Research Paper

Nitric oxide-releasing prodrug triggers cancer cell death through deregulation of cellular redox balance

Anna E. Maciaga, Ryan J. Holland, Y.-S. Robert Cheng, Luis G. Rodriguez, Joseph E. Saavedra, Lucy M. Anderson, Larry K. Keefer

Laboratory of Proteomics and Analytical Technologies, Advanced Technology Program, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Radiation Biology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA
Chemical Biology Laboratory, Frederick National Laboratory for Cancer Research, Frederick, MD, USA

Basic Science Program, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research, Frederick, MD, USA

Abstract

JS-K is a nitric oxide (NO)-releasing prodrug of the O₂-arylated diazeniumdiolate family that has demonstrated pronounced cytotoxicity and antitumor properties in a variety of cancer models both in vitro and in vivo. The current study of the metabolic actions of JS-K was undertaken to investigate mechanisms of its cytotoxicity. Consistent with model chemical reactions, the activating step in the metabolism of JS-K in the cell is the dearylation of the diazeniumdiolate by glutathione (GSH) via a nucleophilic aromatic substitution reaction. The resulting product (CEP/NO anion) spontaneously hydrolyzes, releasing two equivalents of NO. The GSH/GSSG redox couple is considered to be the major redox buffer of the cell, helping maintain a reducing environment under basal conditions. We have quantified the effects of JS-K on cellular GSH content, and show that JS-K markedly depletes GSH, due to JS-K's rapid uptake and cascading release of NO and reactive nitrogen species. The depletion of GSH results in alterations in the redox potential of the cellular environment, initiating MAPK stress signaling pathways, and inducing apoptosis. Microarray analysis confirmed signaling gene changes at the transcriptional level and revealed alteration in the expression of several genes crucial for maintenance of cellular redox homeostasis, as well as cell proliferation and survival, including MYC. Pre-treating cells with the known GSH precursor and nucleophilic reducing agent N-acetylcysteine prevented the signaling events that lead to apoptosis. These data indicate that multiplicative depletion of the reduced glutathione pool and deregulation of intracellular redox balance are important initial steps in the mechanism of JS-K's cytotoxic action.

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Introduction

Diazeniumdiolate-based NO-releasing prodrugs are a growing class of promising cancer therapeutics. O₂-arylated diazeniumdiolates release NO upon nucleophilic aromatic substitution reaction with glutathione (GSH). O₂²-[2,4-Dinitrophenyl] 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K) (structure shown in Fig. 1) has proven effective for inhibiting growth of cancer cells in several in vitro and in vivo models [26,27,22,15,17,31]. JS-K is highly cytotoxic to leukemia and multiple myeloma cell lines and patient-derived multiple myeloma cells, with IC₅₀ values ranging from 0.2 to 1.2 µM [26,27,15]. Mouse models of leukemia or multiple myeloma confirmed the effectiveness of JS-K against these blood cancers in vivo. The prodrug inhibited tumor growth and prolonged survival in a multiple myeloma xenograft model [15]. Importantly, JS-K was very effective against multiple myeloma cells resistant to conventional therapy with dexamethasone, doxorubicin or...
melphalan, with IC50 values for these cell lines of 0.3 to 0.9 μM.

Furthermore, JS-K appeared to be selectively cytotoxic towards cancer cells; no significant toxicity of the drug was observed in bone marrow stromal cells isolated from patients, at concentrations at which it inhibited the proliferation of multiple myeloma cells [15]. Similar selective toxicity towards cancer cells relative to their nonmalignant counterparts was observed in breast [18] and renal cancers [5].

The glutathione (GSH)/glutathione disulfide (GSSG) redox couple plays a central role in cellular protection against genotoxic agents and oxidants, as well as in the control of the thiol/disulfide redox state, which is essential for redox signaling [12]. GSH is the most abundant cellular antioxidant and is also involved in the detoxification of numerous xenobiotics.

