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Mechanism of action for the cytotoxic effects of the nitric oxide prodrug JS-K in murine erythroleukemia cells

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1. Introduction

JS-K (*O*²-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazene-1,2-diolate; Fig. 1A) is an anti-cancer agent that belongs to the diazeniumdiolate family of compounds. It is designed to release nitric oxide (NO) in a sustained and controlled manner within a cell during its reaction with glutathione [1,2]. Because JS-K is also an arylating agent, the anti-cancer effects of the compound may also involve arylation of glutathione or other thiol-containing molecules [3,4]. Previous studies have shown that JS-K is active against several types of human cancers *in vitro* as well as *in vivo* against human tumors given to immunocompromised mice [4–7]. Although studies have linked the anti-cancer effects of JS-K to the induction of apoptosis, the molecular changes initiated by JS-K that trigger the apoptotic response are not completely elucidated. JS-K has been shown to cause prolonged MAPK activation [4], to inhibit ubiquitin E1 activity [8] or β -catenin/TCF signaling [9], and to cause double stranded DNA breaks and a DNA damage response [10]. The multiple effects of JS-K documented in these studies may reflect the different cancer cell types studied, but they may also be related to differences in the response of the cancer cells to either the NO-releasing or arylating capacity of the compound. In this study, we have extended these observations to include erythroleukemia cells.

To better understand the biological effects of JS-K and the importance of both its NO-releasing and arylation capacity, we examined the molecular changes occurring in a retrovirus-transformed murine erythroleukemia cell line (SFFV-MEL) after treatment with JS-K or with 1-chloro-2,4-dinitrobenzene (CDNB), which contains the dinitrophenol ring of

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Contributions. MZK designed, performed and analyzed the majority of the experiments and drafted the manuscript; RJH designed, performed and analyzed experiments; SAL performed experiments and collected data; VIF performed experiments and collected and analyzed data; JAT and CAH provided critical technical support; JES and LKK provided advice for study design and critical reagents; JLC contributed to the design and interpretation of these studies; SKR contributed to the conception and design of the study, directed the research and wrote the final manuscript. All authors read and approved the final manuscript.

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JS-K without the diazeniumdiolate group necessary for NO release [5,11]. CDNB was chosen because its chlorine atom is a comparable leaving group to the diazeniumdiolate group of JS-K in nucleophilic aromatic substitution (S_NAr) reactions [11]. Here we report that both JS-K and CDNB are cytotoxic for SFFV-MEL cells with a low IC_{50} value. Both compounds caused apoptosis of SFFV-MEL cells, consistent with the activation of the tumor suppressor FoxO3a and the subsequent activation of various caspases. Inhibition of Akt kinase and the MAP kinases Erk2 and 5 by these compounds may mediate these effects. These data indicate that JS-K may function as an anti-cancer agent against mouse erythroleukemia cells by activating a tumor suppressor and that the arylating capacity of JS-K may be sufficient to mediate this biological effect.

2. Materials and Methods

2.1. Reagents

JS-K and AcOM-DEA/NO were synthesized as previously described [11, 12]. CDNB was obtained from Alfa Aesar (Ward Hill, MA). DMSO, purchased from Sigma-Aldrich (St. Louis, MO), was used as a solvent. To rule out solvent contribution to the biological effects, each experiment included cells treated with DMSO alone. The final concentration of DMSO was not higher than 0.1 %.

2.2. Cell lines

The cell line used for this study, SFFV-MEL NP7, was previously established from mice injected with helper-free Friend spleen focus-forming virus [13]. The cells were cultured in Dulbeccos modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, and incubated at 37°C in 5% CO₂. For each experiment, cells were plated at a density of 1.5×10^5 cells/ml in complete medium and incubated under 5% CO₂ at 37°C.

2.3. Cell viability assay

Cytotoxicity after treatment was determined using the WST-1 assay (Roche, Mannheim, Germany). Cells were plated in 96-well plates (Corning, Corning, NY; 100 μ l/well) and cultured in complete DMEM. After 48 hours of treatment with different concentrations of tested compounds, absorbance was read at 450 nm using a plate reader (Tecan, Salzburg, Austria).

