critical to the observed biological effects of this series of compounds.

Proliferation of the lung cancer cell line A549, which was resistant to PABA/NO, was significantly inhibited by **4i** and **5b**, whose IC$_{50}$ values were 5.1 and 3.9 μM, respectively, and moderately inhibited by **4a** (IC$_{50}$ = 9.1 μM). Compound **4i** was the most consistent inhibitor of proliferation in the range of cancer cell lines tested, and in all cases, the TGI was less than 10 μM. Intracellular NO release and *S*-glutathionylation by **4i** were at levels comparable to that of PABA/NO, showing that factors other than nitric oxide generation contribute to antiproliferative activity.

Conclusions

A number of bis(diazeniumdiolated) dinitrobenzene derivatives that generate up to 4 mol of NO per mole of prodrug were synthesized and found to have excellent protein *S*-glutathionylating ability in human leukemia HL-60 cells. The antiproliferative activities of a majority of these prodrugs were superior to those of PABA/NO, suggesting them as an improved class of antitumor agents with potent ability to induce *S*-glutathionylation of proteins in cells treated with them.

Experimental Section

NO was purchased from Matheson Gas Products (Montgomeryville, PA). Other starting materials were purchased from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise indicated. NMR spectra were recorded on a Varian UNITY INOVA spectrometer;

chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane. Unless otherwise specified, the solvent used in all NMR experiments was CDCl$_3$. Quantification of NO after treatment of compounds with glutathione (GSH) was carried out by chemiluminescence with a thermal energy analyzer model 502A instrument (Thermedics, Inc., Woburn, MA) or a Sievers 280i nitric oxide analyzer (Boulder, CO). Ultraviolet (UV) spectra were recorded on an Agilent model 8453 or a Hewlett-Packard model 8451A diode array spectrophotometer. Elemental analyses were performed by Midwest Microlab (Indianapolis, IN). Compounds **2a**,[40] **2b**,[41] **2c**,[42] **2d–e**,[20] **2g**,[42] **2h**,[41] **2i**,[43] **2j**,[44] **2k**,[45] **3a**,[20] and **3c**[20] were prepared using reported methods.

Sodium 1-(4-*N*,*N*-Diethylcarbamoylpiperazin-1-yl)diazen-1-ium-1,2-diolate (2f). Compound **2f** was prepared according to ref 18, but the procedure reported for it there was incorrectly labeled as that of "JS-K" in the Materials and Methods section of that paper. The correct procedure for **2f** is as follows (summarized from ref 18). A solution of 5.3 g (0.029 mol) of *N*,*N*-diethylcarbamoylpiperazine in 15 mL of methanol was placed in a Parr bottle and treated with 6.7 mL (0.031 mol) of methanolic sodium methoxide. The solution was purged with 40 psi of NO and stirred at 25 °C for 72 h. The solid precipitate was filtered, washed with ether, and dried to give 4.54 g (60%) of diazeniumdiolate salt: UV (0.1 M NaOH) λ_{max} (ε) 250 nm (9.8 mM^{-1} cm^{-1}); ^{1}H NMR (0.1 M NaOD in D$_2$O) δ 1.13 (t, J = 7.1 Hz, 6H), 3.13–3.16 (m, 4H), 3.27 (q, J = 7.1 Hz, 4H), 3.43–3.47 (m, 4H); ^{13}C NMR (0.1 M NaOD in D$_2$O) δ 29.7, 33.2, 36.5, 39.0 152.7.

