Antitumor Activity of JS-K [O^2-(2,4-Dinitrophenyl) 1-[(4-Ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate] and Related O^2-Aryl Diazeniumdiolates in Vitro and in Vivo

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The literature provides evidence that metabolic nitric oxide (NO) release mediates the cytotoxic activities (against human leukemia and prostate cancer xenografts in mice) of JS-K, a compound of structure $R_2N-N(O)=NO-Ar$ for which R_2N is 4-(ethoxycarbonyl)piperazin-1-yl and Ar is 2,4-dinitrophenyl. Here we present comparative data on the potencies of JS-K and 41 other O^2-arylated diazeniumdiolates as inhibitors of HL-60 human leukemia cell proliferation, as well as in the NCI 51-cell-line screen for six of them. The data show JS-K to be the most potent of the 42 in both screens and suggest that other features of its structure and metabolism besides NO release may contribute importantly to its activity. Results with control compounds implicate JS-K's arylating ability, and the surprisingly low IC_{50} value of the *N*-(ethoxycarbonyl)piperazine byproduct of NO release suggests a role for the R_2N moiety. In addition to the above-mentioned in vivo activities, JS-K is shown here to be carcinostatic in a rat liver cancer model.

Introduction

Compounds of the O^2-aryl diazeniumdiolate family have shown noteworthy anticancer activity in a variety of model systems. An example with particular promise is JS-K[a] (compound **2a**, Scheme 1). It induced monocytic differentiation of HL-60 human leukemia cells in vitro,[1] proved pro-apoptotic through caspase-3 and -9 activation,[2] and blocked angiogenesis by inhibiting proliferation, migration, and cord formation in human umbilical vein endothelial cell cultures,[3] all at micromolar or submicromolar concentrations. Moreover, JS-K halved the growth rate of both human leukemia and prostate cancer subcutaneous xenografts in NOD-SCID mice, inducing extensive necrosis in the tumor mass.[1] It also inhibited hepatoma cell proliferation in vitro[4] and increased the cytotoxicity of cisplatin and arsenite in drug-resistant cells by increasing intracellular drug concentrations.[5]

JS-K was designed to be activated for nitric oxide (NO) release by glutathione *S*-transferase (GST), and this effect may account for part of its mechanism of action.[1] Because the π-isoform is overexpressed in many tumor cells,[6] we postulated that through structural modifications we might be able to increase the efficiency of the diazeniumdiolate's metabolism by GST-π. Even though transition state modeling studies of this material in the enzyme's active site suggested that structural modifications might improve selectivity,[1] JS-K was chosen for further development as an antileukemic drug due to its promising in vivo activity. On the downside, JS-K was found to have limited solubility in water and to react at a significant rate with free glutathione in the absence of GST.

We have continued to synthesize and diversify libraries of O^2-arylated diazeniumdiolates designed to release nitric oxide upon reaction with glutathione catalyzed by GST. Four systematic approaches for changing the JS-K structure have been followed: (a) altering the reactivity of the aryl group without changing the substituent at N-4 of the piperazine moiety; (b) modifying the substituent at the N-4 position while leaving the activated aryl group unchanged; (c) combining approaches a and b by altering the N-4 substituent and installing a less reactive aryl group at the O^2-position of the diazeniumdiolate; and (d) replacing the piperazine ring altogether using different secondary amines as the NO and aryl carriers. As a result, we have found other prodrug candidates that show notable activity against the HL-60 cell line and may provide us with important information toward lead optimization.

In the present work, we compare the in vitro anticancer activity of JS-K with those of numerous structural analogues and then provide further evidence of its in vivo cytotoxic effects.

Results

Previous reports on the pharmacological effects of JS-K (structure **2a**, Scheme 1) have been largely consistent with the interpretation that they are mediated by the nitric oxide (NO) produced on the drug's reaction with glutathione (GSH) or other nucleophiles. NO was detected in good yield on reacting JS-K with GSH (k_{GSH} = 1.0 M^{-1} s^{-1}) as well as on simply

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[a] Abbreviations: B_{12}, hydroxocobalamine; CDNB, 1-chloro-2,4-dinitrobenzene; 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; DMSO, dimethyl sulfoxide; DNP, dinitrophenyl; GI_{50}, 50% growth inhibition; GSH, glutathione; GST, glutathione *S*-transferase; JS-K, O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate; LC_{50}, 50% cell killing; NCI, National Cancer Institute; NO, nitric oxide; NOD-SCID, nonobese diabetic-severe combined immune deficient; PBS, phosphate-buffered saline; PTIO, (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide); TGI, total growth inhibition.

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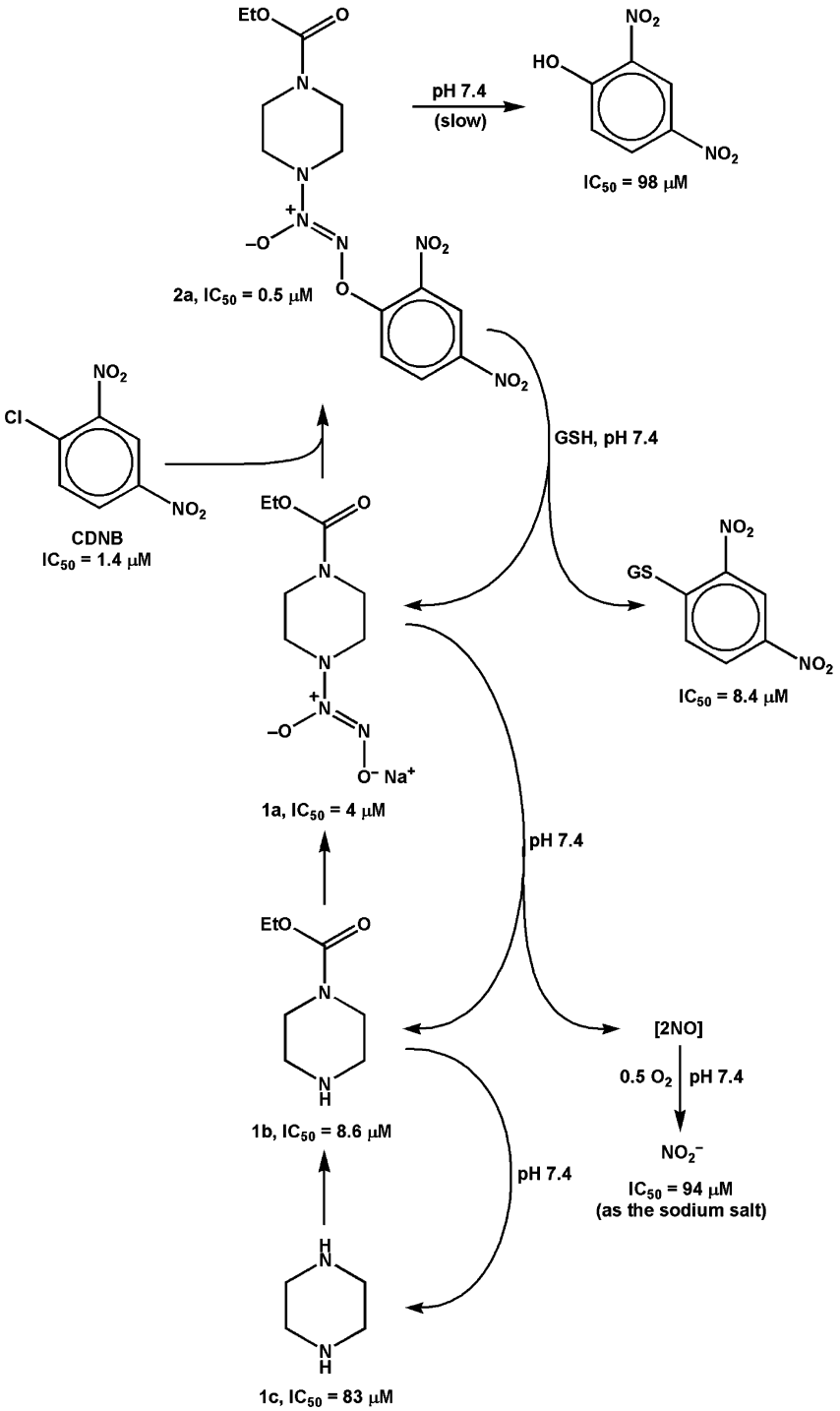
Scheme 1. Synthesis (up arrows) and Presumed Metabolism (down arrows) of JS-K (compound 2a)[a]



[a] IC_{50} values are for inhibition of HL-60 cell proliferation.

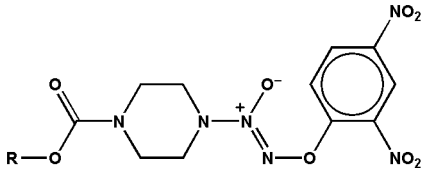
hydrolyzing it in 0.1 M phosphate buffer, pH 7.4 ($k_{hydr} = 4 \times 10^{-5}$ s^{-1}),[1] and JS-K inhibited proliferation of HL-60 human leukemia cells ($IC_{50} = 0.2-0.5$ μM), which are known to be especially sensitive to the cytostatic/cytotoxic effects of NO.[7] But it seemed possible that other features of JS-K's chemistry besides NO release might contribute to its antitumor activity.

In evaluating other possible components of JS-K's mechanism of action, we began by determining the growth-inhibitory activities of JS-K's other presumed metabolites. These are shown in Scheme 1, along with the IC_{50} value for each. Only one of these had an IC_{50} value within an order of magnitude of JS-K's (i.e., $IC_{50} \leq 5$ μM); ion **1a** was 50% inhibitory at 4 μM, again consistent with an NO-mediated mechanism in that it is known to generate up to 2 equiv of NO spontaneously in physiological media.

Importantly, the known arylating agent 1-chloro-2,4-dinitrobenzene (CDNB)[8] had an IC_{50} of 1.4 μM. This control was included so that we could estimate the potential contribution of simple arylation by JS-K as a cytotoxic mechanism. Diazeniumdiolate ions are reported to be similar in nucleofugality to chloride,[9] suggesting that CDNB should be a reasonable tool for addressing this question. The low IC_{50} value observed (1.4 μM) suggests that arylation of cellular nucleophiles may well be an important contributor to JS-K's activity.

It was interesting, and somewhat surprising, that the byproduct of ultimate NO release, *N*-(ethoxycarbonyl)piperazine (**1b**), was about 10-fold more potent in the HL-60 screen than the anthelminthic piperazine (**1c**) and, indeed, with an IC_{50} of 8.6 μM, almost half as active as its diazeniumdiolated derivative, ion **1a**. Furthermore, the BOC analogue *N*-(*tert*-butoxycarbonyl)-piperazine and its ionic diazeniumdiolate derivative had similar activities (IC_{50} values of 9.5 and 9.0 μM, respectively). This suggested that the N-acylated piperazine moiety may also contribute to JS-K's activity.

We prepared and tested a series of analogues represented by structure **2**. Four of these (including JS-K) had IC_{50} values ≤ 5 μM, with the methyl (**2b**), ethyl (**2a**), *n*-propyl (**2c**), *n*-butyl (**2d**) series giving values of 3.5, 0.5, 3.7, and 3.5 μM, respectively.



