Genomics 94 (2009) 32–38

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Gene expression profiling for nitric oxide prodrug JS-K to kill HL-60 myeloid leukemia cells

Jie Liu [a,*], Swati Malavya [b], Xueqian Wang [c], Joseph E. Saavedra [d], Larry K. Keefer [e], Erik Tokar [a], Wei Qu [a], Michael P. Waalkes [a], Paul J. Shami [b,*]

[a] Inorganic Carcinogenesis Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute at NIEHS, Research Triangle Park, NC, USA
[b] Division of Medical Oncology, Department of Internal Medicine, University of Utah, Salt Lake City, UT, USA
[c] Laboratory of Pharmacology, NIEHS, Research Triangle park, NC, USA
[d] Basic Science Program, SAIC-Frederick, USA
[e] Chemical Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute at Frederick, Frederick, MD, USA

ARTICLE INFO

Article history:
Received 16 December 2008
Accepted 26 March 2009
Available online 5 April 2009

Keywords:
JS-K
Nitric oxide donor
HL-60 cells
Gene expression
Confocal analysis

ABSTRACT

The nitric oxide (NO) prodrug JS-K is shown to have anticancer activity. To profile the molecular events associated with the anticancer effects of JS-K, HL-60 leukemia cells were treated with JS-K and subjected to microarray and real-time RT-PCR analysis. JS-K induced concentration- and time-dependent gene expression changes in HL-60 cells corresponding to the cytolethality effects. The apoptotic genes (*caspases*, *Bax*, and *TNF*-α) were induced, and differentiation-related genes (*CD14*, *ITGAM*, and *VIM*) were increased. For acute phase protein genes, some were increased (*TP53*, *JUN*) while others were suppressed (*c-myc*, *cyclin E*). The expression of anti-angiogenesis genes *THBS1* and *CD36* and genes involved in tumor cell migration such as tissue inhibitors of metalloproteinases, were also increased by JS-K. Confocal analysis confirmed key gene changes at the protein levels. Thus, multiple molecular events are associated with JS-K effects in killing HL-60, which could be molecular targets for this novel anticancer NO prodrug.

Published by Elsevier Inc.

Introduction

Nitric oxide (NO) inhibits the growth and induces differentiation of acute myeloid leukemia cells [1,2]. Expression of human monocyte differentiation markers CD11b and CD14, and tumor necrosis factor-α (*TNF*-α) and interleukin-1β (*IL-1*β) transcripts in leukemia cells can be increased by NO [1.2]. Spontaneous NO generating diazeniumdiolate compounds induce apoptosis in acute myeloid leukemia HL-60 and U937 cells [3], with the long half-life (20 h) NO donor DETA-NO being the most active. The major obstacle for the use of NO-generating compounds *in vivo* to treat malignant disease is NO-induced vasodilation [4]. An alternative approach is the use of NO-generating prodrugs that would be activated in the targeted cells, thus avoiding the vascular effects of the drug. For instance, esterase-activated diazeniumdiolates that do not release NO spontaneously but rather are enzymatically activated to release NO are cytotoxic to leukemia cells [5]. However, such a drug design is unlikely to provide enough selectivity because esterases are ubiquitous. Many malignant cells, including leukemia cells, overexpress glutathione *S*-transferases (GST) which result in resistance to cytotoxic compounds through glutathione (GSH) conjugation and cellular efflux via multi-drug resistance (MRP) pumps, or by inhibition of mitogen activated protein kinases (MAPK) [6]. Consequently, GST constitutes an attractive enzyme system for the targeting of cytotoxic agents to cancer cells.

O^2-arylated diazeniumdiolates are designed to release NO upon conjugation with GSH in a reaction catalyzed by GST. O^2-(2,4-dinitrophenyl) 1-[(4-ethoxyxarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K), the lead compound from this family is potently cytotoxic to human myeloid leukemia HL-60 cells *in vitro* and *in vivo* [7]. JS-K also inhibits hepatoma Hep 3B cell proliferation [8], enhances arsenic- and cisplatin-induced cytolethality in arsenic-transformed rat liver cells [9], and induces apoptosis in human multiple myeloma cell lines [10]. In the National Cancer Institute 51-cell panel screen, JS-K was effective against leukemia, renal cancer cells, prostate cancer cells, and brain cancer cells [11,12]. In *in vivo* murine models, JS-K was effective in inhibiting the growth of HL-60 (leukemia) [7], PPC-1 (prostate cancer) [7], JM-1 (hepatoma) [11], and OPM1 cells (myeloma) [10]. Consequently, JS-K is a lead compound establishing a new class of cancer chemotherapeutic agents [12].

Mechanisms for the antineoplastic effects of JS-K are not completely elucidated. In HL-60 cells, JS-K induces differentiation and apoptosis. JS-K-induced apoptosis in HL-60 cells occurs through activation of both the intrinsic and extrinsic pathways [7,13]. Apoptosis

* Corresponding authors.
E-mail addresses: Liu6@niehs.nih.gov (J. Liu), paul.shami@utah.edu (P.J. Shami).

0888-7543/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.ygeno.2009.03.005

induction by JS-K is mediated, at least in part, by cytochrome *c* release and caspase activation [13]. The goal of the present study was to use microarray and real-time polymerase chain reaction (RT-PCR) analysis to identify genes that are modulated by JS-K in killing HL-60 cells. Confocal image analysis was also performed on selected proteins. Results demonstrate that multiple molecular events are likely involved in the antitumor effects of JS-K, including activation of caspases, modulation of cell growth and differentiation genes, increased expression of anti-angiogenesis genes and genes inhibiting tumor cell migration, all of which could be potential molecular targets for the anticancer effects of JS-K.

