



## Radiosensitization of HT-29 Colorectal Cells and Xenografts by the Nitric Oxide Donor JS-K

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### Abstract

**Background:** Nitric oxide (NO) donors have demonstrated radiosensitizing activity against colorectal cancer *in vitro* and *in vivo*. JS-K is an NO donor that releases NO upon activation by glutathione. We evaluated the activity of JS-K *in vitro* and *in vivo* against HT-29 cells.

**Methods:** Cell survival was assessed by colony forming assays in cells subjected to ionizing radiation (IR) [0 - 6 Gray (Gy)] with and without JS-K [0.5 μM]. Mice were inoculated with HT-29 cells and randomized to four treatments: (1) control, (2) IR [2 Gy X 5], (3) JS-K [20 μmol/Kg i.p.], (4) JS-K+ IR.

**Results:** JS-K-treated cells were more sensitive to IR (SF-4 = 0.35) vs. untreated cells (SF-4 = 0.45) and (SF-6 = 0.21 vs. 0.29; respectively). Following 34 days of treatment, the JS-K+IR xenografts had a 9.4% reduction of tumor growth ( $p \leq 0.001$ ) and at 40 days a 14.3% decrease compared to control ( $p \leq 0.0001$ ). Western blot analysis showed an increase in cleaved PARP-1 and AIF with a concomitant decrease in Bcl-2.

**Conclusion:** JS-K demonstrated antitumor activity against HT-29 cells *in vitro* and *in vivo*. Caspase and non-caspase mediated mechanisms of apoptosis were involved in this response.

**Keywords:** Nitric oxide donor; Rectal cancer; Complete pathological response

### Introduction

Patients with adenocarcinoma of the rectum with stage II or local nodal disease are candidates for neoadjuvant chemoradiation (CRT) [1]. This recommendation emanates from multiple advantages in patients receiving pre-operative CRT compared to resection alone (a total mesorectal excision [TME]). For instance, the rate of locoregional recurrence is decreased with the former approach [2]. This modality might also facilitate surgical intervention by allowing the performance of a low anterior resection (LAR) versus and abdominoperineal resection (APR) [3]. Furthermore, data suggest that patients with rectal cancer who achieves a complete obliteration of the tumor (pCR) following neoadjuvant treatment have superior long term outcomes compared to patients who achieve a partial or no response receiving the same form of treatment [4].

The basic challenge with neoadjuvant therapies for the management of adenocarcinoma of the rectum stems from the unpredictable and broad response of this approach. The response of pre-operative CRT can be broadly divided into three groups: 50% of patients achieve a partial response, 25% a pCR and 25% no response [5]. None of the current radiosensitizing agents or radiotherapy schedules have been able to significantly alter these observations [3]. Thus, either a new approach in the delivery of radiotherapy is needed or novel radiosensitizing agents need to be introduced.

Nitric oxide (NO) donors have emerged as novel anti-cancer therapeutic agents [6]. We have demonstrated that the NO donor DETA/NONOate had potent radiosensitizing properties against HT-29 colorectal cancer cells and xenografts [7]. The main limitation in utilizing effective NO donors in oncology originates from adverse effects related to vasodilation culminating in systemic hypotension [8]. An alternative to overcome this limitation is to develop compounds that release anti-cancer agents exclusively in neoplastic tissue.

The compound JS-K is the prodrug: *O*<sup>2</sup>-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl] diazen-1-ium-1,2-diolate, that has shown anti-tumor activity in a variety of rodent cancer models (Figure 1). JS-K was designed to be activated by reaction with glutathione (GSH) [9] to release two moles of NO [10].

