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Short Communication

JS-K has potent anti-angiogenic activity *in vitro* and inhibits tumour angiogenesis in a multiple myeloma model *in vivo*

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Abstract

Objectives Glutathione S-transferases (GSTs) play an important role in multidrug resistance and are upregulated in multiple cancers. We have designed a prodrug class that releases nitric oxide on metabolism by GST. O^2-(2,4-Dinitrophenyl) 1-[(4-ethoxycarbonyl) piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K, a member of this class) has potent antineoplastic activity.

Methods We studied the effect of JS-K on angiogenesis in human umbilical vein endothelial cells (HUVECs), OPM1 multiple myeloma cells, chick aortic rings and in mice.

Key findings JS-K inhibited the proliferation of HUVECs with a 50% inhibitory concentration (IC50) of 0.432, 0.466 and 0.505 μM at 24, 48 and 72 h, respectively. In the cord formation assay, JS-K led to a decrease in the number of cord junctions and cord length with an IC50 of 0.637 and 0.696 μM, respectively. JS-K inhibited cell migration at 5 h using VEGF as a chemoattractant. Migration inhibition occurred with an IC50 of 0.493 μM. In the chick aortic ring assay using VEGF or FGF-2 for vessel growth stimulation, 0.5 μM JS-K completely inhibited vessel growth. JS-K inhibited tumour angiogenesis *in vivo* in NIH III mice implanted subcutaneously with OPM1 multiple myeloma cells.

Conclusions JS-K is a potent inhibitor of angiogenesis *in vitro* and tumour vessel growth *in vivo*. As such, it establishes a new class of antineoplastic agent that targets the malignant cells directly as well as their microenvironment.

Keywords angiogenesis; HUVEC; JS-K; myeloma; nitric oxide

Introduction

Nitric oxide (NO) has significant growth-inhibitory activity against acute myeloid leukaemia (AML) cells.[1–3] The use of compounds that generate NO spontaneously for the treatment of malignancies is precluded by the pleiotropic and potentially toxic effects of NO.[4] A strategy that targets NO to malignant cells is therefore likely to be more clinically applicable. In an effort to develop NO-based therapies for the treatment of malignant diseases, we have developed a class of arylated diazeniumdiolate NO-generating agents. These compounds are pro-drugs that do not release high levels of NO spontaneously.[5] However, they are activated to release NO upon nucleophilic attack by reduced thiols, especially those of the abundant peptide glutathione (GSH). The activation reaction is catalysed by the glutathione *S*-transferases (GST).[5] This drug design strategy seeks to exploit the overexpression of GST in malignant cells as compared with normal tissues.[6,7]

Upon screening a library of arylated diazeniumdiolates for their in-vitro anti-leukaemic activity, we identified O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K) as a lead compound. JS-K inhibits the growth of HL-60 (human myeloid leukaemia) cells with an in-vitro 50% growth-inhibitory concentration (IC50) of 0.25–0.5 μM.[5] JS-K also inhibited the growth of HL-60 cells in a subcutaneous xenograft

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model in NOD/SCID mice.[5] In separate experiments, JS-K was found to be a potent inhibitor of multiple myeloma cell growth, including multi-drug resistant clones.[8] In that study JS-K treatment led to significant survival prolongation of NIH III mice subcutaneously implanted with multiple myeloma OPM1 cells.[8] The purpose of the experiments presented in this paper was to determine the effect of JS-K on angiogenesis. We showed that JS-K is a potent inhibitor of angiogenesis *in vitro* and of tumour vessel growth *in vivo*.

Materials and Methods

Chemicals

JS-K was synthesized as previously described.[9] All other chemicals were from Sigma-Aldrich (St Louis, USA) unless otherwise noted.

Cells

In-vitro angiogenesis experiments were conducted using human umbilical vein endothelial cells (HUVECs; VEC Technologies, New York, USA). For in-vivo experiments OPM1 cells were obtained from Dr Lief Bergsagel (Mayo Clinic, Scottsdale, USA).