Materials and methods

Materials

JS-K was synthesized as previously described [14]. The Clontech Human Cancer Arrays (600 genes) were purchased from Clontech (Palo Alto, CA). All the primers for RT-PCR analysis were synthesized by Sigma-Genosys (The Woodlands, TX). All other chemicals were of reagent grade.

Cell culture and JS-K treatment

Human myeloid leukemia HL-60 cells (ATCC, Manassas, VA) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere. JS-K stock (5 mM) was prepared with dimethyl sulfoxide (DMSO) and diluted in phosphate buffered saline (PBS) before addition to cultures. The final concentration of DMSO added to the cultures was less than 0.1% and the media containing 0.1% DMSO was used as controls. JS-K was added to HL-60 cells at concentrations of 0, 0.25, 0.5, and 1.0 μM, and cells were harvested 2, 4, 6, and 24 h later.

RNA isolation

At the end of 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, and by gel electrophoresis to visualize the integrity of 18S and 28S bands.

Microarray analysis

Approximately 5 mg of total RNA was converted to [a-^{32}P]-dATP-labeled cDNA probe using MuLV reverse transcriptase and the Atlas human cancer cDNA synthesis primer mix, and then purified with a NucleoSpin column (Clontech, Palo Alto, CA). The human cancer membrane array (588 genes, Clontech, Palo Alto, CA) was used for analysis. The membranes were prehybridized with Expresshyb from Clontech for 2 h at 68 °C, followed by hybridization with the cDNA probe overnight at 68 °C. The membranes were then washed four times in 2× SSC/1% SDS, 30 min each, and two times in 0.1× SSC/0.5% SDS for 30 min. The membranes were then sealed with plastic wrap and exposed to a Molecular Dynamics Phosphoimage Screen. Images were analyzed densitometrically using the AtlasImage software (Clontech, version 2.01). Gene expression intensities were first corrected with the external background and then globally normalized as described previously [25].

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) and listed in Table 1. The Power SYBR Green PCR Master Mix (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.

Immuncytochemistry

HL-60 cells were collected by centrifugation and fixed with 3% paraformaldehyde/0.25glutaldehyde in PBS for 10 min, followed by permeabilization with 0.1% Triton X-100 for 10 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, and were incubated at 37 °C for 1 h with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted with 1%BSA in PBS (1:100). After rinsing 3 times in PBS, secondary antibodies [Alexa Fluor 488 conjugated goat anti-mouse IgG (H+L) (Invitrogen) for TIMP1, MYC and CD14 (1:1000) and Alexa Fluor 543 goat anti-rabbit IgG (H+L) (Invitrogen) for CASP3 (1:500) diluted in PBS containing 1% BSA] were added to the cells and incubated at 37 °C for 1 h. Propidium

Table 1
Primer sequences for real-time RT-PCR analysis.

Gene	GenBank number	Forward	Reverse
β-actin	X00351	GTCCACCTTCCAGCAGATGTG	GCATTTGCGGTGGACGAT
BAX	NM_004324	CCGCCGTGGACACAGACT	TTGAAGTTGCCGTCAGAAAACA
CASP3	NM_004346	TGGTTCATCCAGTCGCTTTG	CCCGGGTAAGAATGTGCATAA
CASP8	NM_001228	ACCAGGCAGGGCTCAAATTT	GCACTGGCTGTTTGCTTCAG
JUN	J04111	CCAAAGGCTAGTGCGATGTTTC	GGTCACAGCACATGCCACTT
MYC	V00568	GAGGCGAACACACAACGTCTT	CGCAACAAGTCCTCTTCAGAAA
ITGAM	M82856	CGCCCTCTTCCTTTGAATCTC	AACCACAAGGAAGCCACCAA
CD14	M86511	TAACCTGACACTGGACGGGAAT	CACGCCGGAGTTCATTGAG
CD36	NM_031561	GGCTAAATGAGACTGGGACCAT	CCAGGCCCAGGAGCTTTATT
CCNE1	X75888	GCCCTTAAGTGGCGTCTAAGC	CGTTGACATAGGCCACTTGGA
EGR1	X52541	TGAACGCAAGAGGCATACCA	CCGAAGAGGCCACAACACTT
IL1B	NM_000576	CTTAAAGCCCGCCTGACAGA	TCAGAATGTGGGAGCGAATG
MMP9	NM_004994	GGACGATGCCTGCAACGT	GTACTTCCCATCCTTGAACAAATACAG
MMP11	X57766	CCGCCTCTACTGGAAGTTTGA	TCGGCACAGCCAAAGAAGT
TP53	M14694	CCCAGCCAAAGAAGAAACCA	CAGCTCTCGGAACATCTCGAA
TIMP1	NM003254	TCCCTGCGGTCCCAGATA	GTGGGAACAGGGTGGACACT
TIMP2	NM003255	TCACCCTCTGTGACTTCATCGT	CCATCTGGTACCTGTGGTTCAG
TIMP3	NM000362	AACTTGGGTGAAGGCTGAGTGT	CCTCACCAAGGCCTAACAGATG
TNF	NM_000594	CCTCAACCTCTTCTGGCTCAAA	CTGAATCCCAGGTTTCGAAGTG
THBS1	X14787	CATGCCACGGCCAACAA	GGCCCAGGTAGTTGCACTTG
VIM	X56134	CGCCAACTACATCGACAAGGT	ACTTGCCTTGGCCCTTGAG