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Structural modifications modulate stability of glutathione-activated arylated diazeniumdiolate prodrugs

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ABSTRACT

JS-K, a diazeniumdiolate-based nitric oxide (NO)-releasing prodrug, is currently in late pre-clinical development as an anti-cancer drug candidate. This prodrug was designed to be activated by glutathione (GSH) to release NO. To increase the potency of JS-K, we are investigating the effect of slowing the reaction of the prodrugs with GSH. Herein, we report the effect of replacement of nitro group(s) by other electron-withdrawing group(s) in JS-K and its homo-piperazine analogues on GSH activation and the drugs' biological activity. We show that nitro-to-cyano substitution increases the half-life of the prodrug in the presence of GSH without compromising the compound's in vivo anti-tumor activity.

1. Introduction

JS-K (**1**, Fig. 1), an arylated diazeniumdiolate prodrug, is currently in late pre-clinical development as an anti-cancer drug candidate.[1–5] JS-K was designed to be activated by glutathione (GSH) to release nitric oxide (NO), a potent bioregulatory agent. Structure–activity relation (SAR) studies have shown that a homo-piperazine analogue of JS-K, compound **2** (Fig. 1), exhibits almost identical in vitro activity and better in vivo efficacy.[5] Recently, we have shown that JS-K and **2** are potent cytotoxic agents against human non-small cell lung cancer (NSCLC) cell lines both in vitro and as xenografts in mice.[4,5]

To improve on these lead compounds, we are currently seeking to design JS-K analogues with diminished uncatalyzed reactivity towards GSH, which may subsequently improve their anti-cancer properties. The aryl group 'caging' the NO-generating diazeniumdiolate moiety is a key structural feature that controls this reaction. Herein, we report synthesis and evaluation of arylated diazeniumdiolate prodrugs with several possible combinations of electron-withdrawing groups.

2. Results and discussion

2.1. Synthesis

Arylated diazeniumdiolate prodrugs JS-K and **2** are activated by reaction with GSH to form NO. Earlier, we have reported that compound **2** exhibits anti-proliferative properties comparable to those of JS-K. However, it shows an extended half-life in the presence of GSH.[5,6] We hypothesized that slowing the reaction time with GSH might translate to a prolonged lifetime in the blood stream. This may lead to selective accumulation of the drug in the tumor, thus leading to better anti-tumor efficacy.

To pursue such delayed reaction with GSH, we planned to change the substituents in the aromatic ring of JS-K. SAR studies of JS-K show that both the electron-withdrawing groups in the aromatic ring are important for the activation by GSH and subsequent anti-proliferative activity.[2,6,7] Recently, we reported that adding electron-donating groups like methyl and methoxy in the aromatic ring slows down the reaction with GSH, but their in vivo efficacy was compromised.[5] Thus, the new analogues required two electron-withdrawing groups. We decided to replace one or both nitro group(s) of JS-K with one or two other electron-withdrawing functional group(s) like methoxycarbonyl, fluoro and cyano groups.

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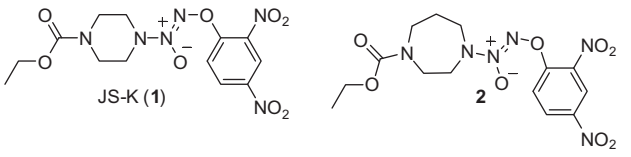
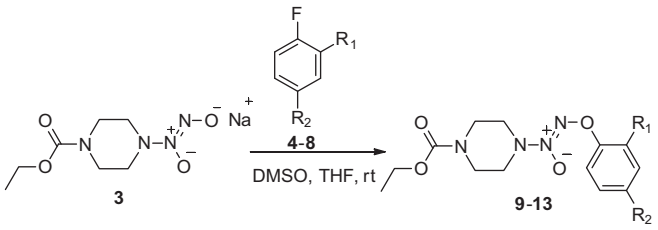


Figure 1. Structures of JS-K and its homo-piperazine analogue **2**.

Table 1
Synthesis of arylated diazeniumdiolate prodrugs **9–13**



Electrophile	R_1	R_2	Product	Yield (%)
4	COOMe	NO_2	**9**	63
5	F	NO_2	**10**	58
6	CN	CN	**11**	85
7	CN	NO_2	**12**	88
8	NO_2	CN	**13**	79

Diazeniumdiolate sodium salt **3**[2] was treated with substituted fluorobenzene derivatives **4–8** in DMSO/THF (Table 1). The reaction proceeded smoothly to afford new arylated diazeniumdiolate prodrugs **9–13** in good yields.

