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Original article

Effects of JS-K, a novel anti-cancer nitric oxide prodrug, on gene expression in human hepatoma Hep3B cells

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ABSTRACT

JS-K is a novel anticancer nitric oxide (NO) prodrug effective against a variety of cancer cells, including the inhibition of AM-1 hepatoma cell growth in rats. To further evaluate anticancer effects of JS-K, human hepatoma Hep3B cells were treated with JS-K and the compound control JS-43-126 at various concentrations (0–100 μM) for 24 h, and cytotoxicity was determined by the MTS assay. The compound control JS-43-126 was not cytotoxic to Hep3B cells at concentrations up to 100 μM, while the LC_{50} for JS-K was about 10 μM. To examine the molecular mechanisms of antitumor effects of JS-K, Hep3B cells were treated with 1–10 μM of JS-K for 24 h, and then subjected to gene expression analysis via real time RT-PCR and protein immunostain via confocal images. JS-K is a GST-α targeting NO prodrug, and decreased immunostaining for GST-α was associated with JS-K treatment. JS-K activated apoptosis pathways in Hep3B cells, including induction of *caspase-3*, *caspase-9*, *Bax*, *TNF-α*, and *IL-1β*, and immunostaining for caspase-3 was intensified. The expressions of thrombospondin-1 (TSP-1) and the tissue inhibitors of metalloproteinase-1 (TIMP-1) were increased by JS-K at both transcript and protein levels. JS-K treatment also increased the expression of differentiation-related genes *CD14* and *CD11b*, and depressed the expression of *c-myc* in Hep3B cells. Thus, multiple molecular events appear to be associated with anticancer effects of JS-K in human hepatoma Hep3B cells, including activation of genes related to apoptosis and induction of genes involved in antiangiogenesis and tumor cell migration.

1. Introduction

Compounds of the O^2-aryl diazeniumdiolate family have shown noteworthy anticancer activity in a variety of model systems [1–4]. JS-K, O^2-(2,4-dinitrophenyl) 1-[(4-ethoxyxarbonyl)piperazin-1-yl] diazen-1-ium-1,2-diolate, is a glutathione/glutathione *S*-transferase-activated nitric oxide (NO) donor with potent antineoplastic activity [4,5]. In the presence of GSH, JS-K's half-life is less than 30 min, but in the absence of GSH, JS-K is stable with a half-life over a week [4]. JS-K induces differentiation and apoptosis in human HL-60 myeloid leukemia cells [5], and induces apoptosis in human multiple myeloma cell lines [6]. JS-K-induced HL-60 cell apoptosis is mediated, at least in part, through cytochrome c release and caspase activation [7]. JS-K also inhibited hepatoma Hep3B cell proliferation through the induction of c-jun via MAP kinase pathways [8]. In the National Cancer Institute 51-cell panel screen, JS-K was effective against renal cancer cells, prostate cancer cells, and brain cancer cells [4]. In intact animals, JS-K was effective against myeloid leukemia and prostate cancer subcutaneous xenografts in NOD-SCID mice [5] and against OPM1 multiple myeloma cells inoculated subcutaneously in mice [6]. JS-K was also effective against JM-1 hepatoma cells implanting intrahepatically in rats [4], and even effective against orthotopic U87 rat gliomas [9]. Taken together, JS-K is a lead anticancer nitric oxide prodrug of the novel class of chemotherapeutics [2].

The molecular mechanisms by which JS-K kills cancer cells are not completely understood but several modes of action have been proposed including 1) as an glutathione S-transferase (GST)-activated nitric oxide donor to inhibit GST activity in a suicide manner, as many cancer cells overexpress GST [4,5]; 2) as an apoptosis activator to initiate tumor cell apoptosis [6,7]; 3) as a cell differentiation modulator to induce differentiation of acute myeloid leukemia cells, with increases in differentiation marker CD14 and IL-1β transcript levels [10,11]; and 4) as an inhibitor of

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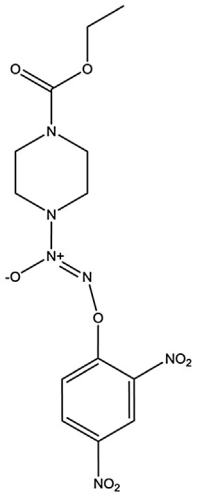
angiogenesis and tumor cell metastasis [12,13]. These mechanisms could be cancer-type specific/selective, and they are not mutually exclusive, but rather work together to exert the anticancer effects in an integrated manner.

