Research Article

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A nitric oxide-releasing prodrug promotes apoptosis in human renal carcinoma cells: Involvement of reactive oxygen species

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Abstract

Background – JS-K is a nitric oxide (NO)-releasing prodrug of the \( \text{O}^2 \)-arylated diazeniumdiolate group that shows pronounced cytotoxicity and antitumor properties in numerous cancer models, including in vitro as well as in vivo. Reactive oxygen species (ROS) induce carcinogenesis by altering the redox status, causing increment in vulnerability to oxidative stress.

Material and methods – To determine the effect of JS-K, a glutathione S-transferase (GST)-activated NO-donor prodrug, on the induction of ROS accumulation, proliferation, and apoptosis in human renal carcinoma cells, we measured the changes of cell proliferation, apoptosis, ROS growth, and initiation of the mitochondrial signaling pathway before and after JS-K treatment.

Results – In vitro, dose- and time-dependent development of renal carcinoma cells were controlled for JS-K, and JS-K also triggered ROS aggregation and cell apoptosis. Treatment with JS-K induces the levels of pro-apoptotic proteins (Bak and Bax) and decrease the number of anti-apoptotic protein (Bcl-2). In fact, JS-K-induced apoptosis was reversed by the antioxidant \( \text{N-acetylcysteine} \), and oxidized glutathione, a pro-oxidant, improved JS-K-induced apoptosis. Finally, we demonstrated that in renal carcinoma cells, JS-K improved the chemosensitivity of doxorubicin.

Conclusion – Our data indicate that JS-K-released NO induce apoptosis of renal carcinoma cells by increasing ROS levels.

Keywords: apoptosis, ROS, JS-K, nitric oxide, renal carcinoma cells

1 Introduction

Renal cell carcinoma (RCC) is a prevalent urogenital system malignant tumor with an incidence of approximately 5–10 per 100,000, representing 2–3% of all adult tumors [1], with one of the highest incidences of urological malignancies. According to the prediction by the American Cancer Society of the United States, almost 73,800 new cases of RCC and nearly 14,700 associated demises were reported in 2019. [2]. RCC is often asymptomatic in its preliminary phases, in that the illness frequently evolves at the time of diagnosis into final stages and exactly 25–30% of patients possess metastatic RCC at diagnosis. Various methods of treating RCC, for instance, conventional immunotherapy, targeted therapy, and radical surveillance, are currently available. Around 40% of patients, regretfully, are immune to radiation therapy and conventional chemotherapy. Such individuals may suffer systemic recurrence, resulting in high toxicity and poor treatment response [3], and a severe effect on disease-related death, with a very low 5-year survival rate (only 10–12%) [4]. Yet more studies on the mechanisms involved in the growth and advancement of RCC are thus need to identify new therapeutic targets.

\( \text{O}^2(2,4-\text{Nitrophenyl})1-[(4-\text{ethoxycarbonyl})\text{piperazine}-1\text{-yl}] \text{diazene-1-ium-1,2-diolate (JS-K, C13H16N6O8, AS-No: 205432-128)}, \) a diazeniumdiolate-based nitric oxide (NO)-donor prodrug, is a glutathione S-transferase (GST)-activated...
compound that can produce more intracellular NO levels (Figure 1) [5]. The optimal activity of JS-K uniquely requires GSTs, with overexpression of GST, and JS-K can produce higher intracellular concentrations of cytotoxic inside tumor cells, thereby reducing the viability of tumor cell. JS-K has no apparent toxicity in normal cells and shows antitumor properties both in vivo and in vitro [6]. In the recent study, the level of intercellular ROS was regulated by NO via outputting numerous responsive nitrogen types (RNS) [7]. A recent study showed that JS-K was extremely effective against nonsmall-cell lung cancer cells through elevation of the basal levels of ROS [8]. JS-K has also been stated to have antitumor activity against prostate cancer cells [9,10], bladder cancer cells [11], Ovarian cancer [12], gastric cancer [13], and nonsmall-cell lung cancer [14]. However, the direct effect of JS-K on proliferation and apoptosis of renal carcinoma cells and the role of ROS have not been characterized. The aim of this study is to explore whether JS-K also has cytotoxic effects on human renal carcinoma cells and to evaluate available mechanisms of ROS.