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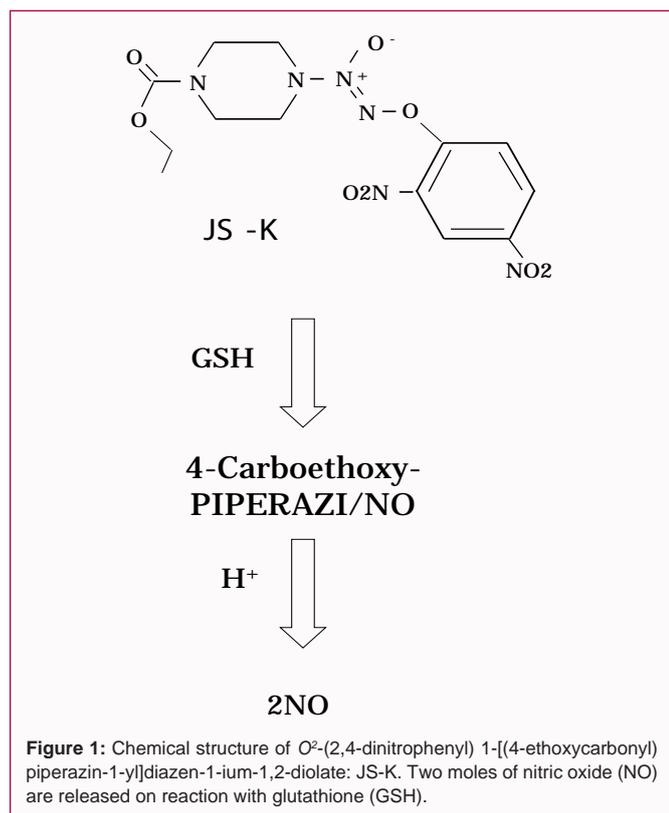
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JS-K has demonstrated potent anti-cancer properties in several cancer cell lines *in vitro* or as xenografts [9]. The effects of JS-K in combination with ionizing radiation (IR) have not been investigated. However, JS-K has been shown to be highly effective against cancer cells characterized by high levels of reactive oxygen species (ROS). We hypothesized that JS-K could act as an effective neoadjuvant prior to radiation therapy in colorectal cancer. In this study, the role of JS-K as a possible neoadjuvant agent in HT-29 colorectal cancer cells and xenografts was investigated. Potential mechanisms of actions were also explored.

## Methods

### Cell culture

HT-29 (HTB-38) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown and handled as previously described [7,11]. All cell lines were maintained at 37°C in ATCC-formulated Leibovitz's L-15 Medium supplemented with 10% heat-inactivated fetal bovine serum and 1% (v/v) L-glutamine.

### Clonogenic survival assays

Clonogenic assays were performed on single-cell suspension of mid-log growing cells as previously described [7,11]. Cells were trypsinized and counted using a Coulter Counter (Casy1, Schärfe System, and Reutlingen, Germany). Plating efficiency (PE) was defined as (colony counted in un-irradiated control)/(cells seeded) as previously described [7,11]. All experiments were repeated at least three times.

### Treatment of cells with JS-K

The nitric oxide donor  $O^2$ -(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K) was prepared as previously described [12]. HT-29 were exposed to

JS-K [0.5 $\mu$ M] in DMSO for 16 h (JS-K pre-treatment). Following pre-treatment cells were subjected to ionizing radiation (IR) at 0 [Gray (Gy)]; control], 2.0 Gy, 4.0 Gy, 6.0 Gy. Cells were then allowed to grow for clonogenic assays and compared to untreated cells. All cells were also collected for Western blot analysis. The dose and timing of JS-K were selected based on previous studies that demonstrated the effects of JS-K against HL-60 cells *in vitro* and *in vivo* [10,13,14].

### *In vivo* studies

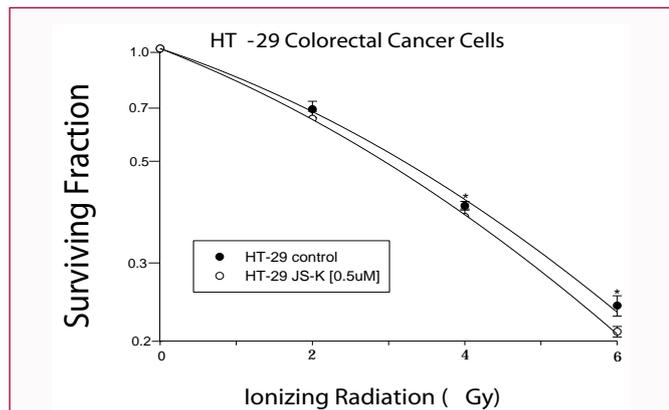
Approval for this protocol was obtained from The Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center. All animal experiments were done in accordance with institutional guidelines for animal welfare and maintained under clean room conditions in sterile rodent micro isolator cages. Animals received sterile rodent chow and water ad libitum. For tumor growth experiments, HT-29 cells ( $1 \times 10^6$ ) were injected s.c. with 27-gauge needle into the right flank region of 6-week old SCID mice as previously described [7,11,15].

