Nitric oxide prodrugs and metallochemotherapeutics: JS-K and CB-3-100 enhance arsenic and cisplatin cytolethality by increasing cellular accumulation

Jie Liu, Chengxiu Li, Wei Qu, Elaine Leslie, Challice L. Bonifant, Gregory S. Buzard, Joseph E. Saavedra, Larry K. Keefer, and Michael P. Waalkes

1Inorganic Carcinogenesis Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute at NIEHS, Research Triangle Park, North Carolina; 2Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute at Frederick, Frederick, Maryland; and 3Basic Research Program, Science Applications International Corporation-Frederick, Frederick, Maryland

Abstract

Development of chemotherapeutic resistance is a major cause of pharmacologic failure in cancer treatment. One mechanism of resistance in tumor cells is the overexpression of glutathione S-transferases (GSTs) that serve two distinct roles in the development of drug resistance via the formation of glutathione conjugates with drugs for their cellular efflux, and the inhibition of the mitogen-activated protein kinase pathway. To target GST-based resistance to chemotherapeutics, a series of nitric oxide (NO)-releasing diazeniumdiolates was synthesized and shown to release NO on reaction with GST and/or glutathione. Two diazeniumdiolates, JS-K \([O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate]\) and CB-3-100 \([O^2-(2,4-dinitrophenyl) 1-[4-(N,N-diethylcarboxamido)piperazin-1-yl]diazen-1-ium-1,2-diolate]\), were studied on their ability in reversing arsenic and cisplatin resistance in a rat liver cell line that is tumorigenic and shows acquired tolerance to arsenic and cisplatin, with overexpression of GSTs. The enhanced cytolethality produced by the NO donors was accompanied by increased accumulation of arsenic and platinum within cells and by enhanced activation of mitogen-activated protein kinase members c-fun-NH-kinase and extracellular signal-regulated kinase. Our data indicate that JS-K and CB-3-100 are promising lead compounds for the possible development of a novel class of adjuvant chemotherapeutic agents potentially capable of reversing arsenic and cisplatin resistance in certain tumor cells.

Introduction

Resistance to anticancer agents is a common reason for treatment failures. One important component of resistance can be the overexpression of glutathione S-transferases (GSTs; refs. 1-3). GSTs are the enzymes that catalyze the conjugation of xenobiotics with cellular reduced glutathione (GSH). Overexpression of GSTs, particularly GST-\(\pi\), often allows tumor cells to gain a selective survival advantage over normal cells by enhanced detoxification through GSH conjugation and/or efflux of the conjugate through the multidrug resistance protein (MRP) pumps (2-5). Overexpression of GSTs can also act as inhibitors of mitogen-activated protein kinases (MAPKs; ref. 3), thereby reducing the cell killing by anticancer drugs. This selection becomes critical in the development of multidrug chemotherapeutic resistance.

To target GST-based chemotherapeutic resistance in GST-overexpressing tumors, nitric oxide (NO) prodrugs of the \(O^2\)-aryl diazeniumdiolate class were designed to act as substrates for GST-\(\pi\). One of these is JS-K \([O^2-(2,4-dinitrophenyl) 1-[4-(ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate]\), already showed to interact with GSTs in vitro and within cells to release NO (6) and to activate MAPK pathways (7). In this study, we studied two \(O^2\)-aryl diazeniumdiolates, JS-K and CB-3-100 \([O^2-(2,4-dinitrophenyl) 1-[4-(N,N-diethylcarboxamido)piperazin-1-yl]diazen-1-ium-1,2-diolate]\), on their ability to overcome drug resistance in the CaSe (Chronic As-Exposed cells) cell line; this is a tumorigenic line derived from rat liver that has been shown to overexpress GST-\(\pi\), MRP1, MRP2, and \(P\)-glycoprotein and to have acquired resistance to arsenite, cisplatin, and other anticancer agents, such as doxorubicin, vinblastine, and actinomycin D (8, 9). We have found that both JS-K and CB-3-100 were effective in increasing cisplatin and arsenic cytolethality, likely by increasing their intracellular accumulation and by enhancing MAPK pathways.