Evidence from many studies suggests that cancer cells are under increased oxidative stress that is associated with oncogenic transformation. Oncogene activation or tumor suppressor inactivation in cancers commonly results in increased generation of reactive oxygen species (ROS) [24,11,20]. Leukemia cells freshly isolated from blood of chronic lymphocytic leukemia or hairy-cell leukemia patients showed increased ROS compared to normal lymphocytes [33,14]. This biochemical property of cancer cells can be exploited for therapeutic benefits. Induction of oxidative stress may be a promising approach for cancer therapy, because cancer cells with increased oxidative stress are likely to be more vulnerable to damage by additional ROS elicited by exogenous agents. NO is a relatively stable free radical that does not readily react with most macromolecules in cells. However, it reacts with oxygen and superoxide, forming reactive nitrogen species (RNS) that are highly damaging to cells.

In this study we present evidence that treatment of human leukemia U937 cells with the NO-releasing prodrug JS-K led to the depletion of GSH, affecting cellular redox potential and triggering MAPK stress signaling and apoptosis. To characterize further the biologic potential of JS-K we performed gene expression analysis using the Affymetrix platform for microarray analysis. This revealed marked alteration in expression of several genes crucial for maintenance of cellular redox homeostasis, as well as cell proliferation and survival.

**Methods**

**JS-K synthesis, chemicals and cell culture**

JS-K was synthesized as described previously [23]. All other chemicals were obtained from Sigma (St. Louis, MO). Leukemia cell line U937 [29] was obtained from the American Type Culture
In vitro metabolism of JS-K

Human leukemia U937 cells were seeded in 75-cm² flasks and incubated overnight at 37° C, 5% CO₂. The cells were treated with 5 µM JS-K and incubated for 10 or 30 min. After treatment, cells were collected by centrifugation at 800g for 10 min, washed with phosphate buffered saline (PBS) and collected again. The pellets were resuspended in 800 µL of 10 mM HCl, then lysed 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 8000g for 10 min, and the supernatant was analyzed by LC/MS using a Thermoquest Surpass (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), glutamine and penicillin–streptomycin, at 5% CO₂, 37° C.

Measurement of cellular glutathione content and cellular reduction potential

U937 cells were cultured at 1 x 10⁶ per mL. Cells were treated in culture medium with 1 µM JS-K for 1 h or for the time periods indicated in Fig. 3. After treatment, cells were collected by centrifugation at 800g for 10 min, washed with phosphate buffered saline and collected again. The pellets were resuspended in 800 µL of 10 mM HCl and lysed by two successive rounds of freezing and thawing. Twenty microliters of a 5% 5-sulfosalicylic acid solution were added to the lysate. The precipitate was removed by centrifugation at 8000g for 10 min, and the supernatant was analyzed by LC/MS using 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 V. Separations were performed on an Agilent Eclipse XDB-C18 5-µm, 4.6 x 150 mm² column at a flow rate of 1 mL/min, under H₂O/acetonitrile/0.1% formic acid gradient conditions. The stability of JS-K in a complete culture medium (RPMI 1640, 10% FBS) was determined with analogical extraction and HPLC methods.

Measurement of mitochondrial membrane potential

U937 cells were seeded on 24-well plates at 2 x 10⁵/well and allowed to grow for 24 h. The cells were then treated in culture medium with either vehicle (DMSO) or 1–10 µM JS-K and incubated at 37 °C, 5% CO₂ for 30 min. The JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical, Ann Arbor, MI) was used according to the manufacturer’s protocol.

Determination of intracellular reactive oxygen/nitrogen species and nitric oxide

The intracellular levels of reactive oxygen and nitrogen species were monitored by the oxidation of the fluorophore 5-(and6)-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) (Invitrogen). U937 cells were incubated with 5 µM DCFH-DA in Hank’s balanced salt solution (HBSS) at 37 °C and 5% CO₂ for 30 min. The cells were collected by centrifugation, the HBSS containing the probe was removed, and the cells were rinsed with HBSS and resuspended in fresh HBSS. Three mL of cell suspension was aliquoted into 15-mL test tubes followed by addition of JS-K or DMSO as a control. After 60 min of incubation at 37 °C, 2,7’-dichlorofluorescein (DCF) fluorescence was measured by using a PerkinElmer Life and Analytical Sciences (Waltham, MA) LS50B luminoscope with the excitation source at 488 nm and emission at 530 nm.