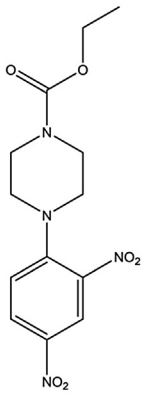
Hepatocellular carcinoma is one of the leading malignant deaths worldwide. Developing effective anticancer agents and exploring the molecular mechanisms for chemotherapeutics are fundamental for hepatocellular carcinoma prevention and treatment. The present study is therefore designed to evaluate the effects of JS-K on human hepatoma Hep3B cells. Real time RT-PCR and confocal images were used to examine JS-K-induced gene expression and cellular protein localization to better understand the molecular events associated with JS-K effects against human hepatoma Hep3B cells. Our results demonstrated multiple molecular events are likely involved in the antitumor effects of JS-K, including caspase activation, anti-angiogenesis, and the inhibition of tumor cell migration. These molecular events could provide new insights on JS-K anticancer effects in hepatoma cells.

2. Materials and methods

Materials. JS-K and JS-43-126 were synthesized as previously described [14]. All the primers for real-time RT-PCR analysis were synthesized by Sigma-Genosys (The Woodlands, TX). Rabbit polyclonal antibody against GST-α was obtained from Novocastra (Newcastle upon Tyne, UK); Monoclonal antibodies against caspase-3, thrombospondin-1, TIMP1, and c-Myc were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of reagent grade.



JS-K



JS-43-126

2.1. Cell culture and JS-K treatment

Human hepatoma3 B cells (ATCC, Manassas, VA) were cultured in Eagle MEM media supplemented with 10% fetal bovine serum (FBS) with penicillin and streptomycin. Cells were cultured at 37 °C in a 5% CO2 humidified atmosphere. JS-K stock solution (10 mM) in dimethyl sulfoxide (DMSO) was serially diluted in phosphate buffered saline (PBS) before addition to the cultures. For cytotoxicity experiments, JS-K and JS-K compound control JS-43-126 were added at the time of culture initiation at concentrations of 1, 3, 10, 30, and 100 μM, and cell viability was determined 48 h later via the MTS assay (below). For gene expression experiment, cells at 70% confluency were treated with JS-K 1, 3, and 10 μM for gene expression and selected protein localization via confocal image analysis.

2.2. Cytotoxic assay

The Promega non-radioactive cell proliferation assay was used to determine acute cytotoxicity. This assay measures the amount of formazan produced by metabolic conversion of Owen's reagent [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt, MTS)] by dehydrogenase enzymes in the mitochondria of metabolically active, and, therefore, viable cells. The quantity of formazan product, as measured by absorbance at 490 nm, is directly proportional to the number of living cells. Data are expressed as percentage of control untreated cells.

2.3. RNA isolation

Twenty four hrs after JS-K treatments, cells were harvested by centrifugation and total RNA was isolated with TRIzol agent (Invitrogen, Carlsbad, CA), followed by purification and DNase-I digestion with RNeasy columns (Qiagen, Valencia, CA). The quality of RNA was determined by the 260/280 ratios (>1.8), and by gel electrophoresis to visualize the integrity of 18S and 28S bands.

2.4. Real-time RT-PCR analysis

Total RNA was reverse transcribed with MMLV reverse transcriptase and oligodT primers. The PCR primers were designed with Primer Express software (Applied Biosystems, Foster City, CA, USA). The SYBR Green DNA PCR kit (Applied Biosystems, Foster City, CA, USA) was used for real-time RT-PCR analysis. Differences in gene expression between groups were calculated using cycle time (Ct) values, which were normalized against β-actin and expressed as relative to control.

2.5. Immuncytochemistry

Both JS-K and JS-43-126 treated Hep3B cells were washed three times with PBS and fixed with 3% paraformaldehyde/0.25glutaldehyde in PBS for 10 min, followed by permeabilization with 0.5% Triton X-100 for 30 min at room temperature. After washing with PBS, cells were blocked for 60 min with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Cells in the coverglass chambers were incubated overnight at 4 °C with each of primary antibodies (GST-α, caspase-3, TSP-1, TIMP1, and c-Myc, as well as CD11b CD14, CD36, and IL-1β) were diluted with 1%BSA in PBS (1:100). After rinsing three times in PBS, secondary antibodies [Alexa Fluor 488 conjugated goat anti-mouse IgG (H + L) (Invitrogen) for TIMP1, c-Myc and CD11b,CD14 IL-1β (1:1000) and Alexa Fluor 543 goat anti-rabbit IgG (H + L) (Invitrogen) for GST-α, TSP-1, and caspase-3, CD36 (1:500) diluted in PBS containing 1% BSA] were added to the cells and incubated at 37 °C for 1 h. Propidium iodide (1 mg/mL) in 1% BSA was used to stain cell nuclei (1:1000) at room temperature for 10 min. After further washing with PBS, cells were transferred to covered Teflon microscope imaging chamber for confocal microscopy. Negative controls were treated similarly except they were not exposed to primary antibody. Negative control experiments showed no signal at the settings used to image specific fluorescence.

2.6. Confocal microscopy

To acquire images, the chamber containing the specimen was mounted on the stage of a Zeiss Model 510 inverted confocal laser-scanning microscope (Carl Zeiss Inc., Thornwood, NY) and viewed