Materials and Methods

Chemicals

JS-K (10) and CB-3-100 (11) were synthesized as detailed below, and their structures are shown in Fig. 1. CB-3-100...
was prepared as outlined below. All other chemicals were of reagent grade and commercially available. NO was purchased from Matheson Gas Products (Montgomeryville, PA). UV spectra were recorded on a Hewlett-Packard Model 8452A diode array spectrophotometer. Nuclear magnetic resonance (NMR) spectra were collected with a Varian XL-200 NMR Spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Elemental analyses were done by Atlantic Microlab, Inc. (Norcross, GA).

**JS-K**
A solution of 5.3 g (0.0286 mol) of \(N,N\)-diethylcarbamoyl piperazine in 15 mL of methanol was placed in a Parr bottle and treated with 6.7 mL (0.0306 mol) of methanolic sodium methoxide. The solution was purged with 40 psi of NO and stirred at 25 °C for 72 hours. The solid precipitate was filtered, washed with ether, and dried to give 4.54 g (60%) of diazeniumdiolate: UV (0.1 mol/L NaOH) λ\text{max} (\varepsilon) 250 nm (9.8 mmol/L cm\(^{-1}\)); \(^1\)H NMR (0.1 NaOD in D\(_2\)O) δ 1.13 (t, 6 H), 3.15 (m, 4 H), 3.27 (d, 4 H), 3.45 (m, 4 H).

**CB-3-100**
The diazeniumdiolate salt of the previous paragraph (1.48 g; 5.8 mmol) was dissolved in 10 mL of 5% aqueous sodium bicarbonate solution and cooled to 0 °C under argon. To the cold solution was added a solution of 608 μL (4.84 mmol) of 2,4-dinitrofluorobenzene in 9.5 mL of t-butyl alcohol. The resulting mixture was allowed to warm to room temperature and stirred for 24 hours. The yellow precipitate that formed was collected by filtration and washed with water to give 1.75 g of crude product. Recrystallization from ethanol gave 1.5 g of analytically pure crystals: M.P. 74 °C to 78 °C; UV (ethanol) λ\text{max} (\varepsilon) 300 nm (12.7 mmol/L cm\(^{-1}\)); \(^1\)H NMR (CDCl\(_3\)) δ 13.17, 41.78, 45.66, 50.33, 117.65, 122.17, 129.08, 137.29, 142.36, 153.81, 163.59.

\[ C_{12}H_{21}N_7O_7 \]

**Calculated:** C43.80, H5.15, N23.83

**Found:** C43.83, H5.18, N23.82

**Cell Culture and Treatments**
CAsE cells, a rat liver epithelial cell line developed by chronic exposure of rat liver TRL 1215 cells to 500 nmol/L arsenite for 24 weeks, have been described previously (8). These cells form malignant tumors on inoculation into nude mice. Cells were cultured in William’s E medium containing 10% fetal bovine serum in the absence of arsenic for at least 2 weeks before the experiments. Cells were cultured in 96-well plates for cytotoxicity evaluation or in 6-well plates for cellular accumulation studies. At 90% confluence, cells were treated with JS-K or CB-3-100 (0, 3, 10, 30, and 100 μmol/L, dissolved in DMSO and diluted in medium), together with various concentrations of arsenic (0 to 200 μmol/L, sodium arsenite, dissolved in distilled water and diluted in medium) or cisplatin (0 to 1000 μmol/L, dissolved in saline and diluted in medium) for up to 24 hours.

**Chemotherapeutic Lethality**
The Fromega non-radioactive cell proliferation assay was used to determine acute cytotoxicity as defined by metabolic integrity. This assay measures the amount of formazan produced by metabolic conversion of Owen’s reagent [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS)] by dehydrogenase enzymes in the mitochondria of metabolically active, and, therefore, viable, cells. The quantity of formazan product, as measured by absorbance at 490 nm, is directly proportional to the number of living cells. Data are expressed as percentage of control untreated cells.