Once the animals developed palpable tumors (50-100 mm<sup>3</sup>) they were randomly assigned to one of four groups (n=4 to 5): (1) control group; (2) IR alone; (3) JS-K alone; and (3) IR plus JS-K. Sterile PBS or 20 $\mu$ mol/Kg of JS-K (100  $\mu$ L total volume) was delivered intraperitoneally everyday for five days (week 0 to 1) by means of a 27-gauge needle attached to a 1-cc tuberculin syringe, immediately following treatment. After each drug delivery, mice from group 2 and 4 were treated with 2.0 Gy of ionizing radiation as previously described [7,11]. Specifically, radiation was delivered to restrained mice using a <sup>60</sup>Co irradiator (Neutron Products, Inc., Dickerson, MD) with a variable collimator to generate a single adjustable collimated dorso-ventral beam of X-rays at a dose rate of 1.0 Gy/min. Lead blocks were used to shield the non-tumor parts of the mice. The dose for JS-K was selected based on our previous observations of the chemo-sensitizing properties of this compound at this dose in HL-60 xenografts [13,14].

Tumor response to a given treatment was assessed as described previously with minor modifications [7,11]. The response to IR was evaluated by determining the rate of tumor growth for each mouse. Tumor growth was compared to the original size of the tumor for each animal at the beginning of the treatment compared to each data point and expressed as a percent in tumor response to a given treatment group. Each treatment group is expressed as the rate of response over time $\pm$ SE.

### Western blot analysis

For western blot analyses cells were pre-treated with JS-K [0.5  $\mu$ M] for 5 h then irradiated at 4.0 Gy or left untreated (control). Total protein was collected 75 h after IR. Western blot analysis of total protein extracts from HT-29 cells was performed as previously described [7,11]. Membranes were exposed to primary antibodies: rabbit anti-Bax, -Bcl-2, -AIF, and -p53 at a 1:1000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA); as well as rabbit anti-survivin, -XIAP, -PARP-1 at a 1:1000 dilution (Cell Signaling Technology, Danvers, MA) for 24 h. After the incubation, the membranes were exposed to horseradish peroxidase-conjugated secondary antibody 1:4000 (anti-IgG, Bio-Rad, Hercules, CA). Levels of beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used to normalize the proteins levels. Relative concentrations were assessed by densitometric analysis of digitized autographic images, using the public domain NIH Image J Program (available on the internet) as previously described [7,11]. Western blot analyses were repeated a



**Figure 2:** JS-K radiosensitization of HT-29. HT-29 cells were exposed to JS-K [0.5  $\mu$ M] for 16 h. Following pre-treatment, cells were subjected to ionizing radiation (IR) at 0 Gy, 2.0 Gy, 4.0 Gy, 6.0 Gy. Cells were then allowed to grow for clonogenic assays as described in the methods. Each point represents the average of percent survival by clonogenic assays of three separate experiments  $\pm$  SE expressed as log 10, \* $p$  < 0.05.

minimum of three times. A representative sample of each Western blot is presented in the results section.

**Statistical analysis**

PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA) was used for statistical analysis. Data are expressed as [means  $\pm$  SE]. Differences in cell survival were assessed by analysis of the variance (ANOVA). The statistical difference in tumor load in HT-29 xenografts receiving the various treatments was also evaluated by ANOVA. Bonferroni comparison test was used to identify significant differences ( $p \leq 0.05$ ) in treatment effects.

**Results**

**JS-K induced cell death in HT-29 cells**

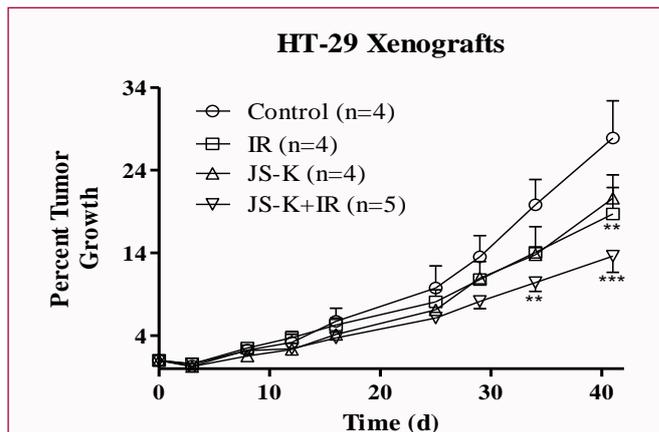
Since colony-formation assays assess all parameters related to cell growth including apoptosis, cell cycle, and necrosis, these assays are ideal to examine the relative effects of drug intervention over time. Consistent with previous observations HT-29 cells are highly resistant to IR [7,11]. Clonogenic assays demonstrated that JS-K-treated cells were more sensitive to IR at 4Gy (SF-4 = 0.35) vs. untreated cells (SF-4 = 0.45;  $p \leq 0.05$ ) and at 6 Gy (SF-6 = 0.21 vs. 0.29;  $p \leq 0.05$ ; (Figure 2).