2

2a: R= Et– (JS-K) IC_{50} = 0.5 μM

2b: R= Me– IC_{50} = 3.5 μM

2c: R= *n*–Pr– IC_{50} = 3.7 μM

2d: R= *n*–Bu– IC_{50} = 3.5 μM

2e: R= Bn– IC_{50} = 17 μM

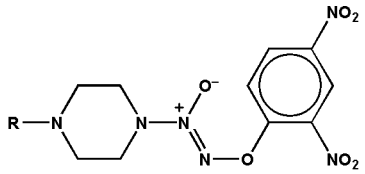
2f: R= *t*–Bu– IC_{50} = 8.1 μM

2g: R=  IC_{50} = 27 μM

Analogues with structure **3** explored further variations on the nature of the substituent attached to the terminal amino group of JS-K's piperazine ring. Secondary amine **3a**, prepared as the hydrochloride salt to prevent its self-destruction by S_NAr displacement of its diazeniumdiolate substituent by a neighboring molecule's basic nitrogen center, had IC_{50} = 5.0 μM. Acetylated analogue **3j** had IC_{50} = 3.9 μM. Very surprisingly, *N*-aryl derivatives **3g** and **3h** were also within the 0.5−5 μM potency range, with values of 1.5 and 3.6 μM, respectively. The results of the previous paragraph, together with the high IC_{50} values associated with such bulky N-subsitituents as benzyl (**2e**) and *tert*-butyl (**2f**) (17 and 8.1 μM, respectively), had led us to expect that activity was a rather sensitive function of the N-substituent's steric requirement, optimized among the derivatives tested thus far by ethoxycarbonyl structure **2a**. Apparently, phenyl and pyrimidin-2-yl are also substituents favorable for activity in this system.

Analogues of structure **4** involved further perturbations of JS-K's amino group. Reducing its size to the minimum, as in the dimethylamino group of **4a**, yielded a reasonably potent analogue with IC_{50} = 2.4 μM. Diethylamino was similar, at 2.2 μM. 3-Carboxamidopiperidine derivative **4d** had IC_{50} = 4.4 μM. Compound **4e** explored the effect of expanding JS-K's piperazine ring by one methylene group; the resulting IC_{50} was 3.4 μM.

Structures **5**−**11** examined the consequences of altering the nature of JS-K's O^2-aryl substituent. No compound in which the 2,4-dinitrophenyl ring was replaced by any other substituent (phenyl, pyridyl, or pyrimidinyl) fell within the IC_{50} range of

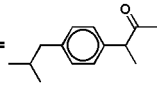


3

3a: R= H– (as the HCl salt) IC_{50} = 5.0 μM

3b: R= Me– IC_{50} = 6.7 μM

3c: R=  IC_{50} = 7.3 μM

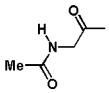
3d: R=  IC_{50} = 3.2 μM

3e: R=  IC_{50} = 3.7 μM

3f: R=  IC_{50} = 7.3 μM

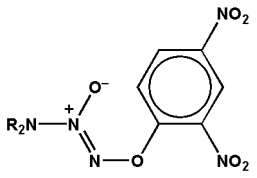
3g: R= Ph– IC_{50} = 1.5 μM

3h: R=  IC_{50} = 3.6 μM

3i: R=  IC_{50} = 8.8 μM

3j: R=  IC_{50} = 3.9 μM

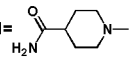
3k: R= $Me_2N–SO_2$– IC_{50} = 5.2 μM

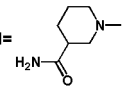


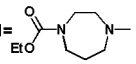
4

4a: R_2N= Me_2N– IC_{50} = 2.4 μM

4b: R_2N= Et_2N– IC_{50} = 2.2 μM

4c: R_2N=  IC_{50} = 5.9 μM

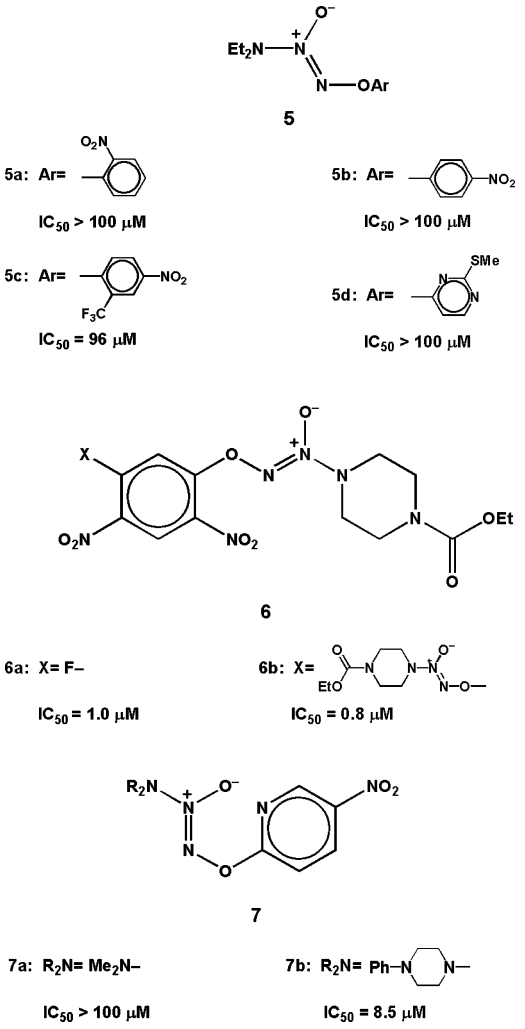
4d: R_2N=  IC_{50} = 4.4 μM

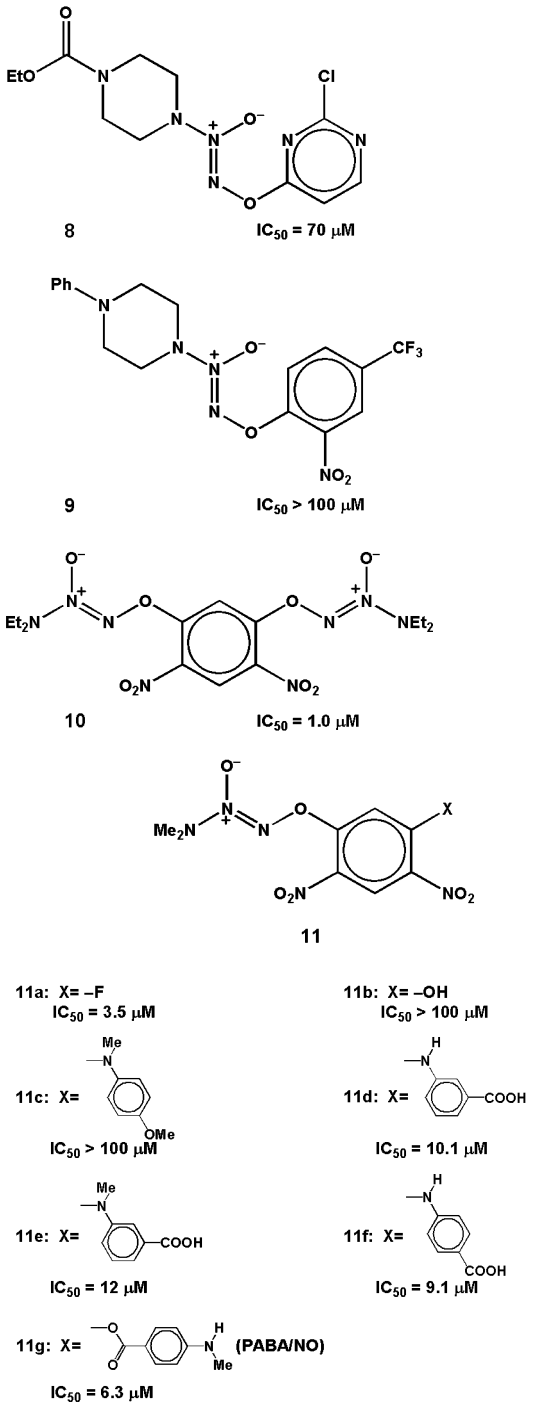
4e: R_2N=  IC_{50} = 3.4 μM

4f: R_2N=  IC_{50} = 7.4 μM

0.5−5 μM. Attaching a second diazeniumdiolate group to the 5 position of JS-K's aryl ring to make the symmetrical compound **6b** yielded the second-most active compound seen in this HL-60 screen, with IC_{50} = 0.8 μM. The 5-fluoro analogue **6a**, from which **6b** was prepared, had IC_{50} = 1.0 μM, as did symmetrically diazeniumdiolated derivative **10**. 5-Fluoro derivative **11a** was also within the reasonably active range, with IC_{50} = 3.5 μM. It appears that placement of a diazeniumdiolate group both ortho and para to nitro substituents is a requisite for maximum (≤ 5 μM) potency on the part of the compounds screened for HL-60 antiproliferative activity in the study reported here.

Results in the NCI 51-Cell-Line Screen. JS-K and its *O*-alkyl variants **2b**−**2e** as well as its homopiperazine analogue **4e** were subjected to the National Cancer Institute (NCI) 51-cell-line screen. The overall dose−response curves revealed that the renal cancer subpanel was the most sensitive to these





analogues. In general, the five analogues tested produced 50% growth inhibition (GI_{50}) and 50% cell killing (LC_{50}) of seven cell lines of the human renal cancer subpanel at concentrations of about 10 and <100 μM, respectively. When concentrations of these analogues increased to 100 μM, 100% cell killing was observed with 786-0, TK-10, and CAKI-1 cell lines, whereas the central nervous system and prostate cancer subpanels were ranked next. It was somewhat surprising that, unlike JS-K that potently and selectively inhibited leukemia HL-60 cells at $GI_{50} = 26$ nM, TGI (total growth inhibition) $= 68$ nM, and $LC_{50} = 3.5$ μM,[1,10] all five analogues showed relatively poor potency in inhibiting the leukemia subpanel. They were unable to produce 50% cell killing of all six leukemia cell lines, including the HL-60 cells, at a concentration of 100 μM. The five analogues showed total growth inhibition of the HL-60 cells at 8.3 μM (**2b**), 8.1 μM (**2c**), 57.5 μM (**2d**), and >100 μM (**4e**).

Analysis of the results from the NCI 51 human cancer cell lines further demonstrated that, as a whole, **2e** (benzyl) and **2d** (*n*-butyl) exhibited similar inhibition patterns against the 51 cell lines, which were weaker than analogues **2b**, **2c**, and **4e** (Table 1). For instance, both **2d** and **2e** needed concentrations of more than 5 and 23 μM in order to reach 50% growth inhibition and total growth inhibition, respectively, of the 51 cell lines, whereas **2b** (methyl), **2c** (*n*-propyl), and **4e** were able to cause 50% growth inhibition and total growth inhibition, respectively, of the 51 cell lines at concentrations as low as 1−2 and 10 μM. These three analogues were even able to produce 50% cell killing of about a third of the 51 cell lines in the concentration range of 1−10 μM and 100% cell killing for a few cell lines at 100 μM.

In Vivo Activity against Rat Hepatoma. Previously we have found that JS-K efficiently inhibited the growth of the human hepatoma cell line Hep3B in culture.[4] Both the MEK inhibitor 2′-amino-3′-methoxyflavone and the nitric oxide scavenger PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) antagonized this growth inhibition. This suggested that generation of nitric oxide and ERK phosphorylation might be involved in JS-K-mediated growth inhibition of the Hep3B hepatoma cells. We therefore tested the effects of JS-K and its inhibitors in vivo on rat liver tumor growth.