The intracellular level of nitric oxide after JS-K treatment was estimated using the NO-sensitive fluorophore 4-amino-5-methylamino-2,7’-difluorofluorescein (DAF-FM) diacetate (Invitrogen). This reagent hydrolyzes after being taken up by the cell, where it reacts with nitrosating agents formed upon 1-electron oxidation of NO to generate a fluorescent species whose intensity is taken as a measure of intracellular NO. Cells were loaded with 2.5 µM DAF-FM diacetate in HBSS at 37 °C and 5% CO₂. After 30 min of incubation the cells were rinsed with HBSS to remove excess probe, resuspended in fresh HBSS, and treated with JS-K at 1 µM final concentration. After 60-min incubation the fluorescence of the benzotriazole derivative formed on DAF-FM’s reaction with aerobic NO was analyzed by using a PerkinElmer Life and Analytical Sciences (Waltham, MA) LS50B luminoscope with the excitation source at 488 nm and emission at 530 nm.
Analytical Sciences LS50B luminescence spectrometer with the excitation source at 495 nm and emission at 515 nm.

**Immunoblotting**

Western blot analysis was performed as previously described [17]. Primary antibodies for phospho-p38, p38, phospho-SAPK/JNK, phospho-ATF2, phospho-c-jun, c-jun, caspases 3, 7, and 8, cytokeratin c, PARP and cleaved PARP, c-myc (Cell Signaling Technology, Danvers, MA) and ATF3 (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

**Microarray analysis**

RNA extraction and transcriptome profiling. Total RNAs from three independent cultures for each treatment time point or control were extracted using TRIzol reagent (Invitrogen) following the manufacturer’s protocol. RNA qualities were checked by the 260 nm/280 nm optical density ratio and Bioanalyzer 2100 (Agilent, Santa Clara, CA) prior to transcriptome analyses. Microarray experiments were performed using Affymetrix (Santa Clara, CA) Human U133 Plus 2.0 GeneChip by the Laboratory of Molecular Technology, Frederick National Laboratory for Cancer Research, Frederick, MD, USA. cRNA probe synthesis, hybridization, and scanning of arrays followed the manufacturers’ default protocols. Probe signal values and detection calls were determined using Affymetrix GeneChip Command and Console Software. Chip-to-Chip variations were normalized by the Robust Multichip Average normalization algorithm.

Multivariate analysis. GeneChip data were exported to BRB-ArrayTools, an open source microarray statistical analysis plug-in for the Microsoft Excel software (Microsoft Professional 2003). The non-supervised hierarchical clustering approach was chosen for the data set exploration.

Class comparison analysis. The class comparison module in BRB-ArrayTools was utilized to identify differentially and statistically significantly expressed genes between sample groups. Microarray data from different comparison pairs were subjected to a two-sample t-test (with random variance model). Type I error correction was applied to all pairwise comparisons. Pairwise analysis was performed using control vs. treated using a fold cut-off of 2. Pathway analysis. Gene lists winnowed from the multivariate analyses were exported to the Ingenuity Pathway Analysis (IPA) web portal (http://www.ingenuity.com) for functional pathway analysis.

**Statistical analysis**

All experiments were performed at least three times, each time at least in triplicate. Results are presented as averages ± SE. Statistical tests of chemical and protein results were carried out using GraphPad Instat version 3.00 (GraphPad Software, San Diego, CA). Pairwise comparisons included the t test, with the Welch correction or application of the Mann–Whitney test as appropriate. Significance of correlations was assessed by the Pearson linear correlation or the Spearman test as appropriate.

**Results**

**GSH-dependent dearylation is a major metabolic pathway for JS-K in the cell**

Cellular redox potential is largely controlled by GSH, which accounts for more than 90% of cellular non-protein thiols [7]. JS-K was designed to release NO upon nucleophilic attack by free thiols (particularly GSH). The first step in the metabolism of JS-K in the cell is the dearylation of the diazeniumdiolate by GSH via a nucleophilic aromatic substitution reaction, liberating the ionic diazeniumdiolate moiety CEP/NO, which spontaneously hydrolyzes, releasing NO (Fig. 1). Subsequent oxidation products of the NO can react with additional equivalents of GSH. A consequence of this metabolism is a rapid depletion of cellular GSH concurrent with a rise in oxidized glutathione (GSSG) (Fig. 1).