2.4. Apoptosis assay

Induction of apoptosis was determined by quantifying cytoplasmic histone-associated DNA fragments using a Cell Death Detection ELISA kit (Roche, Mannheim, Germany) according to the manufacture's protocol. Data are presented as relative DNA fragmentation compared with untreated cells as determined by absorbance at 405 nm with a reference wavelength of 490 nm.

2.5. DNA fragmentation assay

DNA was extracted from cells using the DNeasy Blood and Tissue Kit (Qiagen Sciences, Valencia, CA) and separated on a 1% agarose gel (Lonza, Rockland, ME). DNA fragmentation was visualized by exposing the gel to UV light after staining with ethidium bromide.

2.6. Western blot analysis

Western blot analysis was carried out on cell lysates as previously described [14]. Specific antibodies used in this study were as follows: from Cell Signaling (Danvers, MA): anti-

caspase 9 (#9508), anti-cleaved caspase 9 (#9509), anti-caspase 3 (#9665), anti-cleaved caspase 3 (Ser 253) (#9466), anti-Akt (#4691), anti-PI3-kinase (#4257), anti-p-PI3-kinase p85 (#4228), anti-mTOR (#2983), anti-p-mTOR (#2974), anti-Erk1/2 (#4695), anti-p-Erk1/2 (#4370), and anti-Erk5 (#3372) and anti-p-Erk5 (#3371); from Abcam (Cambridge, MA): anti-caspase 8 (#ab52183), anti-p-Akt (Ser473) (#ab18206) anti-p-Akt (Thr308) (#ab38449); and from Novus Biologicals (Littleton, CO): anti- β -actin (#NB600-501). Each analysis was repeated at least twice and representative blots shown. Quantitation of bands after autoradiography was performed by densitometry utilizing NIH ImageJ software.

2.8 Statistical analysis

To determine statistical significance, Student's *t*-test was performed. *P* values less than 0.05 were considered significant.

3. Results

3.1. Both JS-K and CDN B are cytotoxic for SFFV-MEL cells

The NO prodrug JS-K contains three components: a diazeniumdiolate group, which is necessary for the release of NO, an arylating ring and a carboethoxy-piperazine residue (Fig. 1A). To determine the importance of each component in killing erythroleukemia cells, we exposed SFFV-MEL cells for 48 hrs to various concentrations of JS-K as well as CDN B, which contains an arylating ring analogous to that of JS-K without the diazeniumdiolate group necessary to release NO, and AcOM-DEA/NO, which generates intracellular NO but lacks the arylating ring (Fig. 1A). Exposure of SFFV-MEL cells to 10 μ M of either JS-K or CDN B for 48 hrs completely blocked their survival (Fig. 1B), comparable to reported results on the effects of JS-K on human leukemia cells. Calculated IC₅₀ values were 2.00 μ M for JS-K and 1.75 μ M for CDN B. In contrast to JS-K and CDN B, treatment of SFFV-MEL cells with up to 50 μ M of AcOM-DEA/NO (Fig. 1A), which generates levels of intracellular NO equivalent to JS-K (see Figure S1), demonstrated no significant cytotoxicity (Fig. 1B). Furthermore, the NO scavenger vitamin B12 had no significant effect on the capacity of JS-K or CDN B to kill the cells (Figure S2). Morphological changes consistent with apoptosis or necrosis, including swelling, disintegration of cell membranes and fragmentation into apoptotic bodies (see arrows), could be observed in SFFV-MEL cells treated with 2 μ M of either JS-K or CDN B for 48 hrs (Fig. 1C).

To assure that CDN B was a good mimic for JS-K arylation, the metabolic fate of each compound in SFFV-MEL cells was scrutinized. As early as 15 min after treatment, both CDN B and JS-K were internalized and consumed, resulting in the arylation of nearly identical amounts of GSH (Fig. S3), indicating that both compounds are equally good arylators of cellular thiols. In neither case was the hydrolysis product 2,4-dinitrophenol detected, indicating that it does not play a significant role in the observed cytotoxicity of either compound.