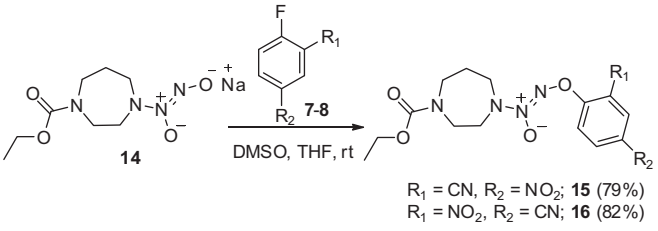
The homo-piperazine analogues of cyano-substituted prodrugs were synthesized by reacting diazeniumdiolate salt **14**[2] with fluorobenzene derivatives **7** and **8** to obtain **15** and **16**, respectively, in good yields (Scheme 1).

2.2. In vitro screening

The new arylated prodrugs **9–13** and **15–16** were screened for in vitro anti-proliferative activity against the H1703 human NSCLC cell line. With the exception of compound **11**, cyano-substituted prodrugs showed good anti-proliferative activity, with IC_{50} values in the low micromolar level, thus comparable to JS-K and compound **2** (Table 2). The rest of the compounds displayed IC_{50} >10 μM. Hence, only the prodrugs **12**, **13**, **15** and **16** were considered for further investigation.

2.3. NO release and GSH reaction kinetics

The GSH reaction kinetics for each of the shortlisted prodrugs were studied to determine the effect of modulating the electron-withdrawing groups on GSH reactivity. The decomposition of the chromophoric diazeniumdiolate functional group in presence of 4 mM GSH in 0.1 M phosphate buffer pH 7.4 was monitored over time (Table 2). Replacing nitro groups of the parent compound (JS-K) with cyano groups significantly increased the half-life of the prodrugs in GSH-containing buffer. The half-life for JS-K under identical conditions was 2.8 min; modifications described here led to a decrease in reactivity with GSH by approximately an order of magnitude, and indeed much more for dicyano compound **11**. Modest to moderate increases to the compound's stability resulted in improved in vitro efficacy, as indicated by the IC_{50} values for reduction in cell numbers for the NSCLC line H1703. However, extending the half-life to over 1 h resulted in a decrease in efficacy, suggesting that the rate of reaction with GSH is important for biological activity.



Scheme 1. Synthesis of prodrugs **15** and **16**.

The prodrugs which were stabilized toward GSH-induced decomposition while maintaining in vitro efficacy, that is, compounds **12**, **13**, **15** and **16**, were evaluated for their NO-releasing potential. GSH-activated nitric oxide yields were measured for these compounds using a chemiluminescence assay. All four compounds were found to release essentially quantitative amounts of NO on reaction with GSH (Table 2).

2.4. In vitro metabolism

As noted, the replacement of a nitro group of JS-K with a cyano group significantly retarded the reaction of each prodrug with GSH, *vide supra*. It was hypothesized that this would result in a slowing of metabolic clearance of the compound. As a proof of concept, the NSCLC cell line H1703 was treated with compound **12**, and the metabolism was followed at various time points via LC/MS. The extracted ion chromatograms in Figure 2 illustrate the decomposition of compound **12**, retention time 10.9 min, over 1 h concomitant with the formation of arylated GSH, retention time 5.8 min, as observed in the GSH reaction kinetics experiments. It is important to note that similar experiments in which cells were treated with JS-K demonstrate a near complete metabolic turnover of JS-K in as few as ten minutes, thus demonstrating the stabilizing effect of these structural modifications on the rate of cellular metabolism.

2.5. In vivo studies

NSCLC cell line H1703, with established in vivo sensitivity to JS-K,[4,5] was chosen for assessment of activity of compound **12** in vivo against xenografted cells in athymic mice. Compound **12** formulated in Pluronic P123[4,5] was administered at 20 μmol/kg via intraperitoneal (ip) injections, five times a week for a four-week period. Treatment with **12** significantly reduced growth of H1703 cells in vivo, when compared with cells in control animals treated with vehicle only (Fig. 3).