Rat hepatoma cell line JM-1 was grown in culture. A million cells suspended in 1 mL of phosphate-buffered saline (PBS)

Table 1. Cytotoxic Concentrations (Means ± SD) of JS-K and Related O^2-Aryl Diazeniumdiolates against the NCI 51 Human Cancer Cell Lines

analogue	cytotoxic concentrations (μM)		
	GI_{50}	TGI	LC_{50}
2a (JS-K)	1.32 ± 0.03	6.61 ± 0.07	29.51 ± 3.31
2b	1.66 ± 0.24	11.75 ± 0.59	51.29 ± 2.57
2c	2.04 ± 0.27	10.23 ± 0.65	40.74 ± 4.36
2d	6.31 ± 1.70	23.44 ± 3.47	63.10 ± 6.03
2e	7.08 ± 1.62	30.90 ± 3.80	77.63 ± 28.18
4e	1.51 ± 0.04	11.75 ± 0.20	51.29 ± 1.55

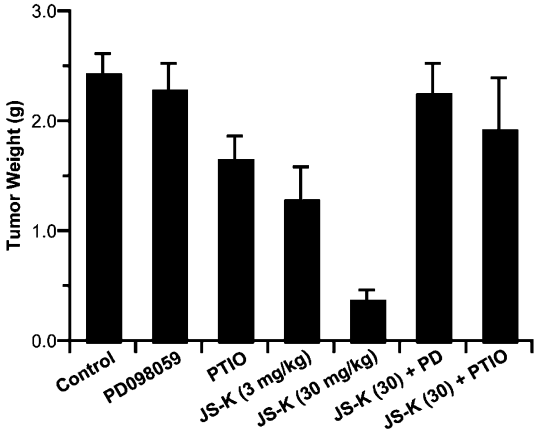


Figure 1. Suppression of tumor growth by JS-K after implanting JM-1 hepatoma cells intrahepatically in rats and then injecting JS-K in dimethyl sulfoxide (DMSO) intraperitoneally 1 week later and every other day thereafter for 2 weeks at the indicated doses. The MEK inhibitor 2′-amino-3′-methoxyflavone (PD) and the NO scavenger PTIO were administered intravenously an hour before JS-K, reversing JS-K's antitumor effect as indicated. The data represent means ± SD, $n = 4$.

were injected under direct vision into the livers of syngeneic Fischer rats through their mesenteric vein. The cells were allowed to grow in the rat livers for 1 week. Then the rats were treated with five intraperitoneal injections every other day of JS-K at a dose of 3 or 30 mg/kg. Animals were sacrificed 3 weeks after JM-1 cell injection. Liver tumors were excised and weighed. Two groups of rats were also intravenously (via tail vein) pretreated with either the MEK inhibitor 2′-amino-3′-methoxyflavone or the nitric oxide scavenger PTIO 1 h before each of the JS-K treatments.

As shown in Figure 1, the total tumor weight was found to decrease with increasing dose of JS-K in vivo. Pretreatment of the animals with either the MEK inhibitor 2′-amino-3′-methoxyflavone or the nitric oxide scavenger PTIO antagonized the tumor growth-inhibitory effect of JS-K. This implied the involvement of both ERK and NO in the growth-inhibitory mechanisms of JS-K action. Exactly how NO might activate MAPKs is currently the subject of intense investigation. It has been shown to include activation of JNK, ERK, and p38[11−14] as well as inhibition of protein tyrosine phosphatases.[15−17] The transient activation of the MAPK pathway has been long associated with the growth-stimulatory action of a variety of mitogens. Recently, it has become clear that prolonged activation of components of the same pathway is an important aspect of several mediators of growth inhibition.[18,19] The prolonged activation of MAPK is proposed in turn to induce AP-1. NO has also been found to induce AP-1 in other cell systems[20,21] and AP-1 induction is associated with apoptosis and growth inhibition in other culture conditions.[22,23]

Discussion

Discovery of JS-K (**2a**) as a promising anticancer lead occurred when a panel of randomly chosen O^2-arylated diazeniumdiolates was screened for activity against the NO-sensitive HL-60 human leukemia cell line. Of special interest in the earlier study was the in vivo activity of JS-K against not only HL-60 but also PPC-1 human prostate cancer xenografts in NOD-SCID mice.[1] The latter cells were not known to be especially NO sensitive, suggesting that JS-K's antitumor effect might involve more than NO-mediated cytotoxicity.

To gain further insight into JS-K's mechanism of action, as well as to search for additional promising lead compounds among its analogues, we have prepared 41 different O^2-aryl diazeniumdiolates and compared them with JS-K for the ability to inhibit proliferation of HL-60 cells in vitro. Five of these were further subjected to the NCI 51-cell-line screen. JS-K emerged as the most potent of the 42 by every criterion examined.

As far as JS-K's mechanism of action is concerned, the 4 μM IC_{50} of JS-K's NO-releasing metabolite **1a** supported the importance of NO-mediated cytotoxicity. This is further supported by our previous work showing that pretreatment of HL-60 cells with the NO-quencher hydroxocobalamin (B_{12}) protected them from JS-K's cytotoxic effects.[2] However, results presented here suggest that other components of the JS-K molecule may contribute to its cytotoxicity. Indeed, the demonstrated ability of JS-K to arylate glutathione (GSH) and other cellular nucleophiles also appears to play a substantial role. JS-K has been shown to be similar in electrophilicity to 1-chloro-2,4-dinitrobenzene,[9] with each one easily transferring its dinitrophenyl (DNP) ring to GSH. The 1.4 μM IC_{50} of the chloro compound showed it to be only slightly less potent than JS-K. These data are in agreement with the notion that JS-K acts through a two-step mechanism: (1) arylation of GSH or other important nucleophilic biomolecules, irreversibly altering their normal function; and (2) spontaneous NO release after the DNP group is no longer present to stabilize the diazeniumdiolate moiety. One could further speculate that with this drug design, GSH arylation makes cells more susceptible to NO attack by rendering thiol groups that act as NO "sinks" unavailable. This is supported by our previous observation that loading HL-60 cells with the GSH precursor *N*-acetyl-L-cysteine protects them from JS-K's cytotoxic effects.[2] The latter observation and the protection of HL-60 cells from JS-K by B_{12} support a central role for NO in JS-K's cytotoxic effect. This action is compounded by the arylation reactions of the DNP component of the molecule. It is hard to predict which pathways affected by JS-K lead to cytotoxicity. Nitric oxide modifies intracellular targets that affect cell growth through multiple mechanisms. These include protein thiol nitrosylation, protein tyrosine nitration (by generating peroxynitrite in the presence of superoxide), ADP ribosylation, inhibition of mitochondrial respiration, inhibition of ribonucleotide reductase, and induction of DNA strand breaks.[24]

The possible involvement of a carbamoylation pathway was suggested by the surprising potency of carbamoylated piperazine **1b** (Scheme 1). At 8.6 μM, it was almost half as active as the corresponding diazeniumdiolate, **1a**. This possibility was further investigated by modifying the alkyl group of the potential carbamoylating moiety. Both shortening and lengthening the C_2 chain in the series **2a**−**2d** decreased the potency by at least 7-fold. Further reductions in potency were seen with the benzyl and *tert*-butyl derivatives, **2e** and **2f**.

Evidence for the possible importance of carbamoylation is weak overall, inasmuch as removing the group altogether, as in decarbamoylated derivative **3a**, led to an IC_{50} of 5.0 μM, comparable to those of **2b**−**2f**. The same was true for acyl analogues **3c**−**3f** and **3i**−**3k**. Comparable activities were also seen when the piperazine ring was replaced by pyrrolidine (**4f**), piperidine derivatives (**4c** and **4d**), or homopiperazine (**4e**) or even by the simple acyclic dimethylamino (**4a**) or diethylamino (**4b**) groups.

An alternative suggestion from these data is that the activity of these compounds depends at least in part on the precise steric arrangement of the amino moiety to which the diazeniumdiolate group is bound. JS-K just happens to be optimal in this regard among the agents tested thus far. However, either decreasing the amino group's steric requirement as described above or increasing it dramatically, as with the *N*-phenyl and *N*-(pyrimidin-2-yl)piperazine derivatives **3g** and **3h** (IC_{50} values of 1.5 and 3.6, respectively), can also sustain significant activity.

A further conclusion arising from our data is that substitution of the aryl ring with nitro groups both ortho and para to the diazeniumdiolate function is required for significant activity. Removal of a nitro group, or replacement of one or both nitro groups by trifluoromethyl or a heterocyclic nitrogen, as in **5a**−**5d**, **7a**, **8**, and **9**, lowered the in vitro potency by at least 2 orders of magnitude. The only exception was **7b**, whose relatively low IC_{50} of 8.0 μM may have been attributable at least in part to the presence of the *N*-phenylpiperazine group, as postulated above for **3g**. However, the *N*-phenylpiperazine effect could apparently not compensate for the lowered electrophilicity of the 2-nitro-4-trifluoromethylbenzene ring in compound **9**, which proved inactive at the 100 μM level.

A referee has suggested the further intriguing possibilities that nucleophilic metabolite **1b** might react with JS-K to activate it for NO release and that the *N*-nitroso derivative of **1b** might play an important mechanistic role. These are interesting ideas that we had not considered, but at the low concentrations/doses administered in our study, such secondary reactions would seem to be relatively unlikely to contribute significantly to the observed pharmacological activities.

While much remains to be elucidated about the mechanism of JS-K's antitumor effects, its demonstrated in vitro and in vivo activities continue to recommend it as a worthy lead in anticancer drug discovery. Of particular note is its activity against human prostate cancer xenografts not known for being susceptible to the cytotoxic potential of NO.

With this in mind, we have examined the action of JS-K against an orthotopic rat liver tumor model. As shown in Figure 1, JS-K significantly slowed the growth of these tumors. In agreement with an NO-mediated mechanism of action, the NO scavenger PTIO blunted JS-K's antitumor effect. The MEK inhibitor 2′-amino-3′-methoxyflavone also reversed JS-K's activity. Ongoing and future studies are aimed at further refining our mechanistic understanding of the pathways responsible for JS-K's promising antitumor effects.

Experimental Section

NO was purchased from Matheson Gas Products (Montgomeryville, PA). Starting materials were purchased from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise indicated. Proton NMR spectra were obtained in chloroform-*d*, acetone-d_6, or DMSO-d_6. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA) or Midwest Microlab, LLC (Indianapolis, IN). Compounds **2a**, **3g**, **3h**, **4a**−**4d**, **5b**, **5c**, **7a**, **7b**, and **8**−**10** were prepared as previously described,[9] as were **1a**,[25] sodium 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO),[26,27] sodium 1-(*N*,*N*-dimethylamino)-diazen-1-ium-1,2-diolate (DMA/NO),[9] **3f**,[3] and **11d**−**11g**.[28]

General Procedures for the Arylation of Diazeniumdiolates. Method A. A solution of 11 mmol of a diazeniumdiolate anion in 20 mL of 5% aqueous sodium bicarbonate is cooled to 0 °C under nitrogen. A solution containing 10 mmol of the activated fluoro- or chloroarene in 10 mL of *tert*-butyl alcohol is added slowly. A precipitate normally forms upon addition. The mixture is allowed to warm gradually to room temperature and then stirred overnight. The product is extracted with dichloromethane and washed subsequently first with cold dilute hydrochloric acid and then with sodium bicarbonate solution. The organic layer is dried over sodium sulfate, filtered through a layer of magnesium sulfate, and evaporated under vacuum to give the crude product. Purification is carried out by recrystallization, flash chromatography, or preparative HPLC.