**JS-K led to a reduction in tumor growth in HT-29 xenografts**

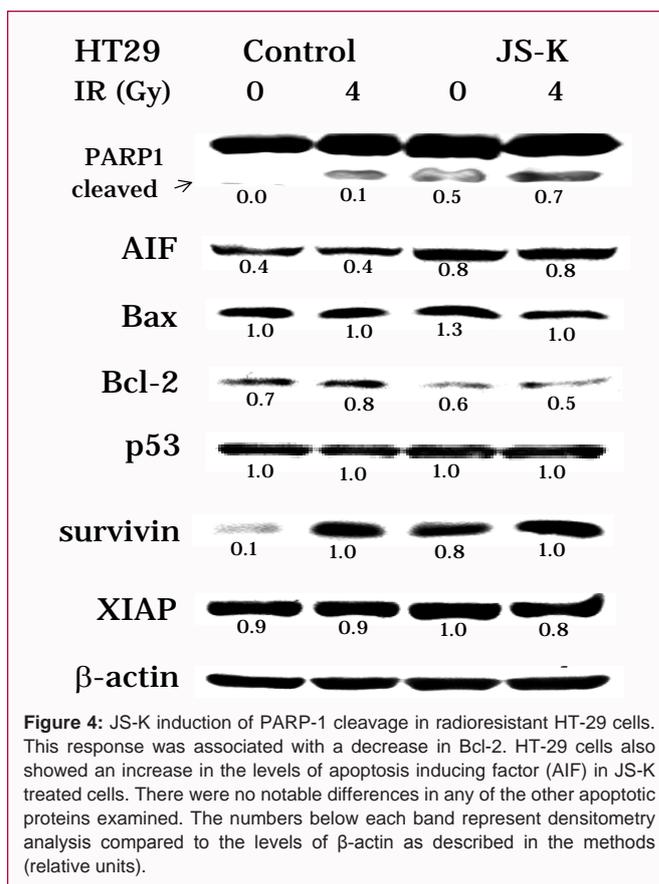
In order to corroborate our observations *in vitro*, we inoculated immunocompromised mice with HT-29 cells. In this study, 17 mice were inoculated with cells and randomized to control (n=4) and three treatment groups (n=4 to 5) with or without IR. Ionizing radiation was delivered exclusively to the xenograft while shielding the rest of the mouse as previously described [7,11]. Mice were pre-treated with JS-K (20  $\mu$ mol/kg i.p.) every day for five days, (week 1) as described in the methods. This was immediately followed by 2.0 Gy IR for five days every day for a total of 10.0 Gy (week 1). Tumor growth is expressed as the average in percent of tumor growth for each treatment over time.

Our *in vivo* results were consistent with the *in vitro* experiments. HT-29 xenografts were highly resistant to IR. There was no difference in tumor growth between JS-K only group compared to control. At the end of the treatment, the IR only group demonstrated a 9.1% reduction in tumor growth compared to control ( $p \leq 0.01$ ; (Figure 3).

Following 34 days of treatment, the JS-K+IR group had a 9.4%



**Figure 3:** JS-K radiosensitization of HT-29 xenografts. SCID mice were inoculated with  $1 \times 10^6$  HT-29 cells. Seventeen SCID mice were randomized to three treatment groups and were compared to control after receiving JS-K only, ionizing radiation (IR) only, and JS-K+IR. Treated mice were subjected to their respective treatment for one week and tumor xenografts were measured twice a week for five weeks. Each point represents the average of the volume of all tumor xenografts  $\pm$  SE. \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  treatment vs. control.



**Figure 4:** JS-K induction of PARP-1 cleavage in radioresistant HT-29 cells. This response was associated with a decrease in Bcl-2. HT-29 cells also showed an increase in the levels of apoptosis inducing factor (AIF) in JS-K treated cells. There were no notable differences in any of the other apoptotic proteins examined. The numbers below each band represent densitometry analysis compared to the levels of  $\beta$ -actin as described in the methods (relative units).

reduction of tumor growth compared to control ( $p \leq 0.001$ ). At the end of the treatment there was a 14.3% reduction in tumor growth in the JS-K+IR treated group compared to control ( $p \leq 0.0001$ ; (Figure 3).

**Western blot analysis suggests caspase and non-caspase mediated pathways of cell death**

Western blot analysis showed an increase in cleaved PARP-1 with JS-K alone compared to untreated cells (0.5 vs. 0.0 [relative units];

respectively) and with JS-K+IR compared to untreated cells (0.1 vs. 0.7; respectively). AIF demonstrated a 2-fold increase with JS-K treatment alone and JS-K+IR compared to untreated cells. JS-K led to a reduction in Bcl-2 in cells receiving no IR (0.6 vs. 0.7; respectively) and a more pronounced effect in cells treated with IR (0.8 vs. 0.5; respectively) (Figure 4).