Growth inhibition assay

The growth inhibition assay was conducted as previously described.[10] In brief, HUVECs (1.5×10^3) were plated in 100 μl of EBM-2 medium (Clonetics, Walkersville, USA). After 24 h (day 0), JS-K was added at the indicated concentrations. On day 0, one plate was stained with 0.5% crystal violet in 20% methanol, rinsed with water and air-dried. The remaining plates were incubated at 37°C. After 24, 48 and 72 h, plates were stained with crystal violet. The stain was eluted with a solution of 0.1 M sodium citrate and ethanol (1 : 1) and absorbance was measured at 540 nm with a microplate reader. Day 0 absorbance was subtracted from the test plates and data were plotted as percentage of control proliferation (vehicle-treated cells).

Cord formation on Matrigel

Cord formation was evaluated as previously described by plating HUVECs on basement membrane matrix preparations (Matrigel; Becton Dickinson, Franklin Lakes, USA) distributed in 96-well plates.[10] HUVECs were treated with the indicated concentrations of JS-K for 24 h. Cells were then harvested, washed, and resuspended in growth factor-supplemented EBM-2 medium before distributing in 96-well plates (2×10^5/100 μl). After 24 h, tube formation was observed using an inverted phase contrast microscope (DM-IRB; Leica Inc.) at 5 × magnification and images were captured with a CCD camera. Quantitation of tube formation was determined by measuring the length of tubes and counting junctions in three random fields from each well (2 wells per data point) using the Bioquant Image Analysis System (Nashville, USA). Data were plotted and the IC50 was derived from the resultant curves.

Cell migration assay

Cell migration was evaluated as previously described.[10] The migration assay was performed in a 96-well disposable chamber (ChemoTx 101-8; Neuroprobe, Gaithersburg, USA). Both sides of the framed filter were coated with 25 μl/well of rat tail collagen Type I (BD Biosciences, Bedford, USA) for 30 min and dried for 1 h in a laminar flow hood. Assay buffer (27–29 μl) for normal negative control or 10 ng/ml vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, USA) as a chemoattractant was added to the wells of the bottom plate to form a small positive meniscus over the wells. The collagen-coated framed filter was then placed over the microplate. HUVECs were harvested and a cell suspension was prepared at 2×10^6/ml. Before addition to the migration plate, cells were mixed with JS-K prepared in media containing 1% bovine serum albumin. Untreated control or JS-K-treated cell suspensions (30 μl) were placed on top of each well of the filter in quadruplets. Plates were incubated at 37°C for 5 h. At the end of the incubation period, the filter was fixed and stained using the Hema 3 staining kit (Fisher Diagnostics, Middletown, USA). The filter was rinsed with distilled water and cells that had not migrated from the top of the filter were removed using a wet Kimwipe. Cells that had migrated were counted from five fields from each well under high power. The number of cells that had spontaneously migrated (negative control wells) was subtracted from the number of cells that had migrated in untreated control or the JS-K treatment variable. Data were expressed as the percent of control migration (vehicle-treated cells). Data were plotted and the IC50 was derived from the resultant curves.

Chick aortic arch assay

Aortic arches were dissected from day 12–14 chick embryos. Periaortic fibroadipose tissue was removed from the aortic arches, which were then cut into 1-mm-long rings. Aortic rings were placed on growth-factor-depleted Matrigel (Becton Dickinson, Franklin Lakes, USA). Rings were cultured at 37°C in a humidified 5% CO_2 atmosphere. VEGF or fibroblast growth factor-2 (FGF-2) at a concentration of 50 ng/ml was used to stimulate vessel growth. JS-K was added at concentrations in the range 0.1–0.5 μM. After 48 h in culture, rings were stained with Calcein AM (Molecular Probes, Eugene, USA) and evaluated for vessel growth using an inverted microscope (DM-IRB, Leica Inc.). As shown, the effects of JS-K on vessel growth were so significant (total inhibition) that no delineation of the outgrowth area for quantitation purposes was conducted.