2.6. Stress signaling and apoptosis

Treatment with compound **12** activated the stress-activated protein kinese/c-jun terminal kinase (SAPK/JNK) stress signaling pathway. SAPK/JNK phosphorylation was observed after 4 h with the drug (Fig. 4), as well as phosphorylation of its downstream effector c-jun. Activating transcription factor 3 (ATF3) was also upregulated. We have shown previously that JNK/ATF3 pathway activation is necessary for triggering apoptosis in the cells treated with diazeniumdiolate-based NO-releasing compounds.[5] In the current experiment apoptosis was activated within 4 h of incubation with the drug, as evidenced by cleavage of PARP and caspases 3 and 7 (Fig. 4).

Table 2
GSH-induced NO-release rates for JS-K and its structural analogues

Compound	NO yield (mol)/mol of compound mean (SE)	λ_{max} Monitored (nm)	k_{obs} (s^{-1}) mean (SE)	$T_{½}$ (min) mean (SE)	IC_{50}[a] (μM) mean (SE)
JS-K	1.96 (0.03)	300	4.08 e^{-3} (1.45 e^{-4})	2.8 (0.1)	1.08 (0.12)
2	2.00 (0.04)	312	3.46 e^{-3} (1.04 e^{-4})	3.3 (0.1)	0.95 (0.16)
13	2.02 (0.06)	273	5.23 e^{-4} (4.20 e^{-5})	22.1 (3.1)	0.73 (0.10)
12	2.04 (0.01)	302	3. e^{-4} (3.72 e^{-5})	33.6 (1.7)	0.55 (0.08)
16	1.96 (0.04)	286	2.71 e^{-4} (3.80 e^{-5})	42.6 (0.6)	0.68 (0.10)
15	2.01 (0.03)	312	1.75 e^{-4} (1.04 e^{-4})	66.0 (4.0)	2.62 (0.18)
11	n.d.	273	1.26 e^{-5} (2.30 e^{-6})	918.0 (69.0)	>20.00

n.d. = not determined.
[a] H1703 human NSCLC cell line.

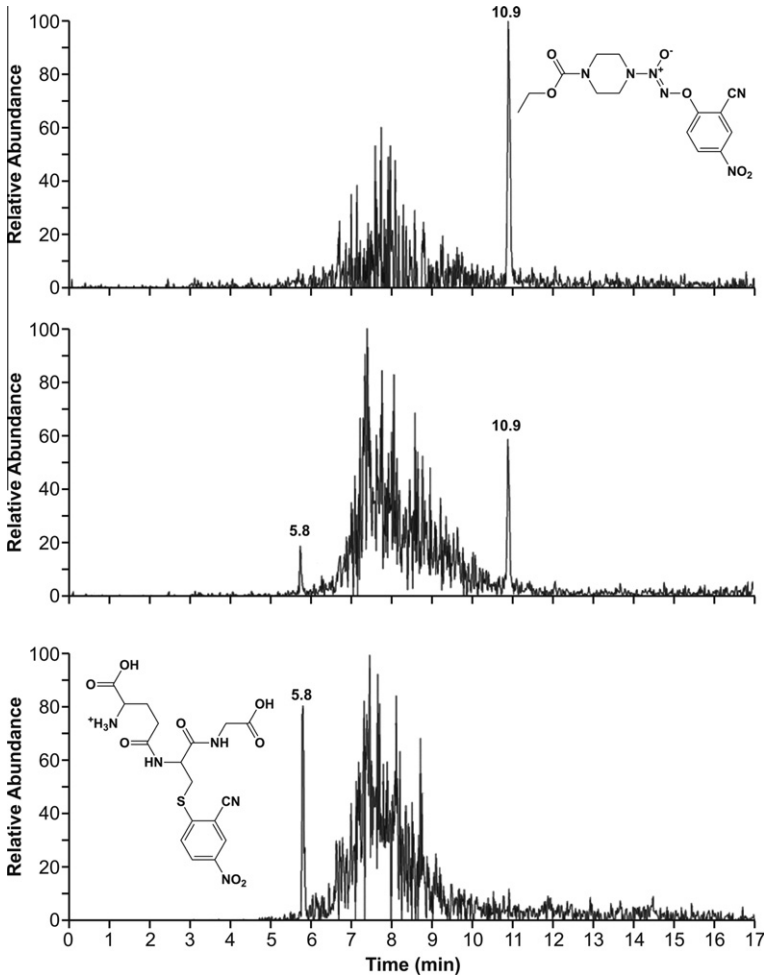


Figure 2. Extracted ion chromatogram showing decomposition of compound **12** in H1703 cells. Cells were treated with 5 μM compound **12** for 5, 30 and 60 min (top, middle and bottom panel, respectively).