***O*^{2}-(2,4-Dinitro-5-fluorophenyl) 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (3b).** A partial solution of 2 g (9.8 mmol) of 1,5-difluoro-2,4-dinitrobenzene in 25 mL of *tert*-butyl alcohol was placed in a round-bottom flask and stirred at room temperature. A solution of 0.70 g (4.4 mmol) of **2b** in 15 mL of 5% sodium bicarbonate was added dropwise at a rapid rate. The reaction mixture was stirred for 30 min, and a thick yellow solid was formed. Water was added, and the product was collected by filtration and recrystallized from methanol to give **3b** (2.2 g, 70%): mp 59–60 °C; UV (ethanol) λ_{max} (ε) 254 nm (12 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.26 (t, J = 7.1 Hz, 6H), 3.65 (q, J = 7.1 Hz, 4H), 7.44 (d, J = 11.7 Hz, 1H), 8.90 (d, J = 7.5 Hz, 1H); ^{13}C NMR δ 11.40, 46.52, 106.31 (d, $^2J_{C-F}$ = 27.6 Hz), 130.71, 132.56, 155.19, 157.29, 160.01. Anal. (C$_{10}$H$_{12}$FN$_5$O$_6$) C, H, F, N.

General Procedure for the Synthesis of Bis(diazeniumdiolates). A slurry of diazeniumdiolate salt (1.3 mmol) in 10 mL of acetone is added slowly to a solution of 1-diazeniumdiolated 5-fluoro-2,4-dinitrobenzene (1 mmol) in 10 mL of acetone that was previously cooled to 0 °C under nitrogen. The reaction mixture is allowed to warm gradually to room temperature. The progress of reaction is monitored by TLC. The product is extracted with dichloromethane and washed with 5% sodium bicarbonate solution. The organic layer is dried over anhydrous sodium sulfate, filtered, and evaporated under vacuum to give the crude product. Purification is carried out by recrystallization or flash chromatography.

1-[1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-{[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diol-2-ato}benzene (4a). A solution of **2c** (282 mg, 1.3 mmol) in 10 mL of acetone was added to a solution of **3a** (289 mg, 1 mmol) in 10 mL of acetone. Purification of the crude material was carried out on silica gel with 7:3 ethyl acetate/hexane as the eluant. The product **4a** was obtained as an oil (410 mg, 84%): UV (ethanol) λ_{max} (ε) 290 nm (22 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.29 (t, J = 7.1 Hz, 3H), 3.28 (s, 6H), 3.61–3.64 (m, 4H), 3.71–3.74 (m, 4H), 4.18 (q, J = 7.1 Hz, 2H), 7.55 (s, 1H), 8.83 (s, 1H); ^{13}C NMR δ 14.6, 41.8, 50.5, 62.1, 105.3, 125.5, 131.8, 131.9, 153.8, 154.2, 155.0. Anal. (C$_{15}$H$_{21}$N$_9$O$_{10}$) C, H, N.

1-[1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-{[(4-*n*-propoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diol-2-ato}benzene (4b). The product **4b** (478 mg, 95%) was obtained from **2d** (254 mg, 1.00 mmol) and **3a** (250 mg, 0.86 mmol). Purification of the crude material was carried out on silica gel with 10:1 dichloromethane/acetone as the eluant: mp 111–112 °C; UV (ethanol) λ_{max} (ε) 290 nm (24 mM^{-1} cm^{-1}); ^{1}H NMR δ

0.97 (t, J = 7.2 Hz, 3H), 1.64–1.73 (m, 2H), 3.28 (s, 6H), 3.62–3.65 (m, 4H), 3.72–3.75 (m, 4H), 4.08 (t, J = 6.8 Hz, 2H), 7.54 (s, 1H), 8.82 (s, 1H); ^{13}C NMR δ 10.2, 22.1, 41.6, 42.0, 50.3, 67.5, 104.7, 125.4, 131.2, 131.4, 153.8, 154.2, 155.0. Anal. (C$_{16}$H$_{23}$N$_9$O$_{10}$) C, H. N: calcd 25.14; found 24.62.