**Cellular Accumulation of Arsenic and Cisplatin**
The CAsE cells were grown to 90% confluence, and then incubated with medium containing 25 μmol/L arsenite or 100 μmol/L cisplatin, in the presence or absence of JS-K (10 and 30 μmol/L) or CB-3-100 (10 and 30 μmol/L) for 2, 4, 8, and 24 hours. At the end of incubation, medium was removed and cells were thoroughly washed 4 times with PBS and harvested. After sonication, cellular protein was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA). The cell suspensions were then completely digested in nitric acid, heated at 70 °C overnight to dryness, and redissolved in distilled water. Total arsenic, which includes both inorganic and organic forms, and platinum were determined using graphite furnace atomic absorption spectrometry (Perkin-Elmer AAnalyst100, Norwalk, CT), and normalized with cellular protein content.

**Western Blot Analysis**
Protein samples (30 μg) derived from the various cell preparations were subjected to SDS-PAGE and transferred...
onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TBS containing 0.05% Tween 20 (TBST) and probed with antibodies against phosphorylated c-jun-NH-kinase 1/2 (JNK1/2) and phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2; Cell Signaling Technology, Beverly, MA). After incubation with secondary antibodies, immunoblots were visualized with the LumiGlo detection method (Cell Signaling Technology).

Statistics
Data are means ± SE of triplicate wells from three experiments using separate cell preparations. For comparison between two groups, the Student t test was used; for comparisons among three or more groups, the data were analyzed by ANOVA, followed by a Ducann’s multiple-range test.

Results
Toxicity of NO Donors
Preliminary experiments indicated that incubating cells with JS-K or CB-3-100 at concentrations below 100 μmol/L for 24 hours did not have significant effects on cell viability (also see Fig. 2 at the zero-arsenite NO-donor-alone points). Thus, 100 μmol/L concentrations or less were used in all subsequent studies. The solubility of JS-K is limited in aqueous solution (≈10 μmol/L) (10); however, this solubility was improved in media containing serum to ≈100 μmol/L without visible precipitation for several hours. In cells cultured at 37°C with serum (10%)-containing media, no apparent precipitation was noted up to 24 hours.

Increasing Arsenite Toxicity with Enhanced Arsenic Accumulation
The effects of JS-K and CB-3-100 on arsenic cytolethality are shown in Fig. 2. Arsenic (as sodium arsenite) induced a concentration-dependent reduction in cell survival. JS-K and CB-3-100 enhanced arsenite cytotoxicity in a concentration-dependent manner, particularly at concentrations above 30 μmol/L. The LC_{50} for arsenite in the absence of the NO donors was 200 μmol/L, and this was decreased to 60 and 50 μmol/L in the presence of 100 μmol/L JS-K and CB-3-100, respectively. To determine whether JS-K and CB-3-100 enhance arsenite toxicity by increasing cellular arsenic accumulation, the concentration of arsenic in CAsE cells was determined at 2, 4, 8, and 24 hours after incubation with a non-toxic concentration of arsenic (25 μmol/L) with or without non-toxic concentrations of JS-K or CB-3-100 (10 to 30 μmol/L). The results clearly showed that co-treatment of cells with the NO donors do not have apparent effects on cellular arsenic uptake at earlier times (2 to 4 hours), but increased cellular arsenic accumulation thereafter (8 and 24 hours) in a concentration-dependent manner (Fig. 3). At 30 μmol/L of NO donors, cellular arsenic levels were elevated 2.8- and 2.6-fold by JS-K and CB-3-100, respectively, at 24 hours.