2.7. Summary

JS-K, an arylated diazeniumdiolate prodrug, has shown promise as a selective anti-cancer agent. To delay the metabolic clearance of the compound we sought to modulate the rate of its reaction with GSH. To this end SAR studies have elucidated the effect of varying electron-withdrawing groups on drug efficacy. While decreasing the strength of the electron-withdrawing groups slows the aromatic substitution reaction with GSH, over-stabilization results in a loss of efficacy. We found that switching one nitro group to a cyano group increases the half-life of the prodrug in the presence of GSH without compromising the compound's in vitro and in vivo anti-tumor activity.

3. Experimental

3.1. Synthesis

3.1.1. General

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. The NMR spectra of compounds **15** and **16** were recorded at 65 °C in DMSO-d_6. Ultraviolet (UV) spectra were recorded on an Agilent Model 8453 or a Hewlett–Packard model 8451A diode array spectrophotometer. Infrared (IR) spectra were

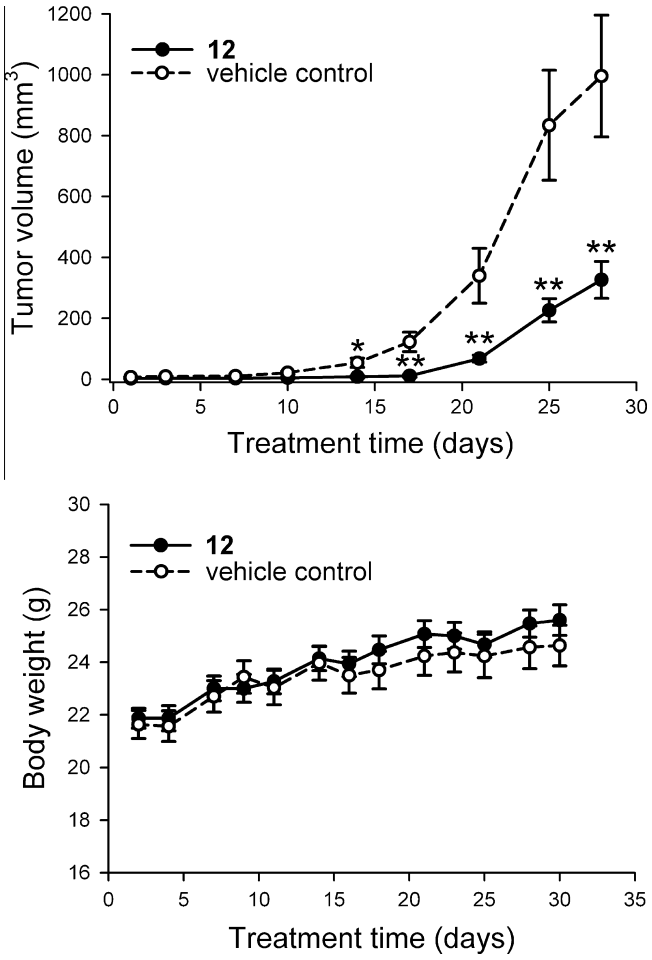


Figure 3. Compound **12** significantly reduced growth of H1703 cells in vivo. The compound was administered ip at 20 μmol/kg five times a week for four weeks, and tumors were measured with a caliper. Values are means (SE); $^{**}P$ <0.01; $^{*}P$ <0.05 by *t*-test with the Welch correction (top panel). Treatment did not affect body weights (bottom panel).

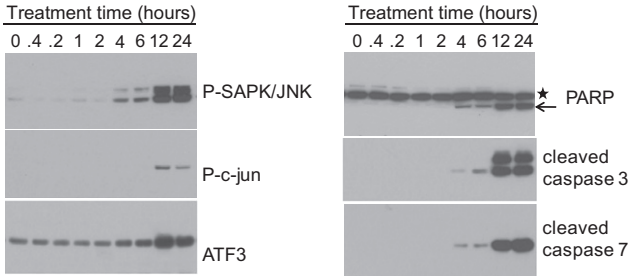


Figure 4. Treatment of H1703 cells with 1 μM compound **12** induced stress signaling and apoptosis in lung adenocarcinoma H1703 cells. Phosphorylation of SAPK/JNK and its downstream effectors c-jun and ATF3 and PARP cleavage/effector caspases 3 and 7 activation are shown by Western blot. The star indicates full length PARP protein, while the arrow indicates the 89-kDa cleaved fragment.