3.2. Both JS-K and CDN B treatment of SFFV-MEL cells results in apoptosis associated with activation of caspases

Since apoptotic morphological changes were observed in SFFV-MEL cells after treatment with JS-K or CDN B, we carried out studies to determine whether the compounds were inducing apoptosis in SFFV-MEL cells. As shown in Fig. 2A, treatment with 2 μ M of either JS-K or CDN B induced significant apoptosis in SFFV-MEL cells within 24 hrs as measured by a specific ELISA assay that measures DNA fragmentation. The level of apoptosis was higher for cells treated with JS-K compared with CDN B. Interestingly, the level of apoptosis did not increase significantly after 48 hrs of exposure to either compound. DNA damage and

its fragmentation to 180–200 base pair pieces due to endogenous DNases could also be detected by 18 hr after treatment with either JS-K or CDNB (Fig. 2B).

Since apoptosis is associated with the activation of caspases, we examined lysates from SFFV-MEL cells treated for various times with 2 μ M of either JS-K or CDNB, for activation of various caspases. As shown in Fig. 2C, treatment with JS-K or CDNB causes activation of caspases 8 and 9, which are involved in extrinsic and intrinsic apoptotic pathways, respectively. Since the anti-caspase 8 antibody used for this experiment detected only full length procaspase protein, activation of caspase 8 was indicated by the disappearance of the full length procaspase 8 protein. Caspase 8 was activated 48 hrs after treatment with CDNB and at 24 hrs after treatment with JS-K (Fig. 2C). Antibody to caspase 9 detects the full-length procaspase 9 as well as its active cleaved form, which increased at 24 hrs after treatment with either CDNB or JS-K (Fig. 2C). Activation of either caspase 8 and 9 can result in the cleavage and activation of caspase 3 [16,17], and as indicated in Fig. 2C, both JS-K and CDNB treatment caused an increase in cleaved and activated caspase 3 after 24 hrs. Compared to CDNB, JS-K caused stronger activation of caspase 3 after 24 h of treatment (Fig. 2C). One of the targets of caspase 3 is PARP protein (poly (ADP-ribose) polymerase), which is involved in the repair of DNA [16,18]. Caspase 3 induces fragmentation of PARP protein and this modification blocks the ability of PARP to repair damaged DNA [18]. As shown in Fig. 2C, PARP is cleaved within 24 hrs of treatment of SFFV-MEL cells with either JS-K or CDNB.

3.3. SFFV-MEL cells treated with JS-K or CDNB show increased expression of nuclear FoxO3a and inactivation of the PI3-kinase/Akt and MAP kinase pathways

FoxO3a is a transcription factor and tumor suppressor that activates genes involved in inducing apoptosis [19,20], as well as protecting cells from oxidative stress [19,21,22]. Erythroid cells such as SFFV-MEL cells express enough FoxO3a in the nucleus to protect them from oxidative stress [23], but not sufficient levels to induce apoptosis. As shown in Fig. 3A, treatment of SFFV-MEL cells with either JS-K or CDNB results in an increase in the amount of FoxO3a in the nucleus, where it can act as a transcription factor for various caspases (Fig. 2C). FoxO3a is kept out of the nucleus due to phosphorylation, and treatment of SFFV-MEL cells with either JS-K or CDNB results in decreased phosphorylation of FoxO3a (Figure 3A). Since FoxO3a is phosphorylated by both the PI 3-kinase/Akt and the MAP kinase pathways [21,24], we analyzed drug-treated SFFV-MEL cells for inactivation of these pathways as measured by phosphorylation of signaling molecules. As shown in Fig. 3B, Akt phosphorylation on both Ser473 and Thr308 decreased to below control levels by 24 hrs after treatment with either CDNB or JS-K. This result is consistent with a decrease in FoxO3a phosphorylation on Ser 253, the residue phosphorylated by Akt kinase, by 24 hrs after treatment with either compound (Figure 3A). Phosphorylation of signaling molecules both upstream and downstream of Akt kinase followed a similar pattern after treatment (Fig. 3B). By 24 hrs after treatment with either CDNB or JS-K, the p85 component of PI 3-kinase, which phosphorylates Akt, as well as mTOR, which is phosphorylated by Akt, showed a decrease in phosphorylation. Treatment with either JS-K or CDNB did not alter total protein levels for Akt, FoxO3a, PI3-kinase or mTOR. The initial increase in phosphorylation of Akt, PI3-kinase and mTOR at 6 hrs after CDNB or JS-K treatment presumably represents an attempt of SFFV-MEL cells to suppress drug-induced cell death.