Method B. To a solution of 7.5 mmol of the diazeniumdiolate in 10 mL of DMSO at 0 °C under a steady stream of nitrogen is added dropwise 6 mmol of the activated aryl compound. The reaction is allowed to warm to room temperature and stirred for 24−72 h. The reaction is quenched with water, extracted with ether, dried over sodium sulfate, and evaporated. The product is either recrystallized or purified by flash chromatography.

Method C. A solution of 1.76 mmol of the activated bromo-, fluoro-, or chloroarene reagent in 3 mL of DMSO is added to a slurry (1.76 mmol) of the diazeniumdiolate in 3 mL of tetrahydrofuran at room temperature under nitrogen. The mixture is stirred for 72 h. The resulting homogeneous solution is treated with 100 mL of water. The precipitate is extracted with ether. The organic layer is dried and evaporated, whereupon the product is purified as described in method A.

Sodium 1-[4-(Methoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-36−**143).** A solution of 12.93 g (0.899 mol) of *N*-(methoxycarbonyl)piperazine[29] in 19.6 mL of 25% sodium methoxide in methanol and 5 mL of additional methanol was placed in a Parr bottle and charged with NO as described above. The product began to form within 2 h but was allowed to stir overnight at room temperature to give 12.3 g (61%) of the diazeniumdiolate sodium salt: mp 183−184 °C; ^{1}H NMR (D_2O/$^-$OD) δ 3.10−3.16 (m, 4 H), 3.36 (s, 3 H), 3.66−3.72 (m, 4 H); ^{13}C NMR (D_2O/$^-$OD) δ 45.49, 54.09, 56.08, 160.14; UV (10 mM NaOH) λ_{max} (ϵ) 250 nm (9.1 mM^{-1} cm^{-1}); half-life 5.1 min in pH 7.4 buffer at 37 °C. Anal. Calcd for $C_6H_{11}N_4O_4Na$: C, 31.86; H, 4.90; N, 24.77; Na, 10.17. Found: C, 31.94; H, 4.88; N, 24.45; Na, 10.21.

***O*^{2}-(2,4-Dinitrophenyl) 1-[4-(Methoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (2b) (JS-36-152).** A solution of 3.76 g (0.0166 mol) of sodium 1-[4-(methoxycarbonyl)piperazin-1-yl]-diazen-1-ium-1,2-diolate in 30 mL of 5% aqueous sodium bicarbonate was treated with 1 equiv of 2,4-dinitrofluorobenzene in *tert*-butyl alcohol according to method A, giving 5.38 g of a glass. The crude material was dissolved in 10 mL of 5:1 dichloromethane: ethyl acetate, whereupon crystalline product formed on standing. The product **2b** was collected by filtration and washed with ether to give 1.8 g of analytically pure material. An additional 780 mg of product was obtained upon flash chromatography of the mother liquor on silica gel on elution with 5:1 dichloromethane:ethyl acetate: mp 144−145 °C; ^{1}H NMR ($CDCl_3$) δ 3.63−3.69 (m, 4 H), 3.65−3.71 (m, 4 H), 3.75 (s, 3 H), 7.66 (d, 1 H, J = 9.25 Hz), 8.43−8.49 (dd, 1 H, J = 2.74 and 9.25 Hz), 8.88 (d, 1 H, J = 2.65 Hz); ^{13}C NMR ($CDCl_3$) δ 42.27, 50.52, 53.10, 117.71, 122.18, 129.08, 137.38, 142.47, 153.67, 155.39; UV (ethanol) λ_{max} (ϵ) 299 nm (12 mM^{-1} cm^{-1}). Anal. Calcd for $C_{12}H_{14}N_6O_8$: C, 38.92; H, 3.81; N, 22.70. Found: C, 39.06; H, 3.85; N, 22.51.

Sodium 1-[4-(*n*-Propoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-36-166). A solution of 7.04 g (0.041 mol) of *N*-(*n*-propoxycarbonyl)piperazine[30] in 8.9 mL (0.041 mol) of 25% methanolic sodium methoxide and 4 mL of methanol was exposed to nitric oxide as described previously to give 6 g (58%) of the diazeniumdiolate sodium salt: mp 191−192 °C; ^{1}H NMR (D_2O/$^-$OD) δ 0.94 (t, 3 H, J = 7.42 Hz), 1.63−1.72 (m, 2 H), 3.12−3.14 (m, 4 H), 3.70 (b, 4 H), 4.08 (t, 2 H, J = 6.54 Hz); ^{13}C NMR

(D_2O/$^-$OD) δ 12.40, 24.58, 45.42, 54.12, 71.06, 159.82; UV (10 mM NaOH) λ_{max} (ϵ) 250 nm (7.9 mM^{-1} cm^{-1}); half-life 4.7 min in pH 7.4 buffer at 37 °C. Anal. Calcd for $C_8H_{15}N_4O_4Na$: C, 37.80; H, 5.95; N, 22.04; Na, 9.04. Found: C, 37.68; H, 5.77; N, 21.79; Na, 9.15.

***O*²-(2,4-Dinitrophenyl) 1-[4-(*n*-Propoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (2c) (JS-36-172).** A solution of 2.35 g (0.0093 mol) of sodium 1-[4-(*n*-propoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate in 5% aqueous sodium bicarbonate was treated with 2,4-dinitrofluorobenzene in *tert*-butyl alcohol according to method A. The product was recrystallized from ether:petroleum ether to give 1.82 g of **2c**: mp 134−135 °C; ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3 H, J = 7.32 Hz); 1.64−1.73 (m, 2 H), 3.62−3.65 (m, 4 H), 3.73−3.75 (m, 4 H), 4.09 (t, 2 H, J = 6.73 Hz); 7.67 (d, 1 H, J = 9.27 Hz); 6.45−8.48 (dd, 1 H, J = 2.73 and 9.27 Hz), 8.88 (d, 1 H, J = 2.73 Hz); ^{13}C NMR ($CDCl_3$) δ 10.33, 22.24, 42.17, 50.52, 67.65, 117.69, 122.15, 129.07, 137.35, 142.45, 153.67, 155.05; UV (ethanol) λ_{max} (ϵ) 252 nm (9.3 mM^{-1} cm^{-1}). Anal. Calcd for $C_8H_{18}N_6O_8$: C, 42.21; H, 4.55; N, 21.10. Found: C, 41.95; H, 4.51; N, 20.85.

Sodium 1-[4-(*n*-Butoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-37-9). A solution of 5.486 g (0.0295 mol) of *N*-(*n*-butoxycarbonyl)piperazine[29] in 6.52 mL (0.03 mol) of 25% sodium methoxide in methanol, 10 mL of additional methanol, and 10 mL of ether was placed in a Parr bottle and charged with NO as described above to give the diazeniumdiolate sodium salt: mp 180 °C (dec); ^{1}H NMR (D_2O/$^-$OD) δ 0.92 (t, 3 H, J = 7.4 Hz), 1.35−1.44 (m, 2 H), 1.61−1.68 (m, 2 H), 3.11−3.14 (m, 4 H), 3.69 (b, 4 H), 4.14 (t, 2 H, J = 6.5 Hz); ^{13}C NMR (D_2O/ $^-$OD) 15.81, 21.37, 33.14, 45.44, 54.14, 69.34, 159.87; UV (10 mM NaOH) λ_{max} (ϵ) 251 nm (9.3 mM^{-1} cm^{-1}); half-life of 5.7 min in pH 7.4 buffer at 37 °C. Anal. Calcd for $C_9H_{17}N_4NaO_4 \cdot {}^1/_3CH_3OH \cdot {}^1/_3H_2O$: C, 39.34; H, 6.72; N, 19.66; Na, 8.07. Found: C, 39.30; H, 7.65; N, 19.81; Na, 8.01.

***O*²-(2,4-Dinitrophenyl) 1-[4-(*n*-Butoxycarbonyl)piperazin-yl]-diazen-1-ium-1,2-diolate (2d) (JS-37-24).** A solution of 2.28 g (0.0085 mol) of sodium 1-[4-(*n*-butoxycarbonyl)piperazin-1-yl]-diazen-1-ium-1,2-diolate in aqueous sodium bicarbonate was arylated with 2,4-dinitrofluorobenzene according to method A to give 2.5 g (71%) of **2d**. The isolated powder was recrystallized from ether:petroleum ether to give 1.75 g of pure material: mp 109−110 °C; ^{1}H NMR ($CDCl_3$) δ 0.95 (t, 3 H, J = 7.05 Hz), 1.35−1.45 (m, 2 H), 1.61−1.68 (m, 2 H), 3.62−3.65 (m, 4 H), 3.70−3.75 (m, 4 H), 4.13 (t, 2 H, J = 6.64 Hz), 7.68 (d, 1 H, J = 9.27 Hz), 8.45−8.48 (dd, 1 H, J = 2.73 and 9.27 Hz), 8.88 (d, 1 H, J = 2.73 Hz); ^{13}C NMR ($CDCl_3$) δ 13.69, 19.09, 30.92, 42.16, 50.49, 55.94, 117.67, 122.12, 129.07, 137.31, 142.42, 153.66, 155.04; UV (ethanol) λ_{max} (ϵ) 299 nm (14.5 mM^{-1} cm^{-1}). Anal. Calcd for $C_{15}H_{20}N_6O_8$: C, 43.69; H, 4.89; N, 20.38. Found: C, 43.62; H, 4.84; N, 20.11.

***O*²-(2,4-Dinitrophenyl) 1-[4-(Benzyloxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (2e) (JS-36-187).** A solution of 4.7 g (0.016 mol) of sodium 1-[4-benzyloxycarbonyl)piperazin-1-yl]-diazen-1-ium-1,2-diolate[25] in aqueous sodium bicarbonate was arylated with 2,4-dinitrofluorobenzene according to method A to give 5.4 g of crude **2e** as an amber oil. The oil was chromatographed on a dry-packed silica gel column and eluted with dichloromethane. The fractions containing the desired product were pooled and concentrated under vacuum to produce a thick oil that crystallized on standing. After trituration with ether, the solid was collected by filtration and dried to give 2.16 g of product **2e**: mp 118−120 °C; ^{1}H NMR ($CDCl_3$) δ 3.63 (b, 4 H), 3.75−3.77 (m, 4 H), 5.16 (s, 2 H), 7.36 (s, 5 H), 7.66 (d, 1 H, J = 9.27 Hz), 8.44−8.47 (dd, 1 H, J = 2.73 and 9.27 Hz), 8.86 (d, 1 H, J = 2.73 Hz); ^{13}C NMR ($CDCl_3$) δ 42.29, 50.44, 67.72, 117.63, 122.09, 126.31, 128.06, 128.56, 129.08, 137.25, 136.01, 142.39, 153.62, 154.71; UV (ethanol) λ_{max} (ϵ) 299 nm (11 mM^{-1} cm^{-1}). Anal. Calcd for $C_{18}H_{18}N_6O_8$: C, 48.43; H, 4.06; N, 18.83. Found: C, 48.61; H, 4.14; N, 18.49.