1-[1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-{[(4-*n*-butoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diol-2-ato}benzene (4c). A solution of **2e** (270 mg, 1.00 mmol) in 10 mL of acetone was added to a solution of **3a** (250 mg, 0.86 mmol) in 10 mL of acetone. Purification of the crude material was carried out by recrystallization from ethanol to give **4c** (480 mg, 93%): mp 113–114 °C; UV (ethanol) λ_{max} (ε) 290 nm (24 mM^{-1} cm^{-1}); ^{1}H NMR δ 0.95 (t, J = 7.3 Hz, 3H), 1.35–1.44 (m, 2H), 1.61–1.68 (m, 2H), 3.28 (s, 6H), 3.61–3.63 (m, 4H), 3.71–3.73 (m, 4H), 4.12 (t, J = 6.7 Hz, 2H), 7.55 (s, 1H), 8.83 (s, 1H); ^{13}C NMR δ 13.7, 19.1, 30.9, 41.6, 42.1, 50.3, 65.9, 104.9, 125.4, 131.4, 131.6, 153.8, 154.2, 155.0. Anal. (C$_{17}$H$_{25}$N$_9$O$_{10}$) C, H. N: calcd 24.46; found 24.01.

1-[1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-{[4-(*N*,*N*-diethylaminocarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diol-2-ato}benzene (4d). A solution of **2f** (255 mg, 1.00 mmol) in 10 mL of acetone was added to a solution of **3a** (250 mg, 0.86 mmol) in 10 mL of acetone. Purification of the crude material was carried out on silica gel with 7:3 ethyl acetate/hexane as the eluant. The product **4d** was obtained as a pale-yellow solid (487 mg, 94%): mp 133–134 °C; UV (ethanol) λ_{max} (ε) 289 nm (25 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.15 (t, J = 7.1 Hz, 6H), 3.23–3.30 (m, 10H), 3.42 (m, J = 5.1 Hz, 4H), 3.63–3.68 (m, 4H), 7.57 (s, 1H), 8.85 (s, 1H); ^{13}C NMR δ 13.2, 41.7, 41.8, 45.6, 50.2, 105.1, 125.5, 131.5, 131.7, 153.8, 154.0, 154.3, 163.6. Anal. (C$_{17}$H$_{26}$N$_{10}$O$_9$) C, H, N.

1-[1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-{[4-(pyrimidin-2-yl)piperazin-1-yl]diazen-1-ium-1,2-diol-2-ato}benzene (4e). A solution of **2g** (200 mg, 0.81 mmol) in 8 mL of acetone was added to a solution of **3a** (184 mg, 0.63 mmol) in 8 mL of acetone. Purification of the crude material was carried out by recrystallization from ethanol to give **4e** (420 mg, 85%): mp 139–140 °C; UV (ethanol) λ_{max} (ε) 290 nm (23 mM^{-1} cm^{-1}); ^{1}H NMR δ 3.28 (s, 6H), 3.69–3.72 (m, 4H), 4.07–4.09 (m, 4H), 6.61 (t, J = 4.8 Hz, 1H), 7.59 (s, 1H), 8.35 (d, J = 4.8 Hz, 2H), 8.86 (s, 1H); ^{13}C NMR δ 41.8, 42.3, 50.5, 105.0, 111.1, 125.6, 131.6, 131.7, 154.0, 154.3, 157.8, 160.8. Anal. (C$_{16}$H$_{19}$N$_{11}$O$_8$) C, H, N.

1-[1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-[(piperidin-1-yl)diazen-1-ium-1,2-diol-2-ato]benzene (4f). The product **4f** (375 mg, 90%) was obtained from **2h** (250 mg, 0.86 mmol) and **3a** (190 mg, 1.13 mmol). The crude compound **4f** was purified by recrystallization from ethanol: mp 134–135 °C; UV (ethanol) λ_{max} (ε) 292 nm (26 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.57–1.63 (m, 2H), 1.80 (m, 4H), 3.28 (s, 6H), 3.61–6.64 (m, 4H), 7.59 (s, 1H), 8.86 (s, 1H); ^{13}C NMR δ 23.2, 24.3, 29.7, 41.7, 51.6, 110.0, 125. 5, 131.5, 154.3. Anal. (C$_{13}$H$_{18}$N$_8$O$_8$) C, H, N.