Enhancing Arsenic Activation of MAPKs
Effect of the NO donors on acute arsenic activation of phosphorylated MAPKs was further examined. The JNK and ERK pathways have been implicated in the regulation of tumor cell killing by various chemotherapeutics, and were suppressed by GST overexpression (3). The levels of phosphorylated JNK1/2 and ERK1/2 were determined by Western blot analysis (Fig. 4). Both JS-K and CB-3-100 treatment enhanced arsenic activation of phosphorylated JNK1/2 and ERK1/2.

Increasing Cisplatin Toxicity with Increased Platinum Accumulation
The effects of JS-K and CB-3-100 on cisplatin cytolethality are shown in Fig. 5. Cisplatin induced a concentration-dependent reduction in cell survival. Both JS-K and CB-3-100 significantly enhanced cisplatin cytotoxicity in a concentration-dependent manner, particularly at concentrations greater than 30 μmol/L. The LC_{50} for cisplatin was 580 μmol/L, and this was decreased to 150 and 100 μmol/L in the presence of 100 μmol/L JS-K and CB-3-100, respectively. To determine whether JS-K and CB-3-100 enhance cisplatin toxicity by increasing cellular platinum accumulation, the concentration of platinum in cells was determined 2, 4, 8, and 24 hours after incubation with a non-cytotoxic concentration of cisplatin (100 μmol/L).
together with non-toxic concentrations of JS-K or CB-3-100 (10 to 30 μmol/L; Fig. 6). The results clearly showed that co-treatment of cells with JS-K and CB-3-100 resulted in concentration-dependent increases in cellular platinum accumulation. Cellular platinum content showed maximal increases of 3.6- and 3.1-fold, respectively, with JS-K and CB-3-100 co-exposure, as compared with cisplatin alone. Again, the effects of NO donors on the uptake of cisplatin at early time points (2 to 4 hours) were not appreciable.

Figure 3. Effects of JS-K (A) and CB-3-100 (B) on cellular arsenic accumulation. CAsE cells were incubated with 12.5 μmol/L sodium arsenite together with 10 and 30 μmol/L JS-K or CB-3-100 for 2, 4, 8, and 24 hours. The cellular arsenic concentration was determined by graphite furnace atomic absorption spectrometry. Points, means of three separate cell preparations; bars, SE. *, significantly different from arsenic-only controls, \( P < 0.05 \).

Discussion

The present study clearly shows that JS-K and CB-3-100 are effective in increasing arsenic and cisplatin toxicity in tumorigenic CAsE cells, apparently by increasing cellular accumulation of arsenic and platinum, and by enhancing activation of MAPK pathways, probably through their interactions with GSTs.

GSTs have long been known to release NO from certain alkyl nitrates (12). JS-K (10) and CB-3-100 were both designed for their interactions with GST. Increased NO release has been shown in vitro using purified GSTs and the molecular interactions of JS-K with GSTs to produce NO have been shown (6, 10). In this regard, it has been shown that NO carriers S-nitrosothiogluthione and dinitrosyl-dithiol-iron complexes are capable of inhibiting GSTs (13, 14) in the cells, and GSTs are also susceptible to inactivation by NO-derived oxidants (15). GSTs may even act as NO storage systems (16, 17). The NO donor, DEA/NO, has been shown to inhibit cytosolic GST activities by S-nitrosylation, particularly for GST-π (18). Thus, targeting GST by designing NO donors is a plausible and feasible strategy for drug development.

Figure 4. Effect of JS-K and CB-3-100 on arsenic activation of MAPKs. CAsE cells were incubated with 50 μmol/L sodium arsenite together with 10 and 30 μmol/L JS-K or CB-3-100 for 3 hours. The levels of phosphorylated JNK1/2 and ERK1/2 were determined by Western blot analysis as detailed in Materials and Methods. Blots, typical result of three independent experiments.