***O*²-(2,4-Dinitrophenyl) 1-[4-(*tert*-Butoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (2f) (CB-3-97).** To a solution of sodium 1-[4-(*tert*-butoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate[25] in 5% sodium bicarbonate at 0 °C under a steady stream of nitrogen gas was added 0.9 equiv of 2,4-dinitrofluorobenzene in *tert*-butyl alcohol (method A). The reaction was warmed to room temperature and stirred for 24 h. Precipitation occurred at such a rapid rate that stirring was impeded throughout the course of the reaction. The yellow mixture was filtered and recrystallized in EtOH: mp 115−117 °C; ^{1}H NMR ($CDCl_3$) δ 1.49 (s, 9 H), 3.60−3.76 (m, 8 H), 7.67 (d, 1 H, J = 9.2 Hz), 8.43−8.46 (dd, 1 H, J = 2.65 and 9.24 Hz), 8.89 (d, 1 H, J = 2.7); ^{13}C NMR ($CDCl_3$) δ 28.29, 42.07, 50.56, 80.91, 117.67, 122.17, 129.07, 137.32, 142.41, 153.72, 154.10; UV (EtOH) λ_{max} (ϵ) 299 nm (13.8 mM^{-1} cm^{-1}). Anal. Calcd for $C_{15}H_{20}N_6O_8$: C, 43.69; H, 4.89; N, 20.38. Found: C, 43.75; H, 4.92; N, 20.44.

***O*²-(2,4-Dinitrophenyl) 1-[4-(Succinimidoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (2g) (CB-4-140).** To a mixture of 1.1 g (3 mmol) of **3a** (see next paragraph) in 15 mL of dichloromethane was added 510 μL (3.7 mmol) of triethylamine to generate the free base. A partial solution of 772 mg (3.02 mmol) of *N*,*N*′-disuccinimidyl carbonate in 15 mL of dichloromethane was added, followed by 510 μL (3.7 mmol) of additional triethylamine. The resulting reaction mixture was stirred at room temperature for 15 h. The mixture was concentrated on a rotary evaporator and the residue was extracted with dichloromethane. The organic solution was washed with 10% hydrochloric acid, followed by 5% sodium bicarbonate solution, and then dried over sodium sulfate, filtered through magnesium sulfate, and evaporated to give a solid. The product was recrystallized from ethanol:acetonitrile to give 143 mg of pure **2g**: mp 162−164 °C; ^{1}H NMR (DMSO-d_6) δ 2.81 (s, 4 H), 3.70−3.79 (m, 8 H), 7.96 (d, 1 H, J = 9.3 Hz), 8.54−8.58 (dd, 1 H, J = 2.75 and 9.31 Hz), 8.88 (d, 1 H, J = 2.7 Hz); ^{13}C NMR (DMSO-d_6) δ 25.28, 42.47, 42.86, 49.27, 118.19, 121.78, 129.71, 136.90, 142.24, 150.05, 152.73, 170.49; UV (acetonitrile) λ_{max} (ϵ) 300 nm (14.2 mM^{-1} cm^{-1}). Anal. Calcd for $C_{15}H_{15}N_7O_{10}$: C, 39.74; H, 3.34; N, 21.63. Found: C, 39.85; H, 3.53; N, 21.61.

***O*²-(2,4-Dinitrophenyl) 1-(Piperazin-1-yl)diazen-1-ium-1,2-diolate, Hydrochloride Salt (3a) (CB-4-35).** To a solution of 1.27 g (0.0031 mol) of **2f** in 150 mL of ethyl acetate was added 6 mL of concentrated hydrochloric acid. The resulting yellow solution was stirred at room temperature for 2 h, during which time a white precipitate was evident. The mixture was filtered and washed with ether to yield a clean product (944 mg, 87% yield) which could be used for subsequent reactions. Recrystallization was achieved in 1:1 methanol:H_2O: mp 184−185 °C; ^{1}H NMR (DMSO-d_6) δ 3.34−3.38 (m, 4 H), 3.85−3.89 (m, 4 H), 7.96 (d, 1 H, J = 9.2 Hz), 8.56−8.59 (ddd, 1 H, J = 0.8, 2.8, and 4.3 Hz), 8.88−8.89 (dd, 1 H, J = 0.8 and 2.8 Hz); ^{13}C NMR (CD_3OD) δ 43.67, 119.24, 122.86, 130.33, 138.88, 144.26, 154.45; UV (ethanol) λ_{max} (ϵ) 297 nm (12.2 mM^{-1} cm^{-1}). Anal. Calcd for $C_{10}H_{13}ClN_6O_6 \cdot 1/6\ H_2O$: C, 34.15; H, 3.82; N, 23.90. Found: C, 34.52; H, 3.92; N, 23.42.

***O*²-(2,4-Dinitrophenyl) 1-(4-Methylpiperazin-1-yl)diazen-1-ium-1,2-diolate (3b) (JS-29-86).** A solution of 50 g (0.5 mol) of 1-methylpiperazine in 100 mL of 25% sodium methoxide (0.46 mol) in methanol and 50 mL of ether was placed in a Parr bottle, degassed, charged with 40 psi of nitric oxide, and stirred overnight at room temperature. The pressure was released and the product was filtered, washed with ether, and dried under vacuum to give 7.54 g of sodium 1-(4-methylpiperazin-1-yl)diazen-1-ium-1,2-diolate: ^{1}H NMR (D_2O) δ 2.29 (s, 3 H), 2.69 (b, 4 H), 3.16−3.19 (m, 4 H); a peak at 3.36 was detected, indicating the presence of sodium methoxide, but the material was used without further purification in the subsequent arylation reaction. The arylation was carried out with 1,5-difluoro-2,4-dinitrobenzene according to method A: mp 149−150 °C; ^{1}H NMR ($CDCl_3$) δ 2.36 (s, 3 H), 2.36−2.66 (m, 4 H), 3.67−3.70 (m, 4 H), 7.66 (d, 1 H, J = 2.7 Hz), 8.44−8.48 (dd, 1 H, J = 2.8, 12.0 Hz) 8.88 (d, 1 H, J = 9.3 Hz); ^{13}C NMR ($CDCl_3$) δ 45.47, 50.43, 53.28, 117.59, 122.17, 129.07, 137.23, 142.23, 153.95; UV (ethanol) λ_{max} (ϵ) 300 nm (16 mM^{-1} cm^{-1}). Anal. Calcd for $C_{11}H_{14}N_6O_6$: C, 40.49; H, 4.33; N, 25.76. Found: C, 40.09; H, 4.29; N, 25.32.

***O*^{2}-(2,4-Dinitrophenyl) 1-(4-Nicotinylpiperazin-1-yl)diazen-1-ium-1,2-diolate (3c) (CB-3-125).** To a solution of **3a** in CH_2Cl_2 was added a slight molar excess of triethylamine to liberate the free base. An equivalent molar amount of nicotinyl chloride was added, followed by another aliquot of triethylamine. The reaction mixture was stirred at room temperature for 30 min, at which time the cloudy solution was washed with water. The organic solution was collected, dried over sodium sulfate, filtered through magnesium sulfate, and evaporated to a yellow film. Recrystallization was achieved with ethanol to yield a beige crystalline material: mp 150−152 °C; ^{1}H NMR ($CDCl_3$) δ 3.51−4.28 (b, 8 H), 7.40−7.45 (m, 1 H), 7.66 (d, 1 H, J = 9.2 Hz), 7.79−7.83 (m, 1 H), 8.44−8.48 (dd, 1 H, J = 2.8 and 9.2 Hz), 8.71−8.75 (m, 2 H), 8.89 (d, 1 H, J = 2.7 Hz); ^{13}C NMR ($CDCl_3$) δ 50.71, 61.22, 117.80, 122.19, 123.71, 129.08, 130.35, 135.22, 137.49, 142.63, 147.96, 151.56, 153.50, 167.94; UV (acetonitrile) λ_{max} (ϵ) 300 nm (16.5 mM^{-1} cm^{-1}). Anal. Calcd for $C_{16}H_{15}N_7O_7$: C, 46.05; H, 3.62; N, 23.49. Found: C, 46.18; H, 3.73; N, 23.42.

***O*^{2}-(2,4-Dinitrophenyl) 1-{4-[2-(4-{2-Methylpropyl}phenyl)-propionyl]piperazin-l-yl}diazen-1-ium-1,2-diolate (3d) (CB-3-132).** To a solution of 112 mg of **3a** in dichloromethane was added 2.4 equiv of triethylamine. The solution was cooled to 0 °C with stirring, and an equimolar amount of 4-isobutyl-α-methylphenylacetic acid chloride was added. The reaction was carried out as described above to yield pure **3d** (232 mg): mp 54−58 °C; ^{1}H NMR ($CDCl_3$) δ 0.86 (d, 6 H, J = 6.6 Hz), 1.41 (d, 3 H, J = 6.8 Hz), 1.76−1.90 (m, 1 H), 2.44 (d, 2 H, J = 7.2 Hz), 2.75−4.12 (m, 8 H), 3.84 (q, 1 H, J = 6.8 Hz), 7.12 (s, 4 H), 7.58 (d, 1 H, J = 9.2 Hz), 8.40−8.46 (dd, 1 H, J = 2.8 and 9.3 Hz), 8.87 (d, 1 H, J = 2.7 Hz); ^{13}C NMR ($CDCl_3$) δ 20.58, 22.31, 30.13, 40.23, 43.25, 43.71, 44.93, 50.01, 50.46, 117.59, 122.16, 126.75, 129.01, 129.94, 137.33, 138.57, 140.72, 142.43, 153.62, 172.26; UV (EtOH) λ_{max} (ϵ) 300 nm (20.9 mM^{-1} cm^{-1}). Anal. Calcd for $C_{23}H_{28}N_6O_7$: C, 55.19; H, 5.64; N, 16.79. Found: C, 55.59; H, 5.87; N, 16.34.

***O*^{2}-(2,4-Dinitrophenyl) 1-[4-(2-Acetoxybenzoyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (3e) (CB-4-69).** Compound **3a** was acylated with an equimolar amount of acetylsalicylic acid chloride as described above. The product was recrystallized from ether:petroleum ether:acetone to yield pale yellow crystals: mp 149−150 °C; ^{1}H NMR (DMSO-d_6) δ 2.25 (s, 3 H), 3.42 (s, b, 2 H), 3.63−3.85 (m, 6 H), 7.25−7.27 (m, 1 H), 7.35−7.39 (m, 1 H), 7.42−7.45 (m, 1 H), 7.50−7.54 (m, 1 H), 7.94 (d, 1 H, J = 9.3 Hz), 8.54−8.58 (dd, 1 H J = 2.7 and 9.3 Hz), 8.87 (d, 1 H, J = 2.7 Hz); ^{13}C NMR (DMSO-d_6) δ 168.72, 165.24, 152.52, 146.47, 142.07, 136.76, 130.45, 129.58, 128.65, 127.70, 126.02, 123.08, 121.64, 118.01, 49.85, 49.74, 44.79, 30.66; UV (acetonitrile) λ_{max} (ϵ) 300 nm (22.1 mM^{-1} cm^{-1}). Anal. Calcd for $C_{19}H_{18}N_6O_9$: C, 48.11; H, 3.82; N, 17.72. Found: C, 48.20; H, 4.02; N, 17.14.