1-[1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-[(2-methylpiperidin-1-yl)diazen-1-ium-1,2-diol-2-ato]benzene (4g). This reaction was carried out as described in the general procedure using **2i** (260 mg, 1.45 mmol) and **3a** (300 mg, 1 mmol). Purification of the crude material was carried out on silica gel with 7:3 ethyl acetate/hexane as the eluant to give **4g** (350 mg, 81%): mp 114–115 °C; UV (ethanol) λ_{max} (ε) 286 nm (22 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.13 (d, J = 6.0 Hz, 3H), 1.44–1.62 (m, 3H), 1.66–1.74 (m, 1H), 1.80 (quintet, J = 6.0 Hz, 2H), 1.87–1.93 (m, 1H), 3.28 (s, 6H), 3.51–3.56 (m, 1H), 3.77–3.85 (m, 1H), 7.68 (s, 1H), 8.85 (s, 1H); ^{13}C NMR δ 16.2, 21.1, 24.9, 31.7, 41.8, 51.2, 55.7, 105.6, 125.4, 131.8, 153.9, 154.3. Anal. (C$_{14}$H$_{20}$N$_8$O$_8$) C, H, N.

1-[1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-[(pyrrolidin-1-yl)diazen-1-ium-1,2-diol-2-ato]benzene (4h). A solution of **2j** (198 mg, 1.3 mmol) in 15 mL of acetone was added to a solution of **3a** (289 mg, 1 mmol) in 10 mL of acetone. Purification of the crude material was carried out on silica gel with 10:1 dichloromethane/acetone as the eluant. The product

4h was obtained as a yellow powder (318 mg, 79%): mp 139–140 °C; UV (ethanol) λ_{max} (ε) 293 nm (22 mM^{-1} cm^{-1}); ^{1}H NMR δ 2.05–2.09 (m, 4H), 3.27 (s, 6H), 3.74–3.78 (m, 4H), 7.58 (s, 1H), 8.86 (s, 1H); ^{13}C NMR δ 23.4, 41.7, 50.5, 104.4, 125.5, 131.1, 131.2, 154.3, 154.7. Anal. ($C_{12}H_{16}N_8O_8$) C, H, N.

1-[1-(N,N-Dimethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-[(2-hydroxymethylpyrrolidin-1-yl)diazen-1-ium-1,2-diol-2-ato]benzene (4i). A solution of **2k** (475 mg, 2.6 mmol) in 15 mL of acetone was mixed with a solution of **3a** (578 mg, 2 mmol) in 10 mL of acetone. Purification of the crude material was carried out on silica gel with 7:3 ethyl acetate/hexane as the eluant. The product **4i** was obtained as an oil (290 mg, 72%): UV (ethanol) λ_{max} (ε) 295 nm (21 mM^{-1} cm^{-1}); ^{1}H NMR δ 2.00–2.20 (m, 5H), 3.28 (s, 6H), 3.71–3.77 (m, 2H), 3.83–3.92 (m, 2H), 4.33–4.37 (m, 1H), 7.58 (s, 1H), 8.86 (s, 1H); ^{13}C NMR δ 23.2, 27.0, 41.7, 52.4, 64.2, 64.5, 104.3, 125.6, 131.0, 131.1, 154.3, 154.4. Anal. ($C_{13}H_{18}N_8O_9$) H, N. C: calcd 36.28; found 35.87.

1-[1-(N,N-Dimethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-[1-(N,N-diethylamino)diazen-1-ium-1,2-diol-2-ato]benzene (4j). The product **4j** (368 mg, 91%) was obtained from **2b** (220 mg, 1.4 mmol) and **3a** (300 mg, 1 mmol). Purification of the crude material was carried out by recrystallization from ethyl acetate/hexane (7:3): mp 130–131 °C; UV (ethanol) λ_{max} (ε) 292 nm (25 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.26 (t, J = 7.0 Hz, 6H), 3.27 (s, 6H), 3.58 (q, J = 7.0 Hz, 4H), 7.59 (s, 1H), 8.88 (s, 1H); ^{13}C NMR δ 11.6, 41.7, 46.9, 104.5, 125.6, 131.2, 131.1, 154.4, 154.5. Anal. ($C_{12}H_{18}N_8O_8$) C, H, N.

