Regulation of Polyamine Analogue Cytotoxicity by c-Jun in Human MDA-MB-435 Cancer Cells

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Abstract
Several polyamine analogues have efficacy against a variety of epithelial tumor models including breast cancer. Recently, a novel class of polyamine analogues designated as oligoamines has been developed. Here, we demonstrate that several representative oligoamine compounds inhibit in vitro growth of human breast cancer MDA-MB-435 cells. The activator protein-1 (AP-1) transcripitional factor family members, c-Jun and c-Fos, are up-regulated by oligoamines in MDA-MB-435 cells, suggesting a possible AP-1-dependent induction of apoptosis. However, the use of a novel c-Jun NH2-terminal kinase (JNK) inhibitor, SP600125, suggests that inhibition of c-Jun activity sensitized tumor cells to oligoamine-induced cell death. To directly test this hypothesis, cells were stably transfected with the dominant-negative mutant c-Jun (TAM67), which lacks the NH2-terminal transactivation domain. Cells overexpressing TAM67 exhibit normal growth kinetics but demonstrate a significantly increased sensitivity to oligoamine cytotoxicity and attenuated colony formation after oligoamine treatment. Furthermore, oligoamine treatment leads to more profound caspase-3 activation and poly(ADP-ribose) polymerase cleavage in TAM67 transfectants, suggesting that c-Jun acts as an antiapoptosis factor in MDA-MB-435 cells in response to oligoamine treatment. These findings indicate that oligoamine-inducible AP-1 plays a prosurvival role in oligoamine-treated MDA-MB-435 cells and that JNK/AP-1 might be a potential target for enhancing the therapeutic efficacy of polyamine analogues in human breast cancer.

Introduction
The polyamines (putrescine, spermidine, and spermine) are naturally occurring polycationic alkylamines that are required for cell growth. Because of the critical role of polyamines in the regulation of cell growth, the polyamine metabolic pathway is an attractive target for antineoplastic strategies (1–3). The primary regulatory enzymes of polyamine biosynthesis include ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase, spermidine synthase, and spermine synthase. The enzymes spermidine/spermine N1-acetyltransferase (SSAT) and spermine oxidase also play a significant rate-limiting role in polyamine catabolism (4–6). The best characterized polyamine biosynthetic pathway inhibitor is α-difluoromethylornithine, an irreversible inhibitor of ODC, which inhibits tumor cell growth in preclinical models, has been tested in phase I/II clinical trials, and is currently being examined as a chemopreventive agent (7–9).

Based on the feedback mechanisms of natural polyamines, whereby they regulate their own synthesis, another useful strategy to develop polyamine metabolism inhibitors is the use of polyamine analogues that can mimic some of the regulatory roles of natural polyamines but are unable to replace the actual function of natural polyamines for cell growth (10–12). Several polyamine analogues have shown antineoplastic activity in a variety of tumor types. For example, N1,N11-diethylnorspermine inhibits polyamine biosynthesis, decreases polyamine transport, and greatly increases SSAT activity in several tumor models (13, 14). Recently, a series of novel oligoamine analogues have been synthesized (15). Some of these new analogues have shown significant inhibitory activity against in vitro and in vivo tumor cell growth (16, 17).