***O*^{2}-(2,4-Dinitrophenyl) 1-[4-(*N*-Acetylglycinyl)piperazin-1-yl]-diazen-1-ium-1,2-diolate (3i) (CB-4-9).** Compound **3a** was acylated with an equimolar amount of acetylglycine succinimidyl ester as described above for similar acylations: mp 131−133 °C; ^{1}H NMR ($CDCl_3$) δ 1.88 (s, 3 H), 3.64 (b, 4 H), 3.68 (b, 4 H), 3.99 (d, 2 H, J = 5.6 Hz), 7.94 (d, 1 H, J = 9.4 Hz), 7.98−8.02 (m, 1 H), 8.55−8.58, 1 H, J = 2.83 and 9.36 Hz), 8.87 (d, 1 H, J = 2.7 Hz); ^{13}C NMR ($CDCl_3$) δ 22.40, 42.32, 49.77, 49.90, 118.14, 121.78, 129.71, 136.90, 142.20, 152.73, 167.37, 169.36; UV (acetonitrile) λ_{max} (ϵ) 300 nm (14.2 mM^{-1} cm^{-1}). Anal. Calcd for $C_{14}H_{17}N_7O_8$: C, 40.88; H, 4.17; N, 23.84. Found: C, 40.75; H, 4.22; N, 23.62.

Sodium 1-(4-Acetylpiperazin-1-yl)diazen-1-ium-1,2-diolate (CB-4-130). A solution of 2.65 g (20.7 mmol) of 1-acetylpiperazine in 5 mL of methanol and 50 mL of ether was placed in a Parr bottle and treated with 4.73 mL of 25% sodium methoxide in methanol. The solution was flushed with nitrogen and charged with 40 psi of nitric oxide. After 24 h, the pressure was released and the white solid was collected by filtration, washed with ether, and dried under vacuum to give 17.6 g of product: NMR (D_2O) δ 2.18 (s, 3 H), 3.13−3.20 (m, 4 H), 3.75−3.77 (m, 4 H). The product was used without further purification for the arylation step.

***O*^{2}-(2,4-Dinitrophenyl) 1-(4-Acetylpiperazin-1-yl)diazen-1-ium-1,2-diolate (3j) (CB-4-132).** A solution of 587 mg (2.8 mmol) of **CB-4-130**, the diazeniumdiolate of 1-acetyl-piperazine, in dilute aqueous sodium bicarbonate was arylated with 1 equiv of 2,4-dinitrofluorobenzene according to method A. The isolated product was recrystallized from ethanol:acetonitrile to give 763 mg (68%) of pure **3j**: mp 153−154 °C; ^{1}H NMR (DMSO-d_6) δ 2.06 (s, 3 H), 3.61−3.68 (m, 8 H), 7.93 (d, 1 H, J = 9.3 Hz), 8.54−8.58 (dd, 1 H, J = 2.75 and 9.38 Hz), 8.87 (d, 1 H, J = 2.7 Hz); ^{13}C NMR (DMSO-d_6) δ 43.88, 49.81, 50.03, 118.09, 121.79, 129.72, 136.87, 142.18, 152.76, 168.45; UV (acetonitrile) λ_{max} (ϵ) 394 nm (16.1 mM^{-1} cm^{-1}). Anal. Calcd for $C_{12}H_{14}N_6O_7$: C, 40.68; H, 3.98; N, 23.72. Found: C, 40.89; H, 4.05; N, 23.62.

***O*^{2}-(2,4-Dinitrophenyl) 1-[4-(*N*,*N*-Dimethylaminosulfonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (3k) (JS-41-92).** To a slurry of 108 mg (0.31 mmol) of **3a** in 5 mL of dichloromethane was added 33 μL (0.31 mmol) of dimethyl sulfamoyl chloride. To the resulting slurry was added 139 μL (0.64 mmol) of triethylamine. The homogeneous solution that formed was stirred for 3 h. The solution was then washed with 1 M hydrochloric acid, dried over sodium sulfate, filtered through magnesium sulfate, and evaporated to give 114 mg of an oil that crystallized on standing. The product was recrystallized from ether:petroleum ether: mp 146−147 °C; ^{1}H NMR ($CDCl_3$) δ 2.87 (s, 6 H), 3.48−3.52 (m, 4 H), 3.71−3.74 (m, 4 H), 7.66 (d, 1 H, J = 9.24 Hz), 8.46−8.49 (dd, 1 H, J = 2.75 and 9.23 Hz), 8.89 (d, 1 H, J = 2.74 Hz); ^{13}C NMR ($CDCl_3$) δ 38.22, 44.90, 50.18, 117.79, 122.19, 129.08, 137.46, 142.56, 153.6; UV (ethanol) λ_{max} (ϵ) 299 nm (10.2 mM^{-1} cm^{-1}). Anal. Calcd for $C_{12}H_{17}N_7O_8S$: C, 34.37; H, 4.09; N, 23.38; S, 7.65. Found: C, 34.22; H, 4.07; N, 22.98; S, 7.75.

Sodium 1-[4-(Ethoxycarbonyl)-1,4-diazacycloheptan-1-yl]diazen-1-ium-1,2-diolate (JS-35-167). A solution of 4 g (0.023 mol) of 1-ethoxycarbonyl-1,4-diazacycloheptane[31] in 4.6 mL of 30% methanolic sodium methoxide, 5 mL of methanol, and 15 mL of ether was placed in a Parr bottle. The homogeneous solution was purged with nitrogen, charged with 50 psi of nitric oxide, and stirred at room temperature overnight. The pressure was released, and the product was collected by filtration, washed with copious amounts of ether, and dried under vacuum to give 1.36 g of diazeniumdiolate sodium salt: mp 149−150 °C; ^{1}H NMR (D_2O/$^-$OD) δ 1.28 (t, 3 H, J = 7.0 Hz), 2.78−2.82 (m, 2 H), 3.19−3.23 (m, 2 H), 3.27−3.29 (m, 2 H), 3.43−3.69 (m, 4 H), 4.17 (q, 2 H, J = 7.0 Hz); ^{13}C NMR (D_2O/$^-$OD) δ 15.51, 26.97, 45.80, 46.60, 56.19, 56.62, 64.21, 159.65; UV (10 mM NaOH) λ_{max} (ϵ) 250 nm (7.88 mM^{-1} cm^{-1}); half-life 45 s in pH 7.4 buffer at 37 °C.

***O*^{2}-(2,4-Dinitrophenyl) 1-[(4-Ethoxycarbonyl)-2,4-diazacycloheptan-1-yl]diazen-1-ium-1,2-diolate (4e) (JS-36-25).** To a solution of 1.15 g (0.0045 mol) of sodium 1-[4-(ethoxycarbonyl)-1,4-diazacycloheptan-1-yl]diazen-1-ium-1,2-diolate in 15 mL of 5% aqueous sodium bicarbonate was added a slurry of 0.527 mL (0.0042 mol) of 2,4-dinitrofluorobenzene in 8 mL of *tert*-butyl alcohol as described in method A. The reaction mixture turned bright yellow, and a precipitate formed during stirring for 1 h. No change was observed beyond 1 h of stirring. The mixture was extracted with dichloromethane and washed subsequently with cold dilute hydrochloric acid and sodium bicarbonate. The organic solution was dried over sodium sulfate, filtered through magnesium sulfate, and evaporated under vacuum to give 1.42 g (79%) of **4e** as a glass that crystallized upon trituration with ether:petroleum ether: mp 110−111 °C; ^{1}H NMR ($CDCl_3$) δ 1.26 (t, 3 H, J = 7.1 Hz), 2.05−2.13 (m, 2 H), 3.45−3.55 (m, 2 H), 3.73−3.82 (m, 2 H), 3.92−3.98 (m, 4 H), 4.10−4.27 (q, 2 H, J = 7.1 Hz), 7.59−7.69 (m, 1 H), 8.43−8.48 (dd, 1 H, J = 2.7 and 9.2 Hz), 8.88 (d, 1 H, J = 2.75 Hz); ^{13}C NMR ($CDCl_3$) δ 14.63, 24.88, 25.71, 45.40, 45.97, 49.71, 51.68, 61.78, 117.26, 117.50, 122.12, 129.18, 141.91, 154.23; UV (ethanol) λ_{max} (ϵ) 308 nm (11.85 mM^{-1} cm^{-1}). Anal. Calcd for $C_{14}H_{18}N_6O_8$: C, 42.21; H, 4.55; N, 21.10. Found: C, 42.16; H, 4.48; N, 20.99.

***O*^{2}-(2-Nitrophenyl) 1-(*N*,*N*-Diethylamino)diazen-1-ium-1,2-diolate (5a) (JS-30-4).** To a solution of 2.79 g (0.018 mol) of sodium 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO)

in 9 mL of DMSO was added a solution of 1.6 mL (0.015 mol) of 2-nitrofluorobenzene in 20 mL of tetrahydrofuran according to method B. The mixture was stirred at room temperature for 48 h. The solution was poured into ice−water and extracted with ether. The ether extract was dried over sodium sulfate, filtered, and evaporated to give 2.88 g of an oil containing mostly diethylnitrosamine and **5a**. The product mixture was chromatographed on silica gel using 2:1 dichloromethane:hexane as the eluant to give 548 mg of crystalline **5a**: mp 34−35 °C; ^{1}H NMR ($CDCl_3$) δ 1.20 (t, 6 H, J = 7.1 Hz), 3.38 (q, 4 H, J = 7.1 Hz), 7.25−7.26 (m, 1 H), 7.49−7.52 (m, 1 H), 7.57−7.53 (m, 1 H), 7.96−8.00 (dd, 1 H, J = 1.6 and 8.16 Hz); ^{13}C NMR ($CDCl_3$) δ 11.47, 47.68, 119.01, 124.51, 125.76, 134.32, 139.16, 149.50; UV (ethanol) λ_{max} (ϵ) 238 nm (8.8 mM^{-1} cm^{-1}). Anal. Calcd for $C_{10}H_{14}N_4O_4$: C, 47.24; H, 5.55; N, 22.04. Found: C, 47.45; H, 5.69; N, 22.23.

***O*^{2}-(2-Methylmercaptopyrimidin-4-yl) 1-(*N*,*N*-Diethylamino)-diazen-1-ium-1,2-diolate (5d) (JS-30-9).** Sodium 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) (3.84 g, 0.025 mol) in DMSO was arylated with 2.3 mL (0.02 mol) of 4-chloro-2-methylmercaptopyrimidine in tetrahydrofuran according to method C to give 4.8 g of a yellow-orange oil. Purification of the crude material was carried out on silica gel with 2:1 dichloromethane: hexane as the eluant. The product **5d** was obtained as an oil: ^{1}H NMR ($CDCl_3$) δ 1.23 (t, 6 H, J = 7.19 Hz), 2.56 (s, 3 H), 3.51 (q, 4 H, J = 7.19 Hz), 6.74 (d, 1 H, J = 5.6 Hz), 8.42 (d, 1 H, J = 5.6 Hz); ^{13}C NMR ($CDCl_3$) δ 11.35, 14.10, 47.15, 100.81, 158.97, 167.82, 173.18; UV (ethanol) λ_{max} (ϵ) 301 nm (17.3 mM^{-1} cm^{-1}). Anal. Calcd for $C_9H_{15}N_5O_2S$: C, 42.01; H, 5.88; N, 27.22; S, 12.46. Found: C, 42.24; H, 6.06; N, 27.44; S, 12.45.