JS-K is relatively stable in complete (RPMI 1640, 10% FBS) cell culture medium, with a half-life of about 5 h. However, it is rapidly and completely metabolized by the cells. Liquid chromatography/mass spectrometry (LC/MS) analysis revealed that 30 min after addition of JS-K to the cell culture the parent compound is not detectable in the lysate; we have found glutathione and cysteine adducts derived from JS-K (Fig. 2), the latter presumably resulting from further metabolism of the glutathione conjugate. The parent compound was also not detected after shorter treatment (10 min); however, in this case only the glutathione adduct was seen (data not shown).

**JS-K has a major impact on cellular redox status**

We quantified GSH and GSSG levels in the U937 leukemia cell line, which grows in suspension as spherical cells. As described under Materials and Methods, we determined the average cell volume to be 4.0 ± 0.5 pl, and measured total amounts of GSH and GSSG. Concentrations per cell and the reduction potential of the GSH redox couple were calculated. Table 1 shows changes in cellular GSH/GSSG concentrations and reduction potential in U937 leukemia cells after JS-K treatment. One-hour treatment with JS-K led to a decrease in total GSH concentration, a decrease in GSH/GSSG ratio, and a rise in reduction potential.

A noteworthy feature of the data in Table 1 is the extent to which low concentrations of JS-K in the medium can generate major changes in cellular GSH/GSSG levels. For example, 1 μM JS-K in the medium dropped the intracellular GSH level by 730 μM, while 4 μM JS-K consumed 1970 μM GSH and increased GSSG by 110 μM. Although the depletion of hundreds of micromoles of GSH by low μM concentrations of JS-K may seem counter-intuitive, a closer look at the stoichiometry provides a rationale. Assuming that the number of moles of JS-K is equally dispersed amongst the cells in suspension, 1.5 fmol and 6 fmol are delivered per cell when JS-K is added to the medium at 1 μM and 4 μM concentrations, respectively. Upon metabolism and NO production, a significant portion of the 13.7 fmol of GSH per cell is consumed, 2.9 fmol from 1 μM treatment and 7.9 fmol from 4 μM treatment, translating to an observable decrease in the cellular GSH concentration.

U937 cells were pretreated with N-acetylcysteine (NAC), a precursor for GSH synthesis and a nucleophilic reducing agent, to determine whether it attenuates the disruption of redox balance. Indeed, pretreatment with NAC prevented the depletion of GSH.

| NAC | Treatment | GSH (μM) | GSSG (μM) | Redox potential (mV) | ΔE (mV) |
|-----|-----------|----------|-----------|---------------------|---------|
| None | DMSO      | 3.43 ± 0.14 | 0.20 ± 0.01 | 227.6               | -10          |
|      | JS-K 1 μM | 2.70 ± 0.16 | 0.21 ± 0.01 | 219.1               | -8.5          |
|      | JS-K 4 μM | 1.46 ± 0.16 | 0.31 ± 0.02 | 197.7               | 29.9          |
| 0.1 mM | DMSO      | 4.30 ± 0.15 | 0.18 ± 0.01 | 233.8               | -24.0         |
|      | JS-K 1 μM | 3.75 ± 0.23 | 0.24 ± 0.01 | 227.2               | 24.0          |
|      | JS-K 4 μM | 2.55 ± 0.22 | 0.37 ± 0.04 | 209.8               | 10.8          |
| 1 mM  | DMSO      | 6.74 ± 0.18 | 0.16 ± 0.01 | 247.6               | -4.2          |
|      | JS-K 1 μM | 6.01 ± 0.26 | 0.18 ± 0.01 | 243.6               | 10.8          |
|      | JS-K 4 μM | 5.40 ± 0.26 | 0.25 ± 0.02 | 236.8               | -15.0         |
associated with JS-K treatment alone and partially prevented the rise in reduction potential (Table 1). Time-dependent study revealed that the rise in reduction potential was significant within minutes of treatment with 1 mM JS-K (Fig. 3), consistent with rapid reaction of the prodrug with GSH. We have shown previously that the rate of reaction with GSH is important for anticancer efficacy of aryl-diazeniumdiolate-based NO-releasing prodrugs [17,19].