measured on a Buck Scientific Infrared Spectrophotometer Model 500. Elemental analyses were performed by Midwest Microlab (Indianapolis, IN). Chromatography was performed on a Biotage SP1 Flash Purification System. Prepacked silica gel flash chromatography columns were purchased from Silicycle (Quebec City, Canada). Compounds **1**[2] (JS-K), **2**,[2] **3**[2] and **14**[2] were prepared using reported methods.

3.1.2. General procedure for arylation of diazeniumdiolate salts

To a partial solution of the diazeniumdiolate **3** or **14** (1 equiv) in DMSO (4 mL/mmol of diazeniumdiolate salt) was added the fluoro compound (**4–8**) (1 equiv) in THF (2 mL/mmol of fluoro compound) at room temperature. The resulting solution was stirred at room temperature overnight. To this homogeneous solution, water was added (8 mL/mmol of diazeniumdiolate), producing a yellow precipitate that was collected by filtration, washed with water, and dried. The crude product was purified by flash column chromatography or by recrystallization.

3.1.3. Compound 9

[JS-59-16] mp 55–56 °C; UV (ethanol) λ_{max} (ε 304 nm 14.0 mM^{-1} cm^{-1}); ^{1}H NMR ($CDCl_3$) δ 1.29 (t, J = 7.1 Hz, 3H), 3.60–3.62 (m, 4H), 3.72–3.75 (m, 4H), 3.99 (s, 3H), 4.18 (q, J = 7.1 Hz, 2H), 7.53 (d, J = 9.0 Hz, 1H), 8.38 (dd, J = 9.0, 2.7 Hz, 1H), 8.80 (d, J = 2.7 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 163.4, 159.7, 155.0, 143.0, 128.7, 128.0, 119.7, 116.1, 62.0, 53.0, 50.8, 14.6. Anal. Calcd for $C_{15}H_{19}N_5O_8$: C, 45.34; H, 4.82; N, 17.63, found: C, 45.41; H, 4.94; N, 17.66.

3.1.4. Compound 10

[JS-59-165] mp 131–133 °C; UV (ethanol) λ_{max} (ε 308 nm 9.36 mM^{-1} cm^{-1}); ^{1}H NMR (DMSO-d_6) δ 1.21 (t, J = 7.1 Hz, 3H), 3.61–3.64 (m, 8H), 4.08 (q, J = 7.1 Hz, 2H), 7.77 (t, J = 8.9 Hz, 1H), 8.14–8.17 (m, 1H), 8.34 (dd, J = 10.9, 2.7 Hz, 1H); ^{13}C NMR (DMSO-d_6) (aromatic region is complex due to 13C–F couplings) δ 155.3, 154.8, 150.5, 149.2, 149.1, 148.0, 143.4, 143.3, 121.7, 121.7, 117.5, 113.6, 113.4, 61.5, 53.0, 50.2, 42.2, 14.9. Anal. Calcd for $C_{13}H_{16}FN_5O_6$: C, 43.70; H, 4.51; N, 19.60: F, 5.32, found: C, 43.50; H, 4.46; N, 19.56; F, 5.01.

3.1.5. Compound 11

[JS-59-145] mp 170–171 °C; UV (ethanol) λ_{max} (ε 275 nm 9.5 mM^{-1} cm^{-1}); IR (film) 3139 cm^{-1}, 3040, 2396, 1720, 1661, 1572, 1431; ^{1}H NMR (DMSO-d_6) δ 1.20 (t, J = 7.4 Hz, 3H), 3.60–3.61 (m, 4H), 3.64–3.66 (m, 4H), 4.07 (q, J = 7.4 Hz, 2H), 7.77 (d, J = 8.6 Hz, 1H), 8.24 (dd, J = 8.6, 2.0 Hz, 1H), 8.56 (d, J = 2.0 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 160.2, 154.8, 139.7, 139.0, 117.4, 116.9, 107.6, 100.8, 61.6, 50.1, 42.2, 14.9. Anal. Calcd for $C_{15}H_{16}N_6O_4{\cdot}0.25H_2O$: C, 51.43; H, 4.80; N, 24.41, found: C, 51.41; H, 4.51; N, 24.18.