Animal studies

NIH III mice, 5–6 weeks old, were purchased from Charles River Laboratories (Wilmington, USA). All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the Dana-Farber Cancer Institute according to accepted standards. The mice were inoculated subcutaneously in the right flank with 3×10^7 OPM1 cells in 100 μl of RPMI 1640 and 100 μl of Matrigel basement membrane matrix (Becton Dickinson, Franklin Lakes, USA). When tumours were palpable, nine mice were

assigned to the treatment group receiving 4 μmol/kg of JS-K intravenously three times per week and eight mice to the control group receiving an equivalent volume of vehicle alone according to the same schedule. JS-K stocks in dimethyl sulfoxide (DMSO) were serially diluted in phosphate-buffered saline (PBS) before injection. The final concentration of DMSO was < 10%. Vehicle control consisted of 10% DMSO in PBS. Caliper measurements of the longest perpendicular tumour diameters were performed every other day to estimate the tumour volume. Mice were sacrificed when tumours reached 2 cm^3 or if the mice appeared moribund. The first day of sacrifice was 21 and 43 days after start of treatment for the vehicle control and JS-K-treated groups, respectively. Upon sacrifice, tumours were dissected out of the mice and stained with a murine specific anti-CD34 rat monoclonal antibody (Abcam Clone MEC14.7) to mark vessels. Vessel density was evaluated visually using light microscopy. Photomicrographs shown are representative of all the sections evaluated. Again here, inhibition of tumour vessel growth by JS-K was so significant that no attempt at quantitation was undertaken using hot-spot analysis or vessel scoring.

Statistics

Student's *t*-test was used to compare results between treated cells and controls. The Bonferroni procedure was used to adjust *P* values for multiple comparisons within each experiment ($P < 0.05$ was considered statistically significant).

Results

Effect of JS-K on HUVEC proliferation

We first determined the effect of JS-K on HUVEC proliferation *in vitro*. HUVEC growth was evaluated 24, 48 and 72 h after adding JS-K. JS-K inhibited HUVEC growth in a dose-dependent fashion (Figure 1). HUVEC growth inhibition by JS-K as a percent of control occurred to the same extent at every time point (Figure 1). The IC50 for HUVEC growth inhibition by JS-K was 0.432, 0.466 and 0.505 μM after 24, 48 and 72 h of exposure, respectively. A JS-K concentration of 1 μM almost completely inhibited HUVEC proliferation (Figure 1).

Effect of JS-K on cord formation

The cord formation assay is indicative of new vessel formation. We therefore determined the effect of JS-K on cord formation *in vitro*. After 24 h exposure to JS-K, a dose-dependent decrease in the number of cord junctions was observed (Figure 2). At the same time points, cord length was significantly diminished as well (Figure 2). The IC50 for JS-K-induced decrease in the number of cord junctions and cord length was 0.637 and 0.696 μM, respectively.

Effect of JS-K on endothelial cell migration

Endothelial cell migration is critical to angiogenesis. We therefore sought to determine the effect of JS-K on HUVEC migration *in vitro*. In this assay, HUVEC were subjected to chemotaxis by VEGF stimulation for 5 h with or without JS-K.

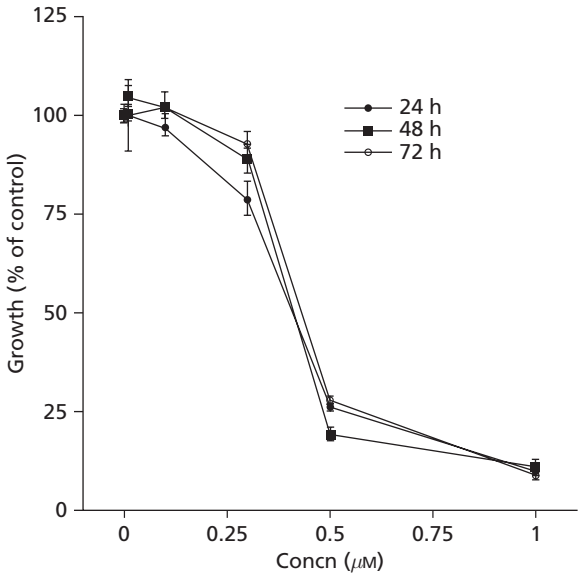

Figure 1 JS-K inhibits HUVEC growth. HUVECs were cultured with the indicated concentrations of JS-K. At the indicated time points cell growth was scored as outlined in the Methods section. JS-K treatment led to a dose-dependent inhibition of HUVEC growth. The IC50 at 24, 48 and 72 h was 0.432, 0.466 and 0.505 μM, respectively. Data are averages ± SEM of three separate experiments.