Depletion of cellular antioxidant defenses allows for the generation of significant quantities of ROS, which have been suggested to induce apoptosis [16]. The release of NO from JS-K in the cells was confirmed by DAF fluorescence assay (Fig. 4(A)). There was also an increase in DCF fluorescence assay (Fig. 4(A)). It may be noted that DCFH-DA reacts with reactive nitrogen species such as peroxynitrite ([10,13]).

Oxidative stress leads to activation of cellular stress signaling and apoptosis

Oxidative stress is widely observed to activate the stress kinases p38 and SAPK/JNK pathways, leading to activation of stress-response genes. Phosphorylation of p38 was very rapidly induced by JS-K. A strong signal was observed as soon as 5 min after treatment with JS-K was initiated and it continued for several hours; at the 16-h time point the signal was significantly reduced (Fig. 5(A)). Importantly, pretreatment with NAC encumbered phosphorylation of p38, suggesting that redox imbalance/oxidative stress is a triggering mechanism in this pathway’s activation (Fig. 5(B)). Phosphorylation of the p38 downstream target ATF2 exhibited the same pattern. SAPK/JNK activation was detected slightly later, about 1 h after JS-K treatment was initiated. Phosphorylation of its downstream target, transcription factor c-jun, was observed after 1 h as well.

We have shown previously that upregulation of activating transcription factor 3 (ATF3) is required for cell killing by aryl-diazeniumdiolate-based NO-releasing prodrugs in non-small cell lung cancer (NSCLC) cells [17]. Here we observed a significant increase in ATF3 protein in the leukemia cells as well, starting 4 h after JS-K treatment was initiated.

To determine whether the induction of ROS/RNS and stress signaling was associated with cell death, cells were treated with JS-K and then analyzed by Western blot for markers of apoptosis. Apoptosis was activated early, initially through the extrinsic pathway, as evidenced by caspase-8 activation within 2 h of treatment with 1 μM JS-K (Fig. 6(A)). Cleaved effector caspase-7 and cleaved PARP signals were also observed within 2 h (Fig. 6(A)).

JS-K-induced U937 cell death is blocked by NAC

As shown in Table 1, pretreatment with NAC increased the basal levels of GSH, and diminished the rise in reduction potential. Moreover, NAC blocked JS-K-induced U937 cell apoptosis (Fig. 6(B)). U937 cells (5 x 10^5/mL) were pretreated with 0.1 or 1 mM NAC for 16 h, followed by 3 h with 1 mM JS-K. Pretreatment with NAC inhibited activation of apoptosis, as evidenced by diminished cleavage of caspases-8 and -3.

Loss of mitochondrial membrane potential after JS-K treatment

Fig. 7(A) shows loss of mitochondrial membrane potential in U937 cells after 4 h incubation with 1 μM JS-K; there was even more significant loss of this membrane potential with 5 μM JS-K (Fig. 7(A)). A similar effect of JS-K was observed previously in H1703 NSCLC cells after 1 h of incubation with 1 μM drug [17]. One-hour treatment did not result in loss of mitochondrial membrane potential in U937 cells, suggesting that the sequence of events could be cell-type specific, and that in U937 cells apoptosis by an extrinsic pathway precedes involvement of mitochondria in the cell death pathway. Collapse of mitochondrial membrane potential has been shown to represent a stage of cell death that is already irreversible [32]. Mitochondrial apoptosis was also activated, as evidenced by presence of cytochrome c in cytosol after 4 h treatment with JS-K (Fig. 7(B)).

Microarray analysis

To provide an assessment of transcripts and pathways altered by JS-K, treated cells were subjected to microarray analysis. Treatment with JS-K resulted in differential expression of 1800 transcripts, suggesting an extensive response to JS-K. Marked up-regulation of expression of JUN and ATF3 (Table 2) confirms the protein results shown in Fig. 5 and indicates regulation of these at the mRNA level. The c-MYC oncogene was downregulated progressively with time (Table 2). We confirmed downregulation of the protein level of this short-lived transcription factor by Western blot. As shown in Fig. 8(B), c-myc protein expression...
was downregulated rapidly, with complete loss of signal after 2 h of treatment with JS-K.