3.1.6. Compound 12

[JS-59-4] mp 149–151 °C; UV (ethanol) λ_{max} (ε 302 nm 13.6 mM^{-1} cm^{-1}); IR (film) 3135 cm^{-1}, 3032, 2397, 1715, 1668, 1594, 1358; ^{1}H NMR ($CDCl_3$) δ 1.29 (t, J = 7.0 Hz, 3H), 3.64–3.67 (m, 4H), 3.73–3.75 (m, 4H), 4.18 (q, J = 7.0 Hz, 2H), 7.51 (d, J = 9.0 Hz, 1H), 8.24 (dd, J = 9.0, 2.3 Hz, 1H), 8.55 (d, J = 2.3 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 161.4, 155.0, 143.1, 129.9, 129.6, 116.1, 113.1, 101.6, 62.1, 50.5, 42.2, 14.6. Anal. Calcd for $C_{14}H_{16}N_6O_6$: C, 46.16; H, 4.43; N, 23.07, found: C, 46.05; H, 4.62; N, 23.08.

3.1.7. Compound 13

[JS-59-131] mp 146–147 °C; UV (ethanol) λ_{max} (ε 275 nm 16.0 mM^{-1} cm^{-1}); IR (film) 3139 cm^{-1}, 3039, 2397, 1715, 1571, 1485, 1264; ^{1}H NMR (DMSO-d_6) δ 1.20 (t, J = 7.1 Hz, 3H), 3.60 (br, 4H), 3.62–3.63 (m, 4H), 4.07 (q, J = 7.1 Hz, 2H), 7.89 (d, J = 8.9 Hz, 1H), 8.24 (dd, J = 8.9, 2.0 Hz, 1H), 8.67 (d, J = 2.0 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 154.8, 151.9, 139.2, 138.0, 130.6, 118.9, 117.2, 107.1, 61.6, 50.1, 42.19, 14.9. Anal. Calcd for $C_{14}H_{16}N_6O_6$: C, 46.16; H, 4.43; N, 23.07, found: C, 46.00; H, 4.71; N, 23.11.

3.1.8. Compound 15

[RN-3-46] UV (ethanol) λ_{max} (ε 310 nm 13.0 mM^{-1} cm^{-1}); IR (film) 3134 cm^{-1}, 3006, 2400, 1709, 1630, 1540, 1485, 1358, 1279; ^{1}H NMR (DMSO-d_6) δ 1.14 (t, J = 7.1 Hz, 3H), 1.90–1.95 (m,

2H), 3.43–3.46 (m, 2H), 3.67–3.70 (m, 2H), 3.94–3.96 (m, 2H), 4.00–4.05 (m, 4H), 7.66 (d, J = 9.3 Hz, 1H), 8.51 (dd, J = 9.3, 2.7 Hz, 1H), 8.74 (d, J = 2.7 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 161.9, 155.3, 142.9, 130.8, 115.8, 113.8, 99.9, 61.2, 50.7, 50.2, 45.5, 44.3, 25.2, 14.9. Anal. Calcd for $C_{15}H_{18}N_6O_6$: C, 47.62; H, 4.80; N, 22.21, found: C, 47.69; H, 4.79; N, 22.01.

3.1.9. Compound 16

[RN-3-101] UV (ethanol) λ_{max} (ε 286 nm 16.2 mM^{-1} cm^{-1}); IR (film) 3147 cm^{-1}, 3002, 2401, 1733, 1589, 1554, 1485,1360; ^{1}H NMR (DMSO-d_6) δ 1.12 (t, J = 6.9 Hz, 3H), 1.87–1.92 (m, 2H), 3.41–3.44 (m, 2H), 3.65–3.68 (m, 2H), 3.90–3.93 (m, 2H), 3.93–3.99 (m, 4H), 7.74 (d, J = 8.8 Hz, 1H), 8.15 (dd, J = 8.8, 2.0 Hz, 1H), 8.57 (d, J = 2.0 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 155.4, 152.4, 138.9, 130.3, 118.5, 117.1, 106.6, 61.2, 50.9, 50.2, 45.7, 44.3, 25.4, 14.8. Anal. Calcd for $C_{15}H_{18}N_6O_6$: C, 47.62; H, 4.80; N, 22.21, found: C, 47.49; H, 4.86; N, 22.19.