Activation of the MAP kinase pathway was also altered by treatment of SFFV-MEL cells with either JS-K or CDNB. As shown in Fig. 3C, Western blot analysis showed differences in phosphorylation of Erk 1 and 2 during treatment of SFFV-MEL cells with either CDNB or JS-K. The highest level of phosphorylation of these proteins was observed after 1 h of treatment (Fig. 3C). Phosphorylation of both Erk 1 and 2 significantly decreased 6 hrs after treatment with CDNB or JS-K. While Erk2 phosphorylation continued to remain low 24 and

48 hrs after treatment, phosphorylation of Erk1 returned to control levels. No difference in total p44/42 Erk 1/2 was observed after treatment with either compound (Fig. 3C). Phosphorylation of Erk5 gradually decreased after JS-K or CDNB treatment, with total Erk5 completely disappearing 24 hrs after treatment with JS-K and 48 hrs after treatment with CDNB (Fig. 3C). Since Erk5 can inhibit the transcriptional activity of FoxO3a by phosphorylating it on Ser 294 [25], the disappearance of Erk5 24-48 hrs after treatment with JS-K or CDNB may play a role in activating FoxO3a. Indeed, phosphorylation of Ser 294 on FoxO3a could not be detected 24 hrs after treatment with JS-K or CDNB (data not shown). We also examined two other MAP kinases, Jun N-terminal kinase 1/2 and p38 MAP kinase, and unlike Erk 1/2 and Erk 5, their phosphorylation was not altered by treatment with either JS-K or CDNB (data not shown).

4. Discussion

JS-K was designed to release nitric oxide upon reaction with glutathione in the presence of glutathione *S*-transferase, an enzyme overexpressed in cancer cells [26]. Previous studies have shown that leukemia cells, compared with normal hematopoietic cells, are particularly sensitive to nitric oxide, and JS-K is effective in killing human leukemia cells *in vitro* and when they are grown as xenografts in NOD-SCID mice [5]. In this study, we show that JS-K is also effective in killing murine erythroleukemia cells *in vitro*, and we use these cells to not only address the mechanism by which JS-K induces cellular cytotoxicity but also to determine the importance of the arylating ring of JS-K in this process. Our data indicate that JS-K induces MEL cells to undergo caspase-associated apoptosis and that this is most likely accomplished by inhibition of both the PI 3-kinase and MAP kinase pathways and the subsequent activation of the tumor suppressor FoxO3a. Further studies allowed us to conclude that the arylating capacity of JS-K is sufficient to induce these biological effects against murine erythroleukemia cells.

FoxO3a is a transcription factor that activates genes that induce apoptosis, and is, thus, considered to be a tumor suppressor [19,20]. In proliferating cells, FoxO3a is inactive due to its phosphorylation, which sequesters it in the cytoplasm. FoxO3a can be phosphorylated by Akt kinase on Ser 253 and Ser 315 [27] and by the MAP kinases Erk 1/2 and Erk 5 on Ser 294, 344 and 425 [28]. Inhibition of either of these kinases permits the translocation of FoxO3a from the cytoplasm to the nucleus where it can activate genes associated with inducing apoptosis. Our studies indicate that treatment of SFFV-MEL cells with either JS-K or CDNB results in inhibition of both the PI-3 kinase/Akt and MAP kinase pathways and the activation of FoxO3a. This is accompanied by increased expression of caspase 3. Thus, the cytotoxicity induced in SFFV-MEL cells by these compounds appears to be the result of a block in either the PI 3-kinase/Akt pathway and/or the MAP kinase pathway, leading to the activation of the tumor suppressor protein FoxO3a and induction of apoptosis. Previous studies from our laboratory have shown that the MEK inhibitor PD98059, which inhibits both Erk 1/2 and Erk5 signaling but not PI3-kinase/Akt signaling, has little effect on the growth of SFFV-MEL cells [14]. This suggests that inhibition of these Erk signaling pathways by JS-K is not sufficient to induce apoptosis and that inhibition of the PI3-kinase/Akt pathway by JS-K, either alone or in conjunction with a block in Erk signaling, is required for mediating the biological effects of this compound.