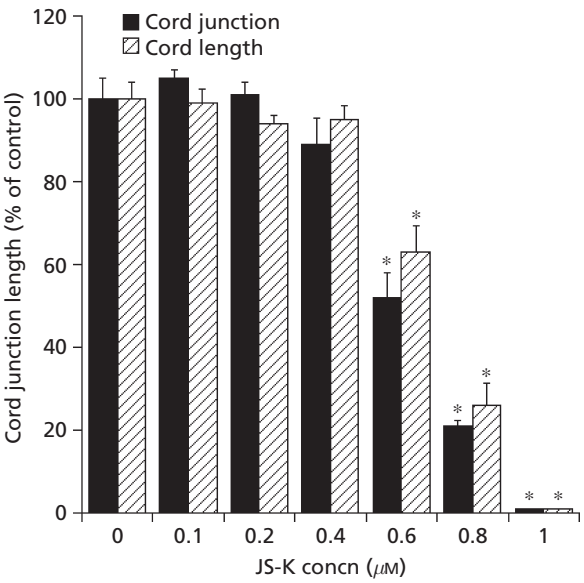

Figure 2 JS-K inhibits cord formation. HUVECs were plated in Matrigel after treatment with the indicated concentrations of JS-K. Twenty-four hours later cord formation was evaluated by scoring cord length and cord junctions. JS-K treatment led to a dose-dependent inhibition of cord formation. The IC50s for cord junction and cord length were 0.637 and 0.696 μM, respectively. Data are averages ± SEM of three separate experiments. *$P < 0.05$ compared with controls.

At concentrations above 0.4 μM, JS-K significantly inhibited HUVEC migration (Figure 3). At that time point, visual inspection revealed no evidence of cell death at any JS-K concentration, indicating that the observed effect was due to

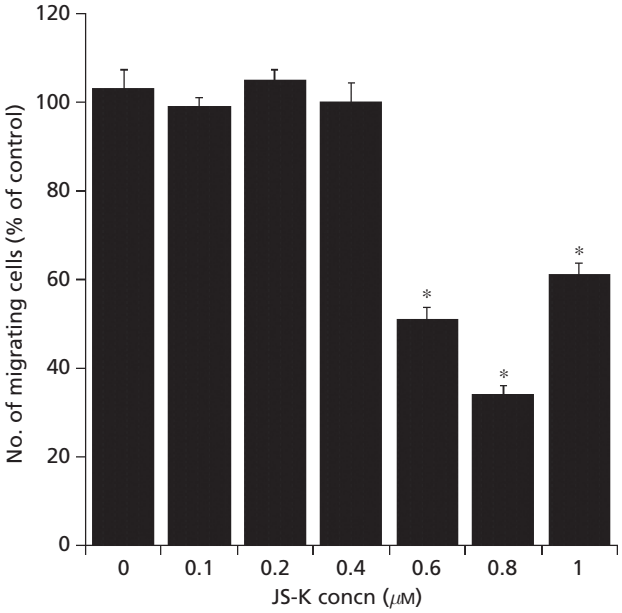

Figure 3 JS-K inhibits HUVEC migration. HUVEC migration at 5 h with the indicated concentrations of JS-K was assayed using 10 ng/ml VEGF as a chemoattractant. JS-K treatment led to a dose-dependent inhibition of HUVEC migration. The IC50 for migration inhibition was 0.496 μM. At the 5-h time point there was no evidence of growth inhibition. Data are averages ± SEM of three different experiments. *$P < 0.05$ compared with controls.

migration inhibition rather than cytotoxicity of the drug towards HUVECs. Cytotoxicity by JS-K at these concentrations was not observed until later time points (24 h or more). The IC50 for migration inhibition was 0.493 μM.

Effect of JS-K in the chick aortic ring assay

In addition to endothelial cells, the chick aortic ring assay includes the surrounding non-endothelial cells such as fibroblasts. Furthermore, in this assay, endothelial cells have not been modified by repeated in-vitro passages. Consequently, the chick aortic ring assay is more reflective of in-vivo angiogenesis. We therefore determined the effect of JS-K on vessel growth in this assay. Two sets of experiments were conducted using FGF-2 or VEGF to stimulate angiogenesis as detailed in the Methods section. Culture of the aortic rings without stimulation led to minimal vessel growth (Figure 4a), while stimulation with 50 ng/ml FGF-2 led to extensive angiogenesis (Figure 4b). FGF-2-induced angiogenesis was diminished by the addition of 0.1 μM JS-K and almost completely abolished when the latter was added at a concentration of 0.5 μM (Figures 4c and 4d, respectively). Similar observations were made when 50 ng/ml VEGF was used to stimulate angiogenesis (not shown).