Preparation of Bis-diazeniumdiolate 6b (CB-4-36). Two equivalents of sodium1-[4-(ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate **1a** was reacted with 1,5-difluoro-2,6-dinitrobenzene according to method A. Purification was carried out using a Flash 40 system utilizing a 10:1 dichloromethane:ethyl acetate solvent system. Two major fractions were collected; the least polar fraction contained the mono adduct **6a** (see experiment below). The second fraction contained the bis-adduct **6b** and it was further purified by recrystallization from ethanol: mp 125−126 °C; ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 6 H, J = 7.1 Hz), 3.58−3.62 (m, 8 H), 3.63−3.75 (m, 8 H), 4.19 (q, 4 H, J = 7.1 Hz), 7.53 (s, 1 H), 8.81 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 14.59, 42.11, 50.55, 62.09, 106.32, 125.39, 132.46, 153.63, 154.97; UV (methanol) λ_{max} (ϵ) 288 nm (24.8 mM^{-1} cm^{-1}). Anal. Calcd for $C_{20}H_{28}N_{10}O_{12}$: C, 40.00; H, 4.70; N, 23.33. Found: C, 40.24; H, 4.54; N, 23.04.

Isolation of Monodiazeniumdiolate 6a (CB-4-39). The first fraction obtained from the experiment above was concentrated under vacuum and the residue was recrystallized from 50% ethanol in water: mp 102−104 °C; ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3 H, J = 7.1 Hz), 3.64−3.67 (m, 4 H), 3.72−3.75 (m, 4 H), 4.19 (q, 2 H, J = 7.1 Hz), 7.43 (d, 1 H, J = 11.5 Hz), 8.92 (d, 1 H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 14.59, 42.14, 50.48, 62.11, 106.60, 106.87, 125.50, 132.86, 154.95, 157.19, 159.93; UV (ethanol) λ_{max} (ϵ) 296 nm (17.5 mM^{-1} cm^{-1}). Anal. Calcd for $C_{13}H_{15}FN_6O_8$: C, 38.81; H, 3.76; N, 20.89. Found: C, 39.08; H, 3.88; N, 20.74.

***O*^{2}-(2,4-Dinitro-5-fluorophenyl) 1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diolate (11a) (JS-41-117).** A partial solution of 4.03 g (0.0197 mol) of 1,5-difluoro-2,4-dinitrobenzene in 50 mL of *tert*-butyl alcohol was placed in a 250-mL round-bottom flask and stirred at room temperature. A solution of 2.92 g (0.023 mol) of sodium 1-(*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (DMA/NO) in 50 mL of 5% sodium bicarbonate was added dropwise at a rapid rate. A thick yellow solid formed as the diazeniumdiolate was added. Upon completion of the addition, the slurry was stirred for 15 min at room temperature. Water was added, and the product was collected by filtration and recrystallized from ethanol to give 2.3 g (40%) of **11a**: mp 129−130 °C; ^{1}H NMR ($CDCl_3$) δ 3.32 (s, 6 H), 7.43 (d, 1 H, J = 7.4 Hz), 8.92 (d, 1 H, J = 7.6 Hz); ^{13}C NMR ($CDCl_3$) δ 41.94, 106.58, 106.86, 125.74, 155.48, 157.58, 160.30; UV (ethanol) λ_{max} (ϵ) 300 nm (22.1 mM^{-1} cm^{-1}). Anal. Calcd for $C_8H_8FN_5O_6 \cdot {}^1/_3H_2O$: C, 32.55; H, 2.96; N, 23.73. Found: C, 32.90; H, 2.84; N, 23.36.

***O*^{2}-(2,4-Dinitro-5-hydroxyphenyl) 1-(*N*,*N*-Dimethylamino)-diazen-1-ium-1,2-diolate (11b) (JS-42-25).** To a partial solution of 510 mg (1.76 mmol) of **11a** in 30 mL of *tert*-butyl alcohol was added 10 mL of 3 M aqueous sodium hydroxide, resulting in a homogeneous solution. No starting material remained after 1.5 h. At this time the reaction mixture was made acidic with 3 M hydrochloric acid and concentrated on a rotary evaporator. The aqueous solution was extracted with dichloromethane and the acidic component (phenol) was extracted into 5% sodium bicarbonate solution. The aqueous layer was acidified with aqueous hydrochloric acid, extracted with dichloromethane, dried over sodium sulfate, filtered through magnesium sulfate, and concentrated under vacuum to give 421 mg (83%) of an orange solid. The product was recrystallized from ether:petroleum ether: mp 105−106 °C; ^{1}H NMR ($CDCl_3$) δ 3.30 (s, 6 H), 7.19 (s, 1 H), 8.97 (s, 1 H), 11.10 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 40.72, 104.58, 124.16, 126.45, 129.45, 155.43, 158.25; UV (ethanol) λ_{max} (ϵ) 279 nm (16.3 mM^{-1} cm^{-1}) and 388 nm (9.5 mM^{-1} cm^{-1}). Anal. Calcd for $C_8H_9N_5O_7$: C, 33.46; H, 3.16; N, 24.39. Found: C, 33.58; H, 3.18; N, 24.21.

2,4-Dinitro-1-fluoro-5-[*N*-methyl-(*p*-methoxyphenyl)amino]-benzene (JS-25-119). To a solution of 1.87 g (0.0092 mol) of 1,5-difluoro-2,4-dinitrobenzene in 10 mL of tetrahydrofuran was added 1 g (0.0095 mol) of anhydrous sodium carbonate. The slurry was flushed with nitrogen followed by the dropwise addition of 1.26 g (0.0092 mol) of *N*-methylanisidine in 10 mL of tetrahyrofuran and stirred overnight at room temperature. The reaction mixture was evaporated to dryness under vacuum and the residue was extracted with dichloromethane, dried over sodium sulfate, and filtered through a layer of magnesium sulfate to give 2.8 g of an orange solid that was recrystallized from ethanol: mp 138−140 °C; ^{1}H NMR ($CDCl_3$) δ 3.38 (s, 3 H), 3.82 (s, 3 H), 6.76 (d, 1 H, J = 13.62 Hz), 6.88−6.92 (m, 2 H), 7.04−7.09 (m, 2 H), 8.57 (d, 1 H, J = 7.78); ^{13}C NMR ($CDCl_3$) δ 43.54, 55.06, 106.94, 107.20, 115.42, 125.87, 126.61, 138.51, 148.49, 156.66, 158.54, 159.35; UV (ethanol) λ_{max} (ϵ) 246 nm (13.5 mM^{-1} cm^{-1}) and 374 nm (13.7 mM^{-1} cm^{-1}). Anal. Calcd for $C_{14}H_{12}N_3O_5F$: C, 52.34; H, 3.76; N, 13.08. Found: C, 52.59; H, 3.80; N, 12.98.

***O*^{2}-[2,4-Dinitro-5-(*N*-methyl-4-methoxyphenylamino)phenyl] 1-(*N*,*N*-Dimethylamino)diazen-1-ium-1,2-diolate (11c) (JS-25-147).** To a solution of 2.56 g (0.020 mol) of DMA/NO in 20 mL of 5% aqueous sodium bicarbonate was added a partial solution of 2.63 g (0.008 mol) of 2,4-dinitro-1-fluoro-5-[*N*-methyl-(*p*-methoxyphenyl)amino]benzene in 75 mL of tetrahydrofuran and 75 mL of *tert*-butyl alcohol. The resulting reaction mixture was stirred at room temperature for 48 h. The tetrahydrofuran was removed on a rotary evaporator and replaced with 200 mL of dichloromethane, and the solution was filtered into a 500-mL separatory funnel. The organic solution was washed with water, dried over sodium sulfate, filtered through magnesium sulfate, and evaporated to give 1.0 g of a red solid. The crude product was recrystallized from ethanol to give 1.44 g of pure **11c** as red-orange crystals: mp 185−187 °C; ^{1}H NMR ($CDCl_3$) δ 3.17 (s, 6 H), 3.38 (s, 3 H), 3.81 (s, 3 H), 6.87−6.91 (m, 2 H), 6.95 (s, 1 H), 7.05−7.09 (m, 2 H), 8.59 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 41.89, 43.21, 55.51, 106.29, 115.23, 125.74, 126.91, 127.86, 133.36, 139.15, 148.10, 153.33, 158.15; UV (ethanol) λ_{max} (ϵ) 250 (6.8 mM^{-1} cm^{-1}), 286 (7.5 mM^{-1} cm^{-1}), 374 (5.9 mM^{-1} cm^{-1}). Anal. Calcd for $C_{16}H_{18}N_6O_7$: C, 47.29; H, 4.46; N, 20.68. Found: C, 47.57; H, 4.85; N, 20.51.

HL-60 Screen: Cell Culture, Viability Assay, and IC_{50} Determination. Human myeloid leukemia HL-60 cells (ATCC, Manassas, VA) were cultured at a density of 1.5×10^5 mL^{-1} in RPMI-1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. Cultures were initiated in 96-well plates in a total volume of 100 μL. Five millimolar stock solutions of each compound were made in DMSO and serially diluted in PBS before addition to the cultures. We established dose−response cultures with concentrations ranging from 0 to 100 μM. The final concentration of DMSO added to the cultures was 0.1% or less. For each experiment, compounds were added to cells in logarithmic phase

growth. Three days after addition of the compounds, cell viability was measured using the CellTiter 96 assay from Promega (Madison, WI) according to the manufacturer's protocol. For each compound screened, there were at least three replicates. The IC_{50} of each compound was determined from growth curves generated by expressing the absorbance of each variable as a percentage of that of untreated controls.

Anticancer Screen of *O*^{2}-Aryl Diazeniumdiolate Analogues against the NCI 51 Human Cancer Cell Lines. The anticancer screening test was conducted as previously described.[10,32] Cells from nine cancer subpanels of 51 human lines were grown in RPMI 1640 culture medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine for 24 h at 37 °C to allow stabilization prior to addition of the *O*^{2}-aryl diazeniumdiolates. The nine human cancer subpanels are leukemia, non-small-cell lung, colon, melanoma, ovarian, renal, prostate, breast, and central nervous system cancer. Stock solutions of the analogues in DMSO were serially diluted with the RPMI 1640 medium and added immediately to the microtiter plates to produce five concentrations of the analogues, i.e., 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M. Each analogue was incubated with cells for 48 h. At the end of incubation, the cells were fixed in situ with 10% trichloroacetic acid, washed five times with water, and dried. Sulforhodamine B (0.4% in 1% acetic acid) was added to each well. After incubation for 10 min at room temperature, unbound sulforhodamine B was removed by washing five times with acetic acid. Then the plates were air-dried. Bound stain was solubilized with Tris buffer, and the optical densities were read at 515 nm. The optical densities generated from sulforhodamine B staining are a function of cell mass and growth rate. The dose−response curve (not shown) was created by plotting the percent growth against the log of concentrations of the corresponding analogue for each cell line and grouped by disease subpanels. The molar concentrations that caused 50% growth inhibition (GI_{50}), total growth inhibition (TGI), and 50% cell killing (LC_{50}) of the 51 cell lines were determined.