1-[1-(N,N-Diethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-{[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diol-2-ato}benzene (5a). A solution of **2c** (390 mg, 1.79 mmol) in 10 mL of acetone was added to a solution of **3b** (480 mg, 1.51 mmol) in 10 mL of acetone. Purification of the crude material was carried out on silica gel with 7:3 ethyl acetate/hexane as the eluant. The product **5a** was obtained as an oil (400 mg, 77%): UV (ethanol) λ_{max} (ε) 291 nm (24 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.27 (t, J = 7.1 Hz, 6H), 1.29 (t, J = 7.2 Hz, 3H), 3.58–3.66 (m, 8H), 3.71–3.75 (m, 4H), 4.18 (q, J = 7.1 Hz, 2H), 7.57 (s, 1H), 8.86 (s, 1H); ^{13}C NMR δ 11.5, 14.5, 42.1, 46.8, 50.4, 62.0, 104.7, 125.5, 131.2, 131.5, 153.9, 154.4, 154.9. Anal. ($C_{17}H_{25}N_9O_{10}$) C, H, N.

1-[4-(Ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-{[4-(N,N-diethylaminocarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diol-2-ato}benzene (5b). A solution of **3c** (172 mg, 0.42 mmol) in 10 mL of acetone was added to a solution of **2f** (154 mg, 0.60 mmol) in 10 mL of acetone. Purification of the crude material was carried out by recrystallization from ethanol to give **5b** (215 mg, 81%): mp 94–95 °C; UV (ethanol) λ_{max} (ε) 288 nm (26 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.16 (t, J = 6.8 Hz, 6H), 1.29 (t, J = 7.0 Hz, 3H), 3.26 (q, J = 7.0 Hz, 4H), 3.42–3.45 (m, 4H), 3.60–3.68 (m, 8H), 3.72–3.74 (m, 4H), 4.19 (q, J = 6.8 Hz, 2H), 7.55 (s, 1H), 8.81 (s, 1H); ^{13}C NMR δ 13.4, 14.5, 41.7, 45.6, 50.2, 50.5, 62.0, 105.9, 125.4, 132.1, 153.7, 153.8, 154.9, 163.5. Anal. ($C_{22}H_{33}N_{11}O_{11}$) C, H, N.

1-[1-(N,N-Diethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-{[(4-(N,N-diethyl-aminocarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diol-2-ato}benzene (5c). Product **5c** (490 mg, 90%) was obtained from **2f** (229 mg, 0.9 mmol) and **3b** (240 mg, 0.75 mmol). Purification of the crude material was carried out on silica gel with 10:1 dichloromethane/acetone as the eluant to give **5c** as a solid: mp 98–99 °C; UV (ethanol) λ_{max} (ε) 291 nm (25 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.15 (t, J = 6.8 Hz, 6H), 1.27 (t, J = 7.1 Hz, 6H), 3.26 (q, J = 7.1 Hz, 4H), 3.43 (m, 4H), 3.60 (q, J = 6.8 Hz, 4H), 3.63–3.67 (m, 4H), 7.58 (s, 1H), 8.87 (s, 1H); ^{13}C NMR δ 11.5, 13.1, 41.7, 45.6, 46.7, 50.1, 104.5, 125.5, 131.0, 131.3, 154.0, 154.5, 163.5. Anal. ($C_{19}H_{30}N_{10}O_9$) C, H, N.

1-[1-(N,N-Diethylamino)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitro-5-{[(2-hydroxymethyl)-pyrrolidin-1-yl]diazen-1-ium-1,2-diol-2-ato}benzene (5d). Product **5d** (400 mg, 87%) was obtained from **2k** (183 mg, 1.00 mmol) and **3b** (412 mg, 1.3 mmol). Purification of the crude material was carried out on silica gel with 10:1 dichloromethane/acetone as the eluant: mp 116–117 °C; UV (ethanol) λ_{max} (ε) 297 nm (21 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.25 (t, J = 7.1 Hz, 6H), 2.01–2.19 (m, 4H), 2.28 (t, J = 5.5 Hz, 1H), 3.58 (q, J = 7.1 Hz, 4H), 3.71–3.79 (m, 2H), 3.83–3.92 (m, 2H), 4.33–4.39 (m, 1H), 7.60 (s, 1H), 8.87 (s, 1H); ^{13}C NMR δ 11.6, 23.2, 27.0, 46.9, 52.4, 64.0, 64.4, 104.2, 125.6, 131.0, 131.1, 154.4. Anal. ($C_{15}H_{22}N_8O_9$) C, H, N.