Effect of JS-K on tumour angiogenesis *in vivo*

To determine whether JS-K affects tumour angiogenesis *in vivo*, we used a murine subcutaneous plasmacytoma model. In this experiment, NIH III mice were implanted subcutaneously with OPM 1 multiple myeloma cells and treated with JS-K at a dose of 4 μmol/kg given intravenously three times a week. We have previously observed that this dose and schedule are safe and effective for the treatment of AML in NOD/SCID mouse xenografts.[5] As previously reported, JS-K treatment of NIH III mice implanted with OPM 1 cells led to significant inhibition of tumour growth and prolongation of survival.[8] Tumour explants obtained from control vehicle-treated mice at 21 days after start of treatment revealed substantial tumour angiogenesis as demonstrated by CD34 staining (Figure 5a). JS-K treatment led to markedly decreased tumour angiogenesis at the 43-day time point (Figure 5b).

Discussion

The experiments we present here show a previously unknown effect of JS-K, namely inhibition of angiogenesis. Using different in-vitro assays, we show that JS-K inhibits three key aspects of angiogenesis – endothelial cell division, endothelial cell migration and new vessel formation. Importantly, JS-K inhibited tumour angiogenesis *in vivo*. In the in-vivo experiment described here and in our previously reported in-vivo experiments, JS-K was administered to mice at a dose of 4 μmol/kg without observable toxicity.[5,8] Such a dose would be expected to lead to peak blood levels of around 17 μM in a 20 g mouse. This concentration is considerably higher than the concentrations at which JS-K was inhibitory in the different angiogenesis assays described in this paper. Consequently, as shown in the in-vivo model presented here, angiogenesis inhibition by JS-K is achievable *in vivo* and therefore is relevant to the clinical development of this drug.

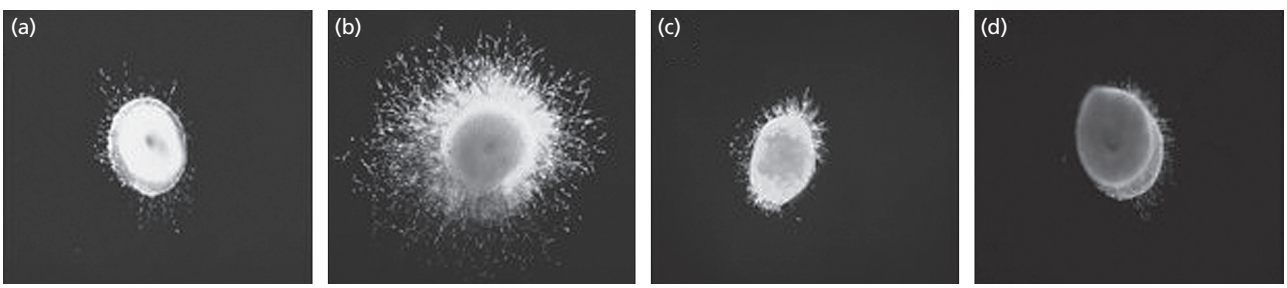

Figure 4 JS-K inhibits vessel growth in the chick aortic ring assay. Chick aortic rings were cultured *in vitro* with different additives as detailed in the Methods section. After two days in culture, rings were evaluated for vessel growth. (a) No additive control. (b) FGF-2 50 ng/ml. (c) FGF-2 50 ng/ml + JS-K 0.1 μM. (d) FGF-2 50 ng/ml + JS-K 0.5 μM. Pictures are representative of two separate experiments. Magnification 50 ×. Similar results were observed when 50 ng/ml VEGF was used to stimulate angiogenesis (not shown).