As expected, the transcriptome from treated cells was enriched in genes implicated in the response to oxidative stress. NF-E2-related factor 2 (NFE2L2, Nrf2) is considered as one of the major transcription factors activated in response to oxidative stress. Upon oxidative stress Nrf2 translocates to the nucleus and upon heterodimerization with other transcription factors (e.g., JunD, ATF4, small MAFs) transactivates the antioxidant response elements of many cytoprotective genes, as well as Nrf2 itself. In our study, NFE2L2 transcript was upregulated 1.4-, 1.6-, and 5-fold at 1.5, 4 and 24 h, respectively. Expression of many Nrf2-regulated genes was altered. NQO1 (NADPH: quinone oxidoreductase 1), a major detoxification enzyme, was upregulated 2, 2.6 and 4-fold, at 1.5, 4 and 24 h, respectively. Antioxidant HMOX1 (heme oxygenase 1) was upregulated 5-fold at 1.5 h and 31-fold at 4 h, then its transcript level decreased. Transcript of the rate-limiting enzyme in glutathione synthesis, GCLM (glutamate-cysteine ligase, modifier subunit) was upregulated 2-fold at 1.5 h and 4-fold at 4 h. Similarly, SLC7A11, a cystine/glutamate antiporter, was upregulated over 2-fold at 1.5 h and over 7-fold at 4 h. These changes appear to be triggered by depletion of GSH from the cell in a cellular attempt to restore its supply.

Complete microarray data have been deposited on the GEO website http://www.ncbi.nlm.nih.gov/geo (GEO Accession number GSE42344).
Discussion

Oxidative stress occurs when there is an imbalance between the rates of ROS generation and removal by scavenging mechanisms. Glutathione is a ubiquitous cellular thiol. Its reduced form (GSH) and oxidized form (GSSG) constitute the major thiol redox system in cells, and proper GSH/GSSG redox balance is crucial for cell function.

Activation of the nitric oxide-releasing prodrug JS-K to release NO through nucleophilic aromatic substitution depletes cells of...
GSH, and increases ROS, which causes further oxidation of GSH and formation of GSSG. We have for the first time quantified loss of GSH and increase in GSSG as a function of treatment of cancer cells with an arylated diazeniumdiolate prodrug. Our results demonstrate that depletion of the cellular glutathione pool is an important first step in the cytotoxic action of JS-K. Thanks to the rapid and complete uptake of the micromolar levels of JS-K from the culture medium, it was on scale to effectively impact millimolar concentrations of GSH in the cell. Depletion of GSH as a therapeutic strategy in sensitizing cancer cells to radiation therapy has been demonstrated [8]. Cancer cells depleted of GSH through inhibition of its synthesis with buthionine sulfoximine (BSO) were much more sensitive to the effects of irradiation.

It will be noted that JS-K is a particularly efficient consumer of GSH, with each molecule imported into the cell being capable of destroying multiple reducing species through GSH-arylation and oxidation of NO to form reactive nitrogen species (Fig. 1). That such significant consumption of GSH by JS-K was indeed operative in these cells was confirmed in our experiments. As stated in the Results, the number of moles of JS-K delivered per cell is in the same scale as the number of moles of GSH. Each mole of JS-K irreversibly arylates a mole of GSH, resulting in release of two moles of NO, the oxidation products of which are capable of consuming more reduced equivalents of GSH (Fig. 1). This consumption of GSH results in a rise in the steady-state level of ROS/RNS, further consuming GSH, with a concurrent increase in GSSG. We have for the first time quantified loss of GSH and increase in GSSG as a function of treatment of cancer cells with an arylated diazeniumdiolate prodrug. Our results demonstrate that depletion of the cellular glutathione pool is an important first step in the cytotoxic action of JS-K. Thanks to the rapid and complete uptake of the micromolar levels of JS-K from the culture medium, it was on scale to effectively impact millimolar concentrations of GSH in the cell. Depletion of GSH as a therapeutic strategy in sensitizing cancer cells to radiation therapy has been demonstrated [8]. Cancer cells depleted of GSH through inhibition of its synthesis with buthionine sulfoximine (BSO) were much more sensitive to the effects of irradiation.