2 Materials and methods

2.1 Reagents and cell culture

JS-K was obtained from Biotechnology, Inc. of Santa Cruz (CA). Beyotime Institute of Biotechnology purchased N-acetylcysteine (NAC) and oxidized glutathione (GSSG) (Shanghai, China). The ACHN and A498 human renal carcinoma cell lines were purchased from the Shanghai Institute of Biochemistry and Cell Biology. ACHN and A498 cells were cultivated in DMEM medium (GIBCO, Grand Island, NY) and PRMI-1640 medium (GIBCO, Grand Island, NY) separately, with 11% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in the air with 5% carbon.

2.2 Cell proliferation assay

The Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay assessed the proliferation of ACHN and A498 cells. Cells (1 × 103/well) were plated for 24 h on 97-well plates and treated for 12, 24, and 48 h using JS-K (5 μM). The Cell Counting Kit-8 (10 μL/per well) reagent was then included in to culture intermediate and incubated for 120 min at 37°C. The microplate reader measured the absorbance of each well at 450 nm (BioTek Instruments, Inc., US). Almost every experiment was performed in triplicate, and the average values were calculated.

2.3 Apoptosis study

Apoptosis was detected using the FITC Annexin V Apoptosis Detection Kit I. The cells were incubated in a conditioned medium by JS-K, then collected, separated by a centrifuge tube, and nurtured with Annexin V-FITC and PI for minutes at room temperature in the darkness. Then, the stained cells were evaluated by flow cytometry within 60 min.

2.4 Caspase-Glo 3/7 assays

We used the caspase-Glo 3/7 assay to gauge the exercise of caspase-3/7 following the manufacturer’s protocol to further analyze cell apoptosis. In short, JS-K treated cells

Figure 1: The structure of JS-K and release of NO.
and conditioned medium were grouped into 97-well dishes (Corning, USA). Then, each well was added with an equal volume of caspase-Glo 3/7 reagent and incubated for 30 min at room temperature in the darkness. A luminometer was used to obtain the luminescence data (Berthold Sirius L, Germany).

### 2.5 Measurement of intracellular ROS

In accordance with the manufacturer’s protocol, we used the Reactive Oxygen Species Assay Kit (Beyotime) via flow cytometry to identify the accumulation of ROS. Cells were shortly treated with JS-K (1, 2, and 5 μM) for 6 h, collected, and centrifuged in a serum-free DCFH-DA reagent-containing medium. However, they were incubated in the dark for 20 min at 37°C. At the excitation source at 489 nm and emission at 526 nm, the DCF fluorescence energy was calculated.

### 2.6 RNS assay

In this study, nitrite ion (NO₂⁻) was described as RNS, as well as the levels of RNS were quantified as per the manufacturer’s instructions using a Nitrite Assay Kit (Beyotime).

### 2.7 Superoxide measurement

The superoxide variable was determined using a Superoxide Assay Kit according to the manufacturer’s protocol. The cells were treated using JS-K (1, 2, and 5 μM) for 6 h, and then, the superoxide detection reagent (200 μL/well) was randomly added and incubated for 20 min at 37°C. In a 96-well plate reader, the solution was read at 450 nm (PerkinElmer, Waltham, MA). The superoxide variable was determined using a Superoxide Assay Kit according to the manufacturer’s protocol. The cells were treated using JS-K 9 for 6 h, and then, the superoxide detection reagent (200 μL/well) was randomly added and incubated for 20 min at normal temperature. In a 96-well plate reader, the solution was read at 450 nm (PerkinElmer, Waltham, MA).

### 2.8 Glutathione content assay

Renal carcinoma cells were incubated at 6 h JS-K (0, 1, 2, and 5 μM) levels. Cells were implanted onto six-well plates, with a density of 3–105 cells (Millipore Scepter TM) per well. There had been two consecutive cycles of precipitation of the cells. The supernatant was extracted by ultracentrifugation at 10,000×g for 15 min. The quantity of glutathione or glutathione disulfide (GSSG) was calculated by GSH and GSSG assay kit as per the manufacturer’s protocol.

### 2.9 Measurement of mitochondrial membrane potential

Cells were grouped at 3–105 cells/well in seven-well plates and subjected to extreme JS-K (0, 1, 2, and 7 μM) concentrations for 3 h. The JC-1 mitochondrial membrane possibility assay kit (Beyotime) is used to evaluate the extent of mitochondrial membrane potential as per the producer’s protocols.

### 2.10 Measurement of ATP production

Cells were incubated for 6 h with JS-K, and afterward, ATP concentrations were calculated using ATP Assay Kit as instructed by producers.