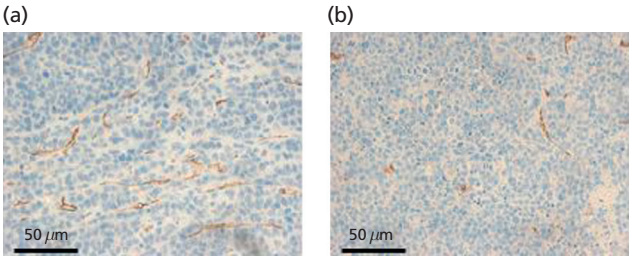

Figure 5 JS-K inhibits tumour angiogenesis in an in-vivo plasmacytoma model. NIH III mice were implanted with OPM1 multiple myeloma cells and treated with vehicle control (a) or JS-K at a dose of 4 μmol/kg thrice weekly (b). Control and JS-K-treated mice were sacrificed 21 and 43 days after starting treatment, respectively. Tumour explants were stained with a CD34 antibody to mark vessels and slides were evaluated under 400 × magnification. JS-K significantly inhibited tumour angiogenesis *in vivo*. Pictures are representative of slides from eight control and nine JS-K-treated mice, respectively.

Another point to make regarding the relevance of our observations is whether JS-K would get activated in endothelial cells *in vivo*. We have used HUVECs in our in-vitro assays; these cells have become a standard in the study of angiogenesis.[11] However, data suggest that tumour endothelial cells are different from normal endothelial cells and therefore could respond differently to cytotoxic or anti-angiogenic agents.[12] JS-K is designed to be activated to release NO upon interaction with GSH in a reaction catalysed by GST.[5] GST is expressed in normal endothelial cells.[13] No data are available comparing levels of expression of GST between normal and tumour endothelial cells. However, since endothelial cells have been shown to express GST,[13] one would expect that JS-K would become activated in the tumour vasculature to inhibit tumour angiogenesis *in vivo*. Furthermore, it is obvious that a single assay of angiogenesis is not enough to demonstrate whether a chemotherapeutic agent is truly an angiogenesis inhibitor.[11] However, the combined analysis of the effect of JS-K in four different in-vitro assays clearly supports the fact that it is an angiogenesis inhibitor. Most importantly, our demonstration that JS-K inhibits tumour angiogenesis *in vivo* in a murine multiple myeloma model certainly makes our observations relevant to the clinical situation.

One obvious question arising in data interpretation is whether our observations are due to a simple cytotoxic effect of JS-K on endothelial cells rather than true inhibition of angiogenesis. Data presented in Figure 2 show that JS-K did not inhibit cord formation or cord junction at a concentration of 0.4 μM. As shown in Figure 1, at this concentration, JS-K would be expected to induce around 50% inhibition of HUVEC growth. If the effect of JS-K on cord formation/cord junction was due to a cytotoxic effect of HUVECs, then one would expect that at a concentration (0.4 μM) that induces 50% growth inhibition in HUVECs one would observe some cord formation/cord junction inhibition. Similarly, data shown in Figure 3 show that JS-K did not inhibit HUVEC migration at a concentration of 0.4 μM. Again, at that concentration JS-K induced almost 50% growth inhibition of HUVECs (Figure 1). If JS-K inhibited HUVEC migration solely through a cytotoxic effect, one would expect to observe some migration inhibition at a concentration of 0.4 μM. Furthermore, migration was evaluated at 5 h. At that point, we did not see any difference in cell density between JS-K-treated HUVECs and controls. Consequently, we believe that the observed effects of JS-K are due to a genuine anti-angiogenic effect rather than simple cytotoxicity.

The mechanism by which JS-K inhibits angiogenesis is yet to be elucidated. JS-K is an NO-generating compound. The effect of NO on angiogenesis has been the subject of much work, as well as controversy. NO plays an important role in the initiation of angiogenesis by inducing vasodilatation through the activation of soluble guanylate cyclase and cGMP production.[14] NO also upregulates the production of VEGF.[15] On the other hand, Pipili-Synetos *et al.*[16] proposed that NO is an endogenous inhibitor of angiogenesis, and Jia *et al.*[17] described the direct anti-angiogenic effect of S-nitrosocaptopril (an S-nitrosothiol type of NO donor compound). In the latter experiments, S-nitrosocaptopril inhibited angiogenesis *in vitro* (proliferation and tube formation of capillary endothelial cells) and *in vivo* (neovasculature formation induced by VEGF on the chick embryo chorioallantoic membrane).[17] In these experiments, the anti-angiogenic effects of S-nitrosocaptopril were ascribed to NO because captopril itself did not affect angiogenesis. If NO is involved in the effects of JS-K on angiogenesis, it is clearly inhibitory in the current context. On the other hand, microarray studies have shown that JS-K upregulates in HL-60 cells the expression of the endogenous angiogenesis inhibitor thrombospondin-1 and its receptor CD36.[18] In the same cells, it also upregulated the expression of several tissue inhibitors of metalloproteinases (TIMPs).[18] Consequently, it is possible that the anti-angiogenic effects of JS-K are mediated by modulating the expression of key factors involved in the angiogenesis process.