Figure 5. Effects of JS-K (A) and CB-3-100 (B) on cisplatin toxicity. CAsE cells were incubated with various concentrations of cisplatin (0 to 1000 μmol/L) together with various concentrations of JS-K or CB-3-100 (0 to 100 μmol/L). The cell viability was assessed 36 hours after treatment. Points, means of three separate cell preparations; bars, SE. *, significantly different from controls, \( P < 0.05 \).
agents (19). In particular, arsenicals are very effective in the treatment of acute promyelocytic leukemia (20). There are also some promising efforts to expand the use of arsenicals to solid tumors. There is, however, the potential for resistance to arsenic chemotherapy to develop after the long-term use of the metalloid, as it does in human and rodent cells chronically exposed to arsenic (9, 21). In this regard, GSH depletion coupled with arsenic exposure reverses acquired arsenic resistance (21-23). Recent studies indicate that conjugation of arsenic with GSH is an important step to enhance arsenic toxicity in a variety of cultured cells (9, 21, 26, 27). Thus, it is likely that increased arsenic cytotoxicity with JS-K or CB-3-100 co-treatment could be coupled to increased cellular accumulation of arsenic, possibly through the inactivation of GSTs and the decreased formation of GSH-arsenic conjugates for cellular efflux. GSTs can also act as inhibitors of MAPKs (3), and the activation of MAPKs is an important mechanism for hepatoma cell killing by JS-K (7). In the present study, both JS-K and CB-3-100 enhanced activation of phosphorylated JNK1/2 and ERK1/2, supporting the role of GST inhibition on MAPK activation (3). In any event, these NO prodrugs are clearly effective in increasing arsenic retention and MAPK activation in tumor cells, and, thereby, increasing the rate of cell killing.

Cisplatin is used extensively as a single chemotherapeutic agent or in combination with other drugs and/or radiation (28). Acquired resistance to cisplatin is a major problem in cancer treatment. A number of mechanisms have been proposed for the development of cisplatin resistance, but the most probable one involves the enhancement of a GSH-Pt adduct for cellular efflux (29, 30). Thus, inactivation of GST and the resulting blockade of the formation of the GSH-Pt complex could reverse cisplatin resistance in tumor cells (31). In a fashion similar to arsenic, the increased sensitivity to cisplatin cytotoxicity by JS-K and CB-3-100 observed in the present study could also be due, at least in part, to the increased accumulation of the platinum compound within cells, as well as activation of the MAPK pathways (data not shown).

The diazeniumdiolate NO donor JS-K has also been shown to induce apoptosis in the HL-60 human myeloid leukemia cells (6), as well as in solid tumor cells, such as the prostate tumor cell line PPC-1, colon tumor cell line DLA-1, the mammary tumor cell line MethA, and Hep 3B hepatoma cells (6, 7). JS-K inhibits xenograft growth of HL-60 and PPC-1 cells when implanted in mice (6). In addition, other NO donors were shown to exert cytostatic effects against leukemia P388 and L1210 cells, and inhibit the development of drug resistance to cyclophosphamide (32). The diazeniumdiolate NO donor DETA/NO improves cisplatin efficacy against head and neck squamous cell carcinoma (33). In this regard, CAsE cells are malignant transformed cells, producing aggressive, malignant tumors on inoculation into nude mice (8). Thus, evidence from a variety of systems with diazeniumdiolate NO donors forecasts potential utility for them as adjuvant to chemotherapeutic attack against actual tumors.

In summary, this study shows that two novel diazeniumdiolates JS-K and CB-3-100 are effective in increasing arsenic and cisplatin toxicity in previously highly resistant cells, apparently by increasing cellular accumulation of the metal and by enhancing activation of the MAPK pathways. Thus, the diazeniumdiolates JS-K and CB-3-100 could be promising candidates for possible development of a new class of adjuvant prodrugs for cancer chemotherapy, particularly against drug-resistant tumors.

Acknowledgments
We thank Dr. Jingbo Pi and Yaxiong Xie for critical review and Catherine Harris for technical assistance.

References
4. Leslie EM, Deely RG, Cole SP. Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. Toxicology 2001;167:3-23.
8. Zhao CG, Young MR, Diwan BA, Coogan TP, Waalkes MP. Association