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SAPK/JNK and p38 stress kinases are important mediators of cell death signaling resulting from a variety of cellular stresses, including ROS/RNS. We have reported previously that SAPK/JNK

| Gene symbol | Description | Fold change 90 min | Fold change 4 h | Fold change 24 h |
|-------------|-------------|-------------------|----------------|----------------|
| PTCD2 | Pentatricopeptide repeat domain 2 | 1.08 | 5.27 | 7.83 |
| HMGCS1 | 3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble) | 1.08 | 7.13 | 13.86 |
| GSTA4 | Glutathione S-transferase A4 | 1.08 | 7.04 | 13.86 |
| SNRPN | Small nuclear ribonucleoprotein polypeptide N | 1.08 | 7.04 | 13.86 |
| INSIG1 | Insulin induced gene 1 | 1.08 | 7.04 | 13.86 |
| EMP1 | Epithelial membrane protein 1 | 1.08 | 7.04 | 13.86 |
| FOXO3 | Forkhead box O3 | 1.08 | 7.04 | 13.86 |
| HMOX1 | Heme oxygenase (decycling) 1 | 1.08 | 7.04 | 13.86 |
| CSAR1 | Complement component 5a receptor 1 | 1.08 | 7.04 | 13.86 |
| IER3 | Immediate early response 3 | 1.08 | 7.04 | 13.86 |
| PALLD | Palladin, cytoskeletal associated protein | 1.08 | 7.04 | 13.86 |
| CDKN1A | Cyclin-dependent kinase inhibitor 1A (p21, Cip1) | 1.08 | 7.04 | 13.86 |
| CD86 | CD86 molecule | 1.08 | 7.04 | 13.86 |

**Table 2**

Most significant alterations in gene expression after JS-K treatment.
activation is a consequence of JS-K treatment in NSCLC cells in vitro and in xenografts [17]. Involvement of the SAPK/JNK pathway in activation of apoptotic cell death has been reported [30], as well as its involvement in ATF3 induction [4]. Kiziltepe and colleagues have shown that activation of SAPK/JNK in multiple myeloma cells in response to JS-K is a critical step in the apoptotic pathway’s activation, and that pretreatment with JNK inhibitor II rescued multiple myeloma cells from apoptosis.

In leukemia U937 cells, however, we observed rapid activation of p38, preceding SAPK/JNK phosphorylation. Phosphorylation and activation of p38 by peroxynitrite have been reported in neuroblastoma cells [21]. Decrease in p38 phosphorylation upon JS-K treatment has been noted in breast cancer cells [28]. The involvement of ROS in JS-K activity was further illustrated by experiments showing that the reducing agent NAC protected against p38 activation and apoptotic cell death.

Microarray analysis provided some useful additional information about this leukemia cell model. Increases in c-jun and ATF3 protein were preceded by changes in gene expression. A number of genes presented marked up- or down-regulation. These included cancer-related genes such as MAF and FOS oncogenes that could be targeted for combination therapy along with JS-K. Similarly, several antioxidant factors were upregulated, which, if individually targeted, could increase the effectiveness of JS-K even further.

Progressive and marked downregulation of the proto-oncogene c-MYC revealed by microarray, and confirmed by immunoblot, is especially notable, in view of its frequent involvement in hematopoietic cancers. In various lymphomas and leukemias it inhibits differentiation and functions as a leukemogenic protein. C-MYC gene amplification and translocation, resulting in its deregulation, have been observed in Burkitt’s lymphoma and acute lymphoblastic leukemia [6]; [2]. Inactivation of the MYC oncogene has also been shown sufficient to induce regression of invasive liver cancers [25].

In summary, we have shown that in U937 leukemia cells a micromolar concentration of the NO-donor prodrug JS-K is sufficient to induce major depletion in GSH, due to its rapid, complete uptake from the culture media and subsequent cascade of reactions with NO-derived species. Marked progressive increase in cellular ROS/RNS and change in cellular reduction potential result, followed by cellular stress signaling, downregulation of c-myc, and initiation of apoptosis. This mechanistic information may be useful for enhancement of the efficacy of JS-K and similar drugs.

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