1,5-Bis[(2-hydroxymethylpyrrolidin-1-yl)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitrobenzene (6). Sodium 2-(hydroxymethylpyrrolidin-1-yl)diazen-1-ium-1,2-diolate, **2k** (745 mg, 4.07 mmol), was dissolved in 5 mL of cold (4 °C) water and placed in a round-bottom flask that had been immersed in an ice–water bath. A solution of 830 mg (4.07 mmol) of 1,5-difluoro-2,4-dinitrobenzene in tetrahydrofuran was prepared, and to this was added 5 mL of *tert*-butyl alcohol. The resulting solution was added to the cold reagent and stirred at 0 °C for 15 min. The ice bath was removed, and the resulting yellow slurry was stirred overnight. Sodium bicarbonate solution (5%) was added, and the resulting precipitate was collected by filtration to give 645 mg of a yellow solid. Flash chromatography on silica gel using 5:1 dichloromethane/ethyl acetate followed by 10:1 dichloromethane/methanol gave 243 mg (12%) of product: mp 131–132 °C; UV (ethanol) λ_{max} (ε) 300 nm (22.5 mM^{-1} cm^{-1}); ^{1}H NMR δ 1.99–2.21 (m, 10H), 3.76–3.81 (m, 4H), 3.83–3.91 (m, 4H), 4.34–4.39 (m, 2H), 7.54 (s, 1H), 8.88 (s, 1H); ^{13}C NMR δ 23.1, 27.2, 52.4, 64.3, 64.4, 103.8, 125.7, 130.9, 154.6. Anal. ($C_{16}H_{22}N_8O_{10}$) C, H, N.

1,5-Bis[(2-carboxylatopyrrolidin-1-yl)diazen-1-ium-1,2-diol-2-ato]-2,4-dinitrobenzene (7). To a solution of **6** (160 mg, 0.33 mmol) in 2 mL of acetonitrile and 2 mL of ethyl acetate were added 564 mg (2.64 mmol) of sodium periodate and 4 mL of water, followed by 4.6 mg (0.02 mmol) of ruthenium trichloride. The mixture was stirred at room temperature for 2 h. The reaction mixture was filtered, diluted with ethyl acetate, and washed with water. The bis-carboxylic acid was extracted from the organic layer with 5% sodium bicarbonate. The aqueous layer was acidified with dilute HCl and extracted with ethyl acetate, dried over sodium sulfate, filtered, and evaporated to give a solid. The solid was taken up in dichloromethane, giving a cloudy solution that was filtered through magnesium sulfate and concentrated under vacuum to give 100 mg (59%) of product: mp 135–136 °C; UV (EtOH) λ_{max} (ε) 295 nm (23 mM^{-1} cm^{-1}); ^{1}H NMR δ 2.02–2.05 (m, 6H), 2.33–2.39 (m, 2H), 3.90 (broad, 4H), 4.71–4.73 (m, 2H), 7.55 (s, 1H), 8.88 (s, 1H), 13.17 (b, 1H); ^{13}C NMR δ 22.0, 27.9, 50.5, 61.8, 102.6, 125.7, 130.3, 154.1, 171.8. Anal. ($C_{16}H_{18}N_8O_{12}$· 0.2EtOAc) C, H, N.