### 2.11 Western blot analysis

Renal carcinoma cells were incubated with a buffer (Beyotime) in radioimmunoprecipitation assay (RIPA) supplemented with 1 mM phenylmethyl sulphonylfluoride (PMSF; Beyotime). Renal carcinoma cells treated with JS-K (0, 1, 2, and 5 μM) were isolated by electrophoresis of sodium dodecyl sulfate polyacrylamide gel. Renal carcinoma lysates were subsequently transferred to the difluoride membranes of polyvinylidene. Bak (Cell Signaling Technology, USA), Bax protein (Cell Signaling Technology, Inc.), Bcl-2 (Cell Signaling Technology), PARP protein receptors (Cell Signaling Technology), and GAPDH (Abcam, Cambridge, MA, USA) were transfected into the neurons and distilled overnight at 4°C. The membranes were then tested with an IgG-HRP supplementary goat-anti-rabbit antibody for 30 min (EarthOx, USA).

### 2.12 Statistical study

All tests were conducted at least three times and at least triplicated each time. The results have been represented...
as average ± SD. The one-way ANOVA statistical analysis was performed using SPSS 18.0. LSD(L) examined the differences; a big variation was inferred for $P < 0.05$, and an exceedingly substantial variance for $P < 0.01$ was inferred.

**Ethical approval:** The experimental protocols were approved by the Ethics Committee of the First Hospital of Huizhou.

## 3 Results

### 3.1 JS-K suppresses proliferation and induces apoptosis in renal carcinoma cells

Renal carcinoma cells were treated with different JS-K (0, 1, 2, and 5 μM) concentrations. Cells treated with JS-K for 24 h were distorted in shape and more cells endured apoptosis than untreated cells (Figure 2a). To analyze the possible inhibition of cell proliferation by JS-K in renal carcinoma cells, a CCK-8 assay was conducted. The data showed that JS-K exhibited highest dose- and time-dependent inhibition of renal carcinoma cell proliferation (Figure 2b and c). IC50 concentrations are $6.59 \pm 0.16$ μM (ACHN cells) and $5.52 \pm 0.14$ μM (A498 cells) at 24 h. Flow cytometry was used for the evaluation of the apoptosis effect of JS-K (0, 1, 2, and 5 μM) in renal carcinoma cells. The findings showed that the apoptosis rate of renal cancer cells expanded in a dose-dependent manner after treatment with JS-K for 24 h (Figure 3a and b).

### 3.2 JS-K increases ROS and RNS levels and decreases the GSH/GSSG ratio in renal carcinoma cells

Overall ROS development, RNS, and superoxide concentrations were inspected after 6 h of treatment with varying ratios of JS-K in renal carcinoma cells. When renal carcinoma cells are treated with S-K, a substantial increase in the total output of ROS (Figure 4a), RNS (Figure 4b), and superoxide (Figure 4c) was observed. To control the impact of JS-K on oxidative stress, we also checked intracellular levels of GSH and GSSG. Its ratio decreased dramatically with JS-K therapy (Figure 4d). Thus, these data show that JS-K can induce an intracellular redox state imbalance, which may stimulate mitochondrial dysfunction and apoptosis mediated by mitochondria.

### 3.3 JS-K reduces mitochondrial membrane potential and ATP levels in renal carcinoma cells

To evaluate the energy generation of mitochondria, the intracellular generation of mitochondrial membrane potential and ATP in renal carcinoma samples transfected with JS-K was evaluated. Upon treatment of cells with varying concentrations of JS-K for 6 h, the mitochondrial membrane potential (Figure 4e) and intracellular ATP (Figure 4f) were reduced in a dose-dependent way.

### 3.4 Effect of JS-K on the production of apoptotic proteins in renal carcinoma cells

Upon treatment with different concentrations of JS-K for 24 h, apoptotic proteins involved in mitochondria-mediated renal carcinoma cell apoptosis were measured. JS-K treatment showed an increase in the exercise of both caspase-9 and cleaved PARP compared to the Western blot negative controls (Figure 4g). Activation of pro-apoptotic proteins such as Bak and Bax was upregulated by the J-SK treatment, while dose-dependent downregulation of the antiapoptotic protein Bcl-2 was seen (Figure 4g).