In Vivo Study in Rats. JM-1 rat hepatoma cells, which were derived from and established to grow in Fischer rats,[33] were a gift (G. Michalopoulos, University of Pittsburgh). Two-month-old male Fischer rats were obtained from Hilltop Labs (Scottsdale, PA). 2′-Amino-3′-methoxyflavone was purchased as PD 98059 from Calbiochem (La Jolla, CA) and PTIO was from Sigma (St. Louis, MO). JM-1 cells were cultured in minimum essential medium (Life Technology, Gaithersburg, MD) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The medium contained 10% fetal bovine serum. Cells were harvested after trypsinization, washed twice with cold PBS, and suspended at a concentration of 10^6 cells/mL in PBS. Five groups (four rats per group) were used for the experiments. One million cells were injected into each rat's liver through the mesenteric vein. A stock solution of JS-K was prepared at a concentration of 10 mg/mL in DMSO. One week after cell transplantation in the rat's liver, JS-K was injected intraperitoneally in three groups five times, at a dose of 0, 3, or 30 mg/kg of rat body weight every other day. Control rats received the same volume of DMSO as those in the other groups. Five milliliters of 75 μM 2′-amino-3′-methoxyflavone or PTIO in 0.37% DMSO was injected intravenously in the remaining two groups through the mesenteric vein 1 h before JS-K (30 mg/kg) treatment. Rats were sacrificed 3 weeks after tumor cell injection and 2 weeks after the start of JS-K treatment. Tumor-bearing livers were removed and the tumors were excised and weighed. The statistical significance of the difference between different treatments was determined by the *t*-test.

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

References

(1) Shami, P. J.; Saavedra, J. E.; Wang, L. Y.; Bonifant, C. L.; Diwan, B. A.; Singh, S. V.; Gu, Y.; Fox, S. D.; Buzard, G. S.; Citro, M. L.; Waterhouse, D. J.; Davies, K. M.; Ji, X.; Keefer, L. K. JS-K, a glutathione/glutathione S-transferase-activated nitric oxide donor of the diazeniumdiolate class with potent antineoplastic activity. *Mol. Cancer Ther.* **2003**, *2*, 409−417.

(2) Udupi, V.; Yu, M.; Malaviya, S.; Saavedra, J. E.; Shami, P. J. JS-K, a nitric oxide prodrug, induces cytochrome *c* release and caspase activation in HL-60 myeloid leukemia cells. *Leukemia Res.* **2006**, *E Published*.

(3) Shami, P. J.; Kaur, G.; Thillainathan, J.; Jia, L.; Saavedra, J. E.; Keefer, L. K. JS-K, a novel nitric oxide (NO) generator, shows potent anti-angiogenic activity. *Blood* **2004**, *104* (Suppl. 1), 931a.

(4) Ren, Z.; Kar, S.; Wang, Z.; Wang, M.; Saavedra, J. E.; Carr, B. I. JS-K, a novel nonionic diazeniumdiolate derivative, inhibits Hep 3B hepatoma cell growth and induces c-Jun phosphorylation via multiple MAP kinase pathways. *J. Cell. Physiol.* **2003**, *197*, 426−434.

(5) Liu, J.; Li, C.; Qu, W.; Leslie, E.; Bonifant, C. L.; Buzard, G. S.; Saavedra, J. E.; Keefer, L. K.; Waalkes, M. P. Nitric oxide prodrugs and metallochemotherapeutics: JS-K and CB-3-100 enhance arsenic and cisplatin cytolethality by increasing cellular accumulation. *Mol. Cancer Ther.* **2004**, *3*, 709−714.

(6) Townsend, D. M.; Tew, K. D. The role of glutathione-*S*-transferase in anti-cancer drug resistance. *Oncogene* **2003**, *22*, 7369−7375.

(7) Shami, P. J.; Sauls, D. L.; Weinberg, J. B. Schedule and concentration-dependent induction of apoptosis in leukemia cells by nitric oxide. *Leukemia* **1998**, *12*, 1461−1466.

(8) Ju, C.; McCoy, J. P.; Chung, C. J.; Graf, M. L. M.; Pohl, L. R. Tolerogenic role of Kupffer cells in allergic reactions. *Chem. Res. Toxicol.* **2003**, *16*, 1514−1519.

(9) Saavedra, J. E.; Srinivasan, A.; Bonifant, C. L.; Chu, J.; Shanklin, A. P.; Flippen-Anderson, J. L.; Rice, W. G.; Turpin, J. A.; Davies, K. M.; Keefer, L. K. The secondary amine/nitric oxide complex ion $R_2N[N(O)NO]^-$ as nucleophile and leaving group in S_NAr reactions. *J. Org. Chem.* **2001**, *66*, 3090−3098.

(10) Jia, L.; Tomaszewski, J. E.; Furchgott, R. F.; Jothianandan, D.; Keefer, L. K.; Shami, P. J. EDRF-like activity and anticancer spectrum of JS-K, a novel diazeniumdiolate nitric oxide donor. *Proc. Am. Assoc. Cancer Res.* **2003**, *44*, 919 (abstract 4619).

(11) Deora, A. A.; Hajjar, D. P.; Lander, H. M. Recruitment and activation of Raf-1 kinase by nitric oxide-activated Ras. *Biochemistry* **2000**, *39*, 9901−9908.

(12) Ingram, A. J.; James, L.; Thai, K.; Ly, H.; Cai, L.; Scholey, J. W. Nitric oxide modulates mechanical strain-induced activation of p38 MAPK in mesangial cells. *Am. J. Physiol. Renal. Physiol.* **2000**, *279*, F243−F251.

(13) Go, Y.-M.; Patel, R. P.; Maland, M. C.; Park, H.; Beckman, J. S.; Darley-Usmar, V. M.; Jo, H. Evidence for peroxynitrite as a signaling molecule in flow-dependent activation of c-Jun NH_2-terminal kinase. *Am. J. Physiol.* **1999**, *277*, H1647−H1653.

(14) Go, Y.-M.; Boo, Y. C.; Park, H.; Maland, M. C.; Patel, R.; Pritchard, K. A., Jr.; Fujio, Y.; Walsh, K.; Darley-Usmar, V.; Jo, H. Protein kinase B/Akt activates c-Jun NH_2-terminal kinase by increasing NO production in response to shear stress. *J. Appl. Physiol.* **2001**, *91*, 1574−1581.

(15) Caselli, A.; Camici, G.; Manao, G.; Moneti, G.; Pazzagli, L.; Cappugi, G.; Ramponi, G. Nitric oxide causes inactivation of the low molecular weight phosphotyrosine protein phosphatase. *J. Biol. Chem.* **1994**, *269*, 24878−24882.

(16) Callsen, D.; Sandau, K. B.; Brüne, B. Nitric oxide and superoxide inhibit platelet-derived growth factor receptor phosphotyrosine phosphatases. *Free Radical Biol. Med.* **1999**, *26*, 1544−1553.

(17) Xian, M.; Wang, K.; Chen, X.; Hou, Y.; McGill, A.; Chen, X.; Zhang, Z.-Y.; Cheng, J.-P.; Wang, P. G. Inhibition of protein tyrosine phosphatases by low-molecular-weight S-nitrosothiols and S-nitrosylated human serum albumin. *Biochem. Biophys. Res. Commun.* **2000**, *268*, 310−314.

(18) Traverse, S.; Gomez, N.; Paterson, H.; Marshall, C.; Cohen, P. Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem. J.* **1992**, *288* (Pt 2), 351−355.

(19) Osada, S.; Osada, K.; Carr, B. I. Tumor cell growth inhibition and extracellular signal-regulated kinase (ERK) phosphorylation by novel K vitamins. *J. Mol. Biol.* **2001**, *314*, 765−772.

(20) Mendes, A. F.; Carvalho, A. P.; Caramona, M. M.; Lopes, M. C. Role of nitric oxide in the activation of NF-kappaB, AP-1 and NOS II expression in articular chondrocytes. *Inflamm. Res.* **2002**, *51*, 369−375.
(21) Ishii, Y.; Ogura, T.; Tatemichi, M.; Fujisawa, H.; Otsuka, F.; Esumi, H. Induction of matrix metalloproteinase gene transcription by nitric oxide and mechanisms of MMP-1 gene induction in human melanoma cell lines. *Int. J. Cancer* **2003**, *103*, 161−168.
(22) Taimor, G.; Rakow, A.; Piper, H. M. Transcription activator protein 1 (AP-1) mediates NO-induced apoptosis of adult cardiomyocytes. *FASEB J.* **2001**, *15*, 2518−2520.
(23) Fan, M.; Goodwin, M. E.; Birrer, M. J.; Chambers, T. C. The c-Jun NH_2-terminal protein kinase/AP-1 pathway is required for efficient apoptosis induced by vinblastine. *Cancer Res.* **2001**, *61*, 4450−4458.
(24) Henry, Y.; Lepoivre, M.; Drapier, J.-C.; Ducrocq, C.; Boucher, J.-L.; Guissani, A. EPR characterization of molecular targets for NO in mammalian cells and organelles. *FASEB J.* **1993**, *7*, 1124−1134.
(25) Saavedra, J. E.; Booth, M. N.; Hrabie, J. A.; Davies, K. M.; Keefer, L. K. Piperazine as a linker for incorporating the nitric oxide-releasing diazeniumdiolate group into other biomedically relevant functional molecules. *J. Org. Chem.* **1999**, *64*, 5124−5131.
(26) Drago, R. S.; Paulik, F. E. The reaction of nitrogen(II) oxide with diethylamine. *J. Am. Chem. Soc.* **1960**, *82*, 96−98.
(27) Keefer, L. K.; Nims, R. W.; Davies, K. M.; Wink, D. A. "NONOates" (1-substituted diazen-1-ium-1,2-diolates) as nitric oxide donors: Convenient nitric oxide dosage forms. *Methods Enzymol.* **1996**, *268*, 281−293.
(28) Saavedra, J. E.; Srinivasan, A.; Buzard, G. S.; Davies, K. M.; Waterhouse, D. J.; Inami, K.; Wilde, T. C.; Citro, M. L.; Cuellar, M.; Deschamps, J. R.; Parrish, D.; Shami, P. J.; Findlay, V. J.; Townsend, D. M.; Tew, K. D.; Singh, S.; Jia, L.; Ji, X.; Keefer, L. K. PABA/NO as an anticancer lead: Analogue synthesis, structure revision, solution chemistry, reactivity toward glutathione, and in vitro activity. *J. Med. Chem.* **2006**, *49*, 1157−1164.
(29) Stewart, H. W.; Turner, R. J.; Denton, J. J.; Kushner, S.; Brancone, L. M.; McEwen, W. L.; Hewitt, R. I.; Subbarow, Y. Experimental chemotherapy of filariasis. IV. The preparation of derivatives of piperazine. *J. Org. Chem.* **1948**, *13*, 134−143.
(30) Toldy, L.; Solyon, S.; Kocka, I.; Toth, G.; Toth, I. Thiourea derivatives with tuberculostatic action. II. Acylthiocarbamides. *Acta Chim. Acad. Sci. Hungaricae* **1971**, *69*, 221−227.
(31) Angier, R. B.; Murdock, K. C.; Curran, W. V.; Sollenberger, P. Y.; Casey, J. P. Antiviral agents. I. Analogues and derivatives of 2-diethylaminoethyl 4-methylpiperazine-1-carboxylate. *J. Med. Chem.* **1968**, *11*, 720−729.
(32) Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A.; Gray-Goodrich, M.; Campbell, H.; Mayo, J.; Boyd, M. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J. Natl. Cancer Inst.* **1991**, *83*, 757−766.
(33) Novicki, D. L.; Jirtle, R. L.; Michalopoulos, G. Establishment of two rat hepatoma cell strains produced by a carcinogen initiation, phenobarbital promotion protocol. *In Vitro* **1983**, *19*, 191−202.

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