Although the NO-releasing capability of JS-K appears to play a necessary role in the cytotoxicity of this compound against a number of different tumor cell lines [4–7], it does not appear to play a major role in the killing of SFFV-MEL cells. CDNB, which lacks the NO-releasing capacity of JS-K, was just as effective in killing SFFV-MEL cells, while AcOM- DEA/NO, which releases high levels of intracellular NO in SFFV-MEL cells, had little effect. Consistent with the idea that the toxicity of JS-K towards SFFV-MEL cells is

not attributable to NO release, the NO scavenger vitamin B12 had no significant effect on JS-K's capacity to kill the cells. The failure of NO to kill SFFV-MEL cells may be because FoxO3a, in addition to its role in apoptosis, plays a protective role against oxidative stress [19,21,22]. In erythroid progenitor cells, FoxO3a expressed in the nucleus induces the expression of antioxidants such as catalase, GADD45 and MnSOD [23,29] without causing apoptosis. Thus, expression of FoxO3a in the nucleus of JS-K-treated erythroleukemia cells used in this study may counteract the oxidative stress-inducing effects of NO released from JS-K [30]. This may explain why both JS-K and CDNB were equally effective in killing SFFV-MEL cells, and why AcOM- DEA/NO was ineffective, and point to a separate role of the arylating ring of both compounds in inducing apoptosis via thiol-S-arylation of proteins [31]. Arylation of key substrates in the PI 3-kinase/Akt and/or the Erk 2/5 pathways may be responsible for blocking activation of these pathways, resulting in the activation of FoxO3a.

5. Conclusions

Our results demonstrate strong activity of JS-K against murine erythroleukemia cells and reveal its involvement in inhibiting signal transduction pathways necessary for cell survival, ultimately leading to activation of a tumor suppressor protein that leads to expression of genes associated with apoptosis. Our data further indicate that the arylating capacity of JS-K is the major contributor to its biological effects against these cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Cytotoxicity of JS-K and CDNB for SFFV-MEL cells

(A) Structures of compounds used in this study. (B) Effects of 48 hr treatment with the various compounds on SFFV-MEL cell survival. Data represent the average from triplicate samples $\pm$ standard deviation; * $p < 0.001$. IC_{50} : JS-K = 2.0 μ M; CDNB = 1.75 μ M; AcOM-DEA/NO = 91.5 μ M. (C) Change in morphology of SFFV-MEL cells 48 hrs after treatment. 20x. Arrows indicate apoptotic cells.

Figure 2. JS-K and CDNB induce caspase-associated apoptosis in SFFV-MEL cells

SFFV-MEL cells were treated with 2 μ M JS-K or CDNB for various times and analyzed for (A) apoptosis as measured by a cell death detection ELISA. Data represent the average from triplicate samples $\pm$ standard deviation * $p < 0.01$; ** $p < 0.001$; (B) DNA fragmentation or (C) expression of various activated caspases and PARP as measured by Western blot. Quantitation was performed by densitometry and expression values were normalized to β -actin. Normalized values relative to that of untreated cells (set to 1) are shown. Values for caspases 9 and 3 are for the cleaved form.

Figure 3. JS-K and CDNB alter expression of nuclear FoxO3a and the PI 3-kinase/Akt and MAP kinase pathways

SFFV-MEL cells were treated with 2 μ M JS-K or CDNB for various times and analyzed by Western blot. (A) Nuclear localization and phosphorylation of FoxO3a. Quantitation was performed by densitometry and expression values were normalized to β -actin. Normalized values relative to that of untreated cells (set to 1) are shown. (B) Activation of the PI 3-kinase/Akt pathway. For quantitation of phosphorylated proteins, expression values were normalized to total Akt, PI3-kinase or mTOR. (C) Activation of the MAP kinase pathway. For quantitation, p-Erk1 and 2 expression values were normalized to total Erk-1 or Erk-2, while phospho and total Erk5 expression values were normalized to β -actin.