### 3.5 Impacts of NAC and GSSG on JS-K-induced cell growth suppression and apoptosis

In the inclusion or exclusion of antioxidants NAC (100 μM) or pro-oxidant GSSG (5 μM), renal carcinoma cells were treated with 5 μM JS-K for 24 h, separately, to evaluate the function of ROS in JS-K-induced cell proliferation inhibition and also apoptosis. The review shows that NAC restored the JS-K-induced inhibitory activity as well as renal cancer cell apoptosis and effectively reinstated ROS levels (Figure 5). Conversely, GSSG enhanced the inhibitory effect and apoptosis inserted by JS-K and thereby promoted the JS-K-mediated ROS production (Figure 5).

### 3.6 JS-K augments the chemosensitivity of doxorubicin in renal carcinoma cells

Renal carcinoma cells have been co-treated with JS-K and doxorubicin to assess the impacts of JS-K on the chemical
sensitivity of doxorubicin. Compared to the treatment with JS-K alone or doxorubicin alone, combination action with JS-K and doxorubicin resulted in much more potent cell viability inhibition (Figure 6a). There was also a synergistic impact of the combined therapy with JS-K and doxorubicin on the promotion of caspase-3/7 in renal carcinoma cells (Figure 6b).

4 Discussion

The previous studies have shown a steady increase of this cancer globally. Moreover, renal cell carcinoma is resistant to chemotherapy and radiotherapy. Therefore, it is essential to obtain an insight into the mechanisms of molecules of tumor cell apoptosis in primary renal cell carcinoma lesions to improve treatment and prognosis for this disease [15,16].

Whenever an imbalance exists between both the generation rate of ROS and the rate of ROS removal by scavenging mechanisms, oxidative stress occurs in some way. Glutathione is a ubiquitous cellular thiol that has two types: the main thiol redox mechanism in cells is depleted (GSH) and oxidized (GSSG) and correct GSH/GSSG redox equilibrium is essential for the cell activity [17]. Thus, we hypothesized that JS-K-released nitric oxide would promote apoptosis of renal carcinoma cells by triggering ROS. Thus, this research shows that by the ROS production, JS-K conferred a cytotoxic effect and triggered renal carcinoma cell apoptosis. The development of renal carcinoma cells reduced with increasing JS-K concentrations, and apoptosis improved in a dose-dependent method. Findings show that NAC reversed the
The cytotoxic effect of JS-K and was improved by GSSG. ROS is significant for normal cellular processes. The deregulating level of ROS leads to the growth of numerous human diseases, including cancer. In contrast with normal tissue, due to rapid cell metabolism, cancer cells have elevated ROS levels. In cancer cells, the high ROS levels differentiate them from normal cells and can promote tumorigenesis. However, high ROS levels are also an Achilles’ heel for cancer cells because the elevated ROS renders cancer cells highly prone to oxidative stress-induced cell death that can be used by targeted cancer therapy to control [18,19]. For cell proliferation and apoptosis, ROS is a vital signaling molecule [20]. The stimulation of intracellular signal transduction, including the numerous cellular processes, such as inflammation, progression of the cell cycle, and invasion, is also involved in ROS [21].

It has been confirmed that various physiological phenomena and apoptosis-inducing chemicals result in oxidative stress by ROS generation, indicating a strong linkage between apoptosis and oxidative stress [22,23].
Figure 4: Effect of JS-K on ROS, RNS, mitochondrial membrane potential, GSH/GSSG ratio, ATP production, and apoptotic-related proteins in renal carcinoma cells. Treatment of JS-K renal carcinoma cells (0, 1, 2, and 5 μM) for 360 min, and then detection of intracellular maximum ROS (a), RNS (b), superoxide (c), and GSH/GSSG ratio (d) levels. Upon 360 min of JS-K treatment, measurements were made for mitochondrial membrane potential (e) and ATP output (f and g). Western blot was used to identify concentrations of apoptotic-related proteins such as Bak, Bax, and PARP after cells were treated with JS-K (0, 1, 2, and 5 μM) for 24 h. For at least three separate assays, findings are reported as mean ± SD.
Figure 5: Effects of NAC and GSSG on JS-K-induced cell growth suppression and apoptosis. Cells were pre-cultured for 24 h with 100 μM NAC or 5 μM GSSG, and then incubated for 24 h both with and without 5 μM JS-K. Cell survival was assessed by CCK-8 assay (a), and flow cytometry (c) was evaluated for cell apoptosis. Cells were pretreated with 100 μM NAC or 5 μM GSSG for 24 h and subsequently treated with or without 5 μM JS-K for 6 h, and ROS output was measured (b) and Caspase-3/7 activation was analyzed (d). The values are represented as mean ± SD for at least three independent experiments. A single asterisk (*) shows a substantial difference (P < 0.05), and a double asterisk (**) (P < 0.01) shows an exceedingly big variation.