Nitric Oxide Release. Chemiluminescence detection and quantification of NO evolving from the reactions of PABA/NO and its analogues were conducted by preparing 10 mM stock solutions of these compounds in pH 7.4 phosphate buffer. A pH 7.4 0.1 M phosphate buffer (3 mL) containing 50 μM diethyltriaminepentaacetic acid and GSH (4 mM) was sparged into the chemiluminescence detector with inert gas until a steady detector response was established. The stock solution of the diazeniumdiolate prodrug (20–100 μL, 0.31–1.87 μM) was injected into this GSH solution, and the NO release profile was followed at 37 °C. Nitric oxide release was monitored until baseline signal was achieved (25 min to 5 h). The resulting curve was integrated to quantify the amount of NO released per mole of compound.

Intracellular NO Release. The intracellular level of nitric oxide after bis(diazeniumdiolate) prodrug treatment was quantified using the NO-sensitive fluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate) (Invitrogen, Carlsbad, CA). HL-60 cells were loaded with 2.5 μM DAF-FM diacetate in Hanks' balanced salt solution (HBSS) at 37 °C and 5% CO_2. After 30 min of incubation the cells were collected by centrifugation, rinsed with HBSS to remove excess of probe, and resuspended in fresh HBSS. The compounds were added to the cells at 5 μM final concentration. After 40 min of incubation the fluorescence of the benzotriazole derivative formed on DAF-FM's reaction with aerobic NO was analyzed using a Perkin-Elmer LS50B luminescence spectrometer with the excitation source at 495 nm and emission at 515 nm.

Cell Culture and Cytotoxicity Assays. Cell lines HL-60, U-937, H441, and A549 were obtained from American Type Culture Collection (ATCC, Manassas, VA), and HCT-116, HCT-15, HT29, OVCAR-3, OVCAR-5, and PC-3 were from the NCI−Frederick DCTD tumor/cell line repository (Frederick, MD). Cells were maintained in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA), supplemented with 10% fetal calf serum (Gemini Bio-Products, Sacramento, CA), 100 U/mL penicillin/streptomycin, and 2 mM glutamine at 37 °C and 5% CO_2.

The CellTiter 96 nonradioactive cell proliferation assay (MTT assay; Promega, Madison, WI), performed according to the manufacturer's protocol, was used to measure cell proliferation. Cells were seeded in 96-well plates at a density of 10^4 per well and allowed to grow for 24 h before addition of the drugs. Bis(diazeniumdiolates) were prepared as 10 mM stock solution in dimethyl sulfoxide (Sigma, St. Louis, MO). Increasing drug concentrations in 10 μL of phosphate-buffered saline (PBS) were added to 100 μL of the culture medium and incubated at 37 °C for 72 h. Each compound concentration was represented in six repeats, and the screening was performed as at least two independent experiments.

***S*-Glutathionylating Activities of Bis(diazeniumdiolates).** HL-60 cells in complete growth medium (RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin) were treated with 1 μM compound, or an equal volume of dimethyl sulfoxide as a control, for 15 min. Cells were collected by centrifugation, washed with PBS, and prepared for immunoblotting by lysing in lysis buffer (25 mM Hepes buffer, pH 7.2, containing 150 mM NaCl, 10 mM $MgCl_2$, 1% Nonidet P40, 0.25% sodium deoxycholate, 10% glycerol, 2.5 mM EDTA, and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Proteins in cell lysates were separated under nonreducing conditions on 4−12% NuPage bis-Tris gels (Invitrogen Life Technologies, Carlsbad, CA) and transferred to PVDF membranes (Invitrogen) at 30 V for 1.5 h. After overnight blocking in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the membranes were probed with anti-glutathione monoclonal antibodies (1:1000, Virogen, Watertown, MA) in 5% milk in TBS-T for 3 h at room temperature. After repeated washings in TBS-T, the blots were incubated for 60 min at room temperature with antimouse secondary antibody, 1:1000, conjugated with horseradish peroxidase (GE Healthcare, Piscataway, NJ), in 5% milk in TBS-T, and then, after washing, developed using the chemiluminescence ECL kit (GE Healthcare, Piscataway, NJ). To show equal loading, the blots were stripped with stripping buffer (Thermo Scientific, Rockford, IL) and reprobed with anti-β-actin rabbit polyclonal antibodies (Abcam, Inc., Cambridge, MA).

Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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