Arylation reactions (the reaction of an aryl ring with free protein thiols) have been shown to affect protein function.[19] The structure of JS-K includes a diazeniumdiolate moiety as well as a dinitrophenyl ring. Consequently, arylation could contribute to JS-K's mechanism of action. Indeed, we have previously shown that protein arylation plays a role in the anti-leukaemic effects of JS-K.[20] It is therefore likely that JS-K induces arylation of key proteins in endothelial cells with resultant inhibition of angiogenesis. On the other hand, the JS-K derivative PABA/NO has been shown to induce extensive protein glutathionylation in ovarian cancer cells.[21] Similar to arylation, glutathionylation of thiols can have an inhibitory effect on protein function.[22] Whether JS-K exerts its effects on endothelial cells (and therefore angiogenesis) through arylation or glutathionylation in addition to NO release remains to be proven. However, similar to leukaemic cells, since JS-K has multiple biologic effects, it is likely to exert its action on endothelial cells through multiple mechanisms, including NO release.

Arylated diazeniumdiolates constitute a large group of NO-generating compounds that share a common backbone. Our lead optimization efforts have allowed the identification of JS-K as the most active antineoplastic agent of this family, as determined by screening their ability to inhibit HL-60 cell proliferation.[20] Even though they vary in their direct cytotoxic potency, arylated diazeniumdiolates share the common

properties of NO generation and arylation of thiols. No data are currently available on the effect of other arylated diazeniumdiolates on angiogenesis. Consequently, it is not possible to tell whether the anti-angiogenic properties of JS-K are unique to this compound or constitute a class effect. We have previously determined that any substitution on the dinitrophenyl ring of JS-K diminished its direct cytotoxic effects.[20] Any conclusion as to whether a similar effect would be observed with JS-K's anti-angiogenic properties is speculative at this point.

Using a murine subcutaneous plasmacytoma model, we show here that JS-K inhibits tumour angiogenesis *in vivo*. We have previously demonstrated that JS-K is directly cytotoxic to multiple myeloma cells by activating both the intrinsic and extrinsic apoptosis pathways.[8] Furthermore, JS-K overcomes the survival and growth advantages imparted to multiple myeloma cells by exogenous interleukin-6 and insulin growth factor-1 (IGF-1), or by adherence of multiple myeloma cells to bone marrow stromal cells.[8] It is established that multiple myeloma cells thrive on the normal microenviroment of the tumour cell.[23] Part of this is due to recruitment of new vessels. Bone marrow angiogenesis has also been shown to correlate negatively with survival of multiple myeloma patients.[24,25] Our previous work and results presented here show that JS-K has anti-myeloma activity by a direct cytotoxic effect as well as by affecting the interaction of the malignant cells with their microenvironment. This dual effect makes JS-K a very potent new agent for the treatment of multiple myeloma. Besides multiple myeloma, we have shown that JS-K has significant anti-neoplastic properties *in vivo* against AML, prostate cancer and hepatoma cells.[5,20] In these models, JS-K was directly cytotoxic to the malignant cells. The in-vitro angiogenesis assays we used here are not specific to a particular malignancy. It is therefore likely that JS-K would inhibit angiogenesis in different tumour models, including the ones we have studied *in vivo* so far.

Conclusions

We show here that JS-K potently inhibits several key elements of the angiogenesis process. Furthermore, it inhibits tumour angiogenesis *in vivo*. Consequently, JS-K establishes a new class of cancer chemotherapeutic agents acting by a direct cytotoxic effect as well as inhibition of tumour angiogenesis. As such, JS-K shows great promise for medicinal development for the treatment of different malignant diseases.