3.2. NO-release by chemiluminescence

Chemiluminescence detection and quantification of NO evolving from the reactions of each prodrug were conducted using a Sievers 280i Nitric Oxide Analyzer (NOA). A solution of pH 7.4 phosphate buffer containing 4 mM GSH at 37 °C was sparged with inert gas until a steady detector response was established. The NO release profile was followed over time after injecting each prodrug to start the reaction. The resulting curve was integrated to quantify the amount of NO released/mol of compound.

3.3. GSH reaction kinetics

Kinetic experiments were performed at 37 °C on a standard UV–visible spectrophotometer. Reactions were initiated by addition of substrate after temperature equilibration. Typical substrate concentrations were ~15 μM with 4 mM GSH in 0.1 M phosphate buffer, pH 7.4, containing 50 μM diethylenetriaminepentaacetic acid (DTPA). For prodrugs **12**, **13**, **15** and **16** the rate was derived by fitting the data to an exponential curve typical for first order processes. Prodrug **11** was evaluated with initial rate calculations.

3.4. In vitro metabolism

The H1703 cells were plated in 75-cm^2 flasks and incubated overnight at 37 °C. The cells were treated with 5 μM of compound **12** and incubated for 5, 30 and 60 min. At each time point the cells were lysed via scraping in 800 μL of 10 mM HCl followed by successive rounds of freezing and thawing. To the lysate was added 200 μL of a 5% 5-sulfosalicylic acid solution. The precipitate was removed by centrifugation at 8000×g for 10 min, and the supernatant was analyzed by LC/MS.

The system used for analysis is a Thermoquest Surveyor HPLC coupled with a Finnigan LCQ deca mass spectrometer. Positive ions were generated with an atmospheric pressure chemical ionization (APCI) source with a capillary voltage of 15 V and a corona discharge of 4 μA. Separations were performed on an Agilent Eclipse XDB-C18 5-μm 4.6 × 150 mm column at a flow rate of 1 mL/min under H_2O/acetonitrile/0.1% formic acid gradient conditions.

3.5. Biological evaluation

3.5.1. Cell culture and proliferation assay

Human NSCLC cell line H1703 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to the supplier's protocol. For proliferation assay cells were seeded at 1 × 10^4 per well in 96-well plates and allowed to adhere for 24 h. Compounds were prepared as 10 mM stock solution in DMSO. Increasing drug concentrations in 10 μL of PBS were added to 100 μL of the culture medium for 48 h. MTT assay (Promega, Madison, WI) was performed according to the manufacturer's protocol. Each concentration was represented in eight repeats, and the screening was performed as two independent experiments. IC_{50} values were calculated by using Sigma Plot software (Systat Software, Inc., San Jose, CA).

3.5.2. In vivo treatments

All animals used in this project were cared for and used humanely according to the following policies: the U.S. Public Health Service Policy on Humane Care and Use of Animals (1996), the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (1996), and the U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research and Training (1985). All NCI-Frederick animal facilities and the animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. H1703 cells were injected at 5 × 10^6 s.c. into a flank of 7-week-old female athymic NCr-nu/nu mice (Charles River Laboratories, Inc. Wilmington, MA). The drug injections were initiated when the tumors reached 2 × 2 × 2 mm^3 (typically four weeks). Compound **12** was formulated in Pluronic P123 (BASF, Florham Park, NJ) as micelles.[4,5] Animals were treated five times a week for four weeks with ip injections of either vehicle (2.25% Pluronic P123 in PBS) or compound **12** (20 μmol/kg in vehicle). Tumors were measured with a caliper twice a week, and the tumor volumes were calculated using a formula for ellipsoid volume, $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. The non-parametric Mann–Whitney test was used for statistical comparison of tumors volume at each time points. Body weights were taken three times a week.

3.5.3. Immonoblotting

Western blot analysis was performed as described previously.[4] Primary antibodies to caspase 3, caspase 7, PARP, phosphorylated SAPK/JNK, phosphorylated c-jun (Cell Signaling Technology, Danvers, MA) and ATF3 (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

3.5.4. Statistical analysis

Statistical tests were carried out by using Instat version 3.00 (GraphPad Software Inc., San Diego, CA). Pairwise comparisons included the t test, with the Welch correction or Mann–Whitney test as appropriate.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.02.045.

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