## Discussion

The present study was undertaken to determine the role of JS-K as a potential radiosensitizing agent against HT-29 cell *in vitro* and *in vivo*. The anticancer properties of JS-K are centered on two principles. The first one is based on the fact that cancer cells often over-express glutathione S-transferase [16], which allows drug specific enzymatic action in tumor tissue, while sparing potential systemic side effects. For instance, JS-K was a potent anticancer agent against HL-60 leukemia cells and caused a substantial reduction of implanted xenografts without hypotensive events [10,13]. The second principle has to do with the anti-cancer activity of nitric oxide. Nitric oxide donors have emerged as novel potential therapeutic agents in the past few years [6]. We have previously demonstrated that the nitric oxide donor DETA/NONOate had an additive apoptotic effect with ionizing radiation in HT-29 cells and xenografts [7].

JS-K is one of a myriad of diazeniumdiolate compounds [17]. JS-K has demonstrated promising broad-spectrum anti-cancer activity [9]. JS-K has been shown to have potent anti-neoplastic activity against many tumor types *in vitro* [9]. Additionally, *in vivo* JS-K has proved activity against different cancers such as leukemia xenografts in mice [10], murine prostate cancer xenografts [10], orthotopic models of liver cancer in rats [13], non-small cell lung xenografts in mice [18], glioma xenografts in rats [19], and multiple myeloma xenografts in mice [20]. Furthermore, JS-K has shown synergism in combination with other chemotherapeutic modalities including: bortezomib [20], sodium arsenite [21], cytarabine [14] and cisplatin [22]. The role of JS-K in combination with IR has not been investigated.

In the present report, we demonstrated that JS-K has additive effects *in vitro* and *in vivo* against HT-29 colorectal cancer cells in combination with IR. We showed a significant reduction of colony formation in HT-29 cells receiving JS-K compared to cells treated with IR alone (by 10% and 8% at 4 and 6 Gy; respectively). These results are in agreement with our previous observations of an additive effect in apoptosis in HT-29 cell with the NO donor DETA/NONOate [7].

Similarly, *in vivo* JS-K in combination with IR led to a reduction in tumor growth in immune compromised mice; with HT-29 xenografts receiving combination treatment compared to the control group, a 9.3% difference in tumor growth was observed at 34 days of treatment. At the end of the treatment, a 14.3 % difference was observed in mice receiving combination treatment compared to control and a 5.2% difference in mice treated with IR alone. This is also in agreement with our previous observations *in vivo* in HT-29 xenografts treated with DETA/NONOate [7].

While JS-K was designed to be activated for NO release on reaction with GSH, multiple mechanisms have been elucidated and multiple molecular events are thought to lead to the anti-cancer properties of JS-K including caspase activation, cell growth modulation, as well as an anti-angiogenic mechanism and cell migration [21]. The present report suggests a caspase-mediated mechanism of apoptosis by a reduction of Bcl-2 leading to PARP-1 cleavage activation as previously

described [21]. Non-caspase mediated mechanisms of action were also suggested via apoptosis inducing factor protein augmentation as reported in other studies with JS-K in multiple myeloma cells [20].

Microarray and real-time RT-PCR have suggested multiple pathways involved in the cell death induced by JS-K against HL-60 cells including apoptosis, differentiation, and suppression of angiogenesis [21]. Further, JS-K has been shown to induce DNA strand breaks in multiple myeloma cells [20]. In HL-60 cells, JS-K caused DNA strand breaks only at high concentrations (10  $\mu$ M). However, when combined with cytarabine, it acted synergistically at sub micromolar concentrations to induce DNA damage in HL-60 cells [14].

Because HT-29 cells have been shown to have low glutathione S-transferase activity compared to Caco-2 cells (206 $\pm$ 54 vs. 3680 $\pm$ 236 nmol/mg/protein/min) by other investigators [23], it is likely that mechanisms beyond GST-mediated pathways accounted for our observations in HT-29 cells by JS-K *in vitro* and *in vivo*. Additional studies are required to determine whether there is augmentation in DNA-induced damage directly by JS-K in HT-29 cells in combination with IR.

The limitations of our study stem from its preliminary nature. The numbers of mice included in our experimental protocol are small and the differences in the clonogenic studies are modest compared to other nitric oxide donors (i.e., DETA/NONOate) [7]. However, these findings show unambiguously that JS-K has additive effects in combination with IR and incorporates a second NO donor as a possible novel radiosensitizing agent in the management of rectal cancer refractory to conventional radiochemotherapeutic interventions.

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