Declarations

Conflict of interest

Paul J. Shami is a founder, member of the Board of Directors and Chief Medical Officer of JSK therapeutics Inc.

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References

1. Magrinat G *et al*. Nitric oxide modulation of human leukemia cell differentiation and gene expression. *Blood* 1992; 80: 1880–1884.
2. Shami PJ *et al*. Nitric oxide modulation of the growth and differentiation of freshly isolated acute non-lymphocytic leukemia cells. *Leuk Res* 1995; 19: 527–533.
3. Shami PJ *et al*. Schedule and concentration-dependent induction of apoptosis in leukemia cells by nitric oxide. *Leukemia* 1998; 12: 1461–1466.
4. Moncada S *et al*. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991; 43: 109–142.
5. Shami PJ *et al*. JS-K, a glutathione S-transferase-activated nitric oxide donor with potent anti-neoplastic activity. *Mol Cancer Ther* 2003; 2: 409–417.
6. Baines P *et al*. Multidrug resistance in leukaemia. *Baillieres Clin Hematol* 1992; 5: 943–960.
7. Sargent JM *et al*. Evidence for the involvement of the glutathione pathway in drug resistance in AML. *Adv Exp Med Biol* 1999; 457: 205–209.
8. Kiziltepe T *et al*. JS-K, a GST-activated nitric oxide generator, induces DNA double strand breaks, activates DNA damage response pathways, and induces apoptosis in human multiple myeloma cells. *Blood* 2007; 110: 709–718.
9. Saavedra JE *et al*. The secondary amine/nitric oxide complex ion $R_2N[N(O)NO]^-$ as nucleophile and leaving group in S_NAr reactions. *J Org Chem* 2001; 66: 3090–3098.
10. Kaur G *et al*. Antiangiogenic properties of 17-(dimethylaminoethylamino)-17-demethoxy-geldanamycin: an orally bioavailable heat shock protein 90 modulator. *Clin Cancer Res* 2004; 10: 4813–4821.
11. Auerbach R *et al*. Angiogenesis assays: a critical overview. *Clin Chem* 2003; 49: 32–40.
12. de Vos FY *et al*. Endothelial cell effects of cytotoxics: balance between desired and unwanted effects. *Cancer Treat Rev* 2004; 30: 495–513.
13. Bruneel A *et al*. Proteomic study of human umbilical vein endothelial cells in culture. *Proteomics* 2003; 3: 714–723.
14. Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 1992; 6: 3051–3064.
15. Kimura H *et al*. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. *Blood* 2000; 95: 189–197.
16. Pipili-Synetos E *et al*. Evidence that nitric oxide is an endogenous antiangiogenic mediator. *Br J Pharmacol* 1994; 111: 894–902.

17. Jia L *et al*. Anti-angiogenetic effects of S-nitrosocaptopril crystals as a nitric oxide donor. *Eur J Pharmacol* 2000; 391: 137–144.
18. Shami PJ *et al*. Gene expression profiling for nitric oxide prodrug JS-K to kill HL-60 myeloid leukemia cells. *Genomics* 2009; 94: 32–38.
19. Cohen SD, Khairallah EA. Selective protein arylation and acetaminophen-induced hepatotoxicity. *Drug Metab Rev* 1997; 29: 59–77.
20. Shami PJ *et al*. Antitumor activity of JS-K [O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate] and related O^2-aryl diazeniumdiolates *in vitro* and *in vivo*. *J Med Chem* 2006; 49: 4356–4366.
21. Townsend DM *et al*. A glutathione S-transferase p-activated prodrug causes kinase activation concurrent with S-glutathionylation of proteins. *Mol Pharmacol* 2006; 69: 501–508.
22. Mieyal JJ *et al*. Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. *Antioxidants Redox Signaling* 2008; 10: 1941–1988.
23. Podar K *et al*. Best practice and research. The malignant clone and the bone-marrow environment. *Clin Haematol* 2007; 20: 597–612.
24. Rajkumar SV *et al*. Prognostic value of bone marrow angiogenesis in multiple myeloma. *Clin Cancer Res* 2000; 6: 3111–3116.
25. Kumar S *et al*. Prognostic value of bone marrow angiogenesis in patients with multiple myeloma undergoing high-dose therapy. *Bone Marrow Transplant* 2004; 34: 235–239.