Figure 6: JS-K enhances the chemosensitivity of doxorubicin in renal carcinoma cells. (a) Cell viability was assessed following 12 h treatment with JS-K (5 μM) and doxorubicin (0.2 μM) using a CCK-8 assay. (b) Expression of caspase-3/7 in cells of renal carcinoma following treatment at multiple drug levels and time courses. The result for at least three separate experiments is expressed as mean ± SD. A single asterisk (*) shows a substantial difference (P < 0.05), and a double asterisk (**) (P < 0.01) shows an exceedingly huge difference.
The intracellular redox state is sustained inside a narrow range under standard circumstances, but under pathological conditions, this balance may be disrupted to decrease or increase values [24]. JS-K-released NO has been reported to result in extremely high intracellular concentrations of ROS [6]. To inhibit the epithelial–mesenchymal transition as well as cancer development in prostate cancer cells [25], excessive ROS generation has been reported. In renal carcinoma cells, larger ROS, RNS, superoxide alterations, cell inhibition rates, and apoptosis rates have originally been identified, suggested that these cells might be highly sensitive to JS-K [9]. Indeed, we found significantly increased levels of ROS/RNS along with the increased apoptosis and inhibition of cell proliferation in two renal carcinoma cell lines following the treatment with JS-K. In addition, the antioxidant NAC reversed the antitumor effects of JS-K, whereas the pro-oxidant GSSG enhanced these effects, suggesting that ROS induction may be a way by which JS-K exerts its effects in renal carcinoma cells. Moreover, the outcome indicated that co-treatment with JS-K improved the renal carcinoma cell’s chemosensitivity to doxorubicin.

GSH is an important antioxidant. The consumption of glutathione can result in redox imbalance, which is related to mitochondrial dysfunction and apoptosis [26]. We demonstrated that the GSH level was significantly reduced by JS-K but increased the GSSG production in renal carcinoma cells, which lead to a reduction in the GSH/GSSG ratio. Moreover, findings have shown that the manufacturing of ATP could be controlled during apoptosis by the reduction of mitochondrial membrane potential [8]. Our data showed that, in a dose-dependent way, JS-K reduced the mitochondrial membrane latent and also ATP concentrations. In addition, upregulation of cleaved-caspase-9 has been observed, and it has a significant role in the family of cysteine aspartic protease (C) [27,28]. PARP is a caspase-9 precursor that helps in DNA repair, and cleaved-PARP is considered an indicator of cell proliferation apoptosis [29,30]. Bcl-2, Bax, and Bak are essential components of apoptosis activation of mitochondrial stress, and the Bcl-2 and Bax balance is essential for cell existence [31,32]. In this study, we showed that therapy with JS-K enhanced the expression in renal carcinoma cells of pro-apoptotic proteins, for instance, cleaved-Caspase-9, cleaved-PARP, Bax, and Bak, but JS-K reduced the expression of the antiapoptotic protein Bcl-2. For instance, JS-K therapy has also improved caspase-3 and caspase-7 activities since they are either partly or entirely accountable for PARP cleavage. It has also showed that treatment using JS-K increased caspase-3/7 activation in renal carcinoma cells, indicating that aggregation of ROS induced by JS-K can encourage mitochondria-mediated apoptosis in cells of renal carcinoma.

5 Conclusion

In particular, a major element for the progression of the disease is redox imbalance due to excessive or inadequate ROS. We have shown that ROS is strongly correlated with the proliferation of renal carcinoma cells as well as that the mitochondria-dependent apoptosis pathway is stimulated by excessive ROS accumulation. In this study, the impacts of JS-K on human renal carcinoma cells have been evaluated and JS-K is indicated to considerably get rid of cell proliferation and to induce apoptosis in human renal carcinoma cells as a result of the high ROS buildup. We indicated that the development of ROS is necessary for the initiation of the mitochondria-dependent apoptosis pathway by JS-K (Figure 7). We theorize that there had been a disparity in redox reactions to support well-being in renal carcinoma cells. The effect of nitrites from NIL sources on renal carcinoma cells had also been studied, and findings equivalent to those
induced with JS-K therapy were seen. Although more research will be required to comprehend the dynamic underlying mechanisms in the JS-K–induced production of ROS, collected data indicate that JS-K induces renal carcinoma cell apoptosis, and this is regulated, at least to some extent, via the renal carcinoma cell ROS-related pathway.

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