In our previous studies, several early polyamine analogues were reported to induce programmed cell death in human breast cancer cells, but the cell death mechanisms have been uncertain (18). For example, N1,N11-diethylnorspermine-related superinduction of SSAT with subsequent depletion of natural polyamine pools was noted in specific human breast cancer cell lines (19), a finding that has been confirmed by conditional overexpression of SSAT in breast cancer MCF-7 cells (20). Although superinduction of SSAT seems to be directly associated with growth inhibition by some polyamine analogues, other polyamine analogues do not highly induce SSAT but can still inhibit tumor cell growth (21, 22). These results suggest that polyamine analogue-induced cell death occurs through multiple agent-specific and cell type-specific mechanisms. Several important growth-associated or cell cycle-associated genes or pathways have been reported to be affected by specific polyamine analogues (17, 23, 24). However, little is known thus far about the exact mechanisms by which other polyamine analogues inhibit growth and induce apoptosis in tumor cells.
An important mediator that is widely involved in regulating cellular proliferation, differentiation, and apoptosis is the activator protein-1 (AP-1) transcription factor family (25). AP-1 proteins are homodimers or heterodimers composed of leucine zipper proteins that belong to the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra-1, and Fra-2), Jun dimerization partners (JDP1 and JDP2), and activating transcription factors (ATF2, LRF1/ATF3, and B-ATF) subgroups (26, 27). The major upstream regulator of AP-1 is the c-Jun NH2-terminal kinase (JNK), one subgroup of the mitogen-activated protein kinase (MAPK) family. After exposure of cells to several cytokines or extracellular stresses, JNK is activated and subsequently phosphorylates its major target, c-Jun, and related molecules. This in turn leads to the enhanced transcriptional regulation of c-Jun and its dimerization partners with subsequent effects on the promoter regions of downstream target genes (27).

In our previous work, we noted that a new oligoamine analogue, SL-11144, inhibited growth and induced apoptosis in several human breast cancer cell lines (17). In some cases, this was associated with induction of the Jun protein. In this study, we have examined the mechanisms of cytotoxicity of several oligoamines using the human MDA-MB-435 cancer cell line as a model system. Our data demonstrate that these oligoamines significantly inhibit cell growth and activate AP-1 members, c-Jun and c-Fos. By using a selective JNK inhibitor and the stable transfection of dominant-negative AP-1 members, c-Jun and c-Fos, we provide evidence that c-Jun plays a protective role in oligoamine-induced cell death. However, further evidence is needed to support this hypothesis because JNK activation may concurrently regulate a variety of other downstream genes or pathways with diverse functions (27).

**Results**

**Growth Inhibition by Oligoamines**

The MDA-MB-435 cells were chosen as a model system as they exhibit highly metastatic and aggressive behavior in vivo compared with other human breast cancer cell lines (28–30). The in vitro sensitivities of MDA-MB-435 cells to SL-11144, SL-11159, and SL-11172 (Fig. 1) were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) growth inhibition assay. Tumor cells were treated with increasing concentrations of oligoamines for 24 and 48 h, respectively. All three agents showed significant growth inhibitory effects against MDA-MB-435 cells in a time- and dose-dependent manner (Fig. 2).

**Oligoamines Up-Regulate c-Jun and c-Fos**

AP-1 is an important signaling complex in the regulation of cellular proliferation, differentiation, apoptosis, and metastasis (31). Therefore, we first examined the impact of oligoamines on the expression of AP-1 family members, c-Jun and c-Fos, in MDA-MB-435 cells. All three oligoamines significantly induced the phosphorylation of c-Jun and enhanced the protein level of both c-Jun and c-Fos in a dose-dependent manner within 24 h of treatment (Fig. 3). The phosphorylation of c-Jun can occur within 6 h of oligoamine exposure (data not shown). These results suggested that up-regulation of the AP-1 family might play an active role in the mediation of oligoamine-induced growth inhibition in MDA-MB-435 cells.

**SP600125 Blocks c-Jun Activation and Promotes the Cytotoxicity of Oligoamine**

SP600125 is a novel selective JNK1, JNK2, and JNK3 inhibitor that can inhibit the phosphorylation of c-Jun (32, 33). To examine whether the inhibition of c-Jun phosphorylation could affect oligoamine-induced cell death, MDA-MB-435 cells were treated with SP600125 or oligoamines alone or simultaneously. By Western blot, we determined that cotreatment with oligoamines and SP600125 significantly blocked the phosphorylation and decreased the expression of c-Jun compared with that seen with oligoamine treatment alone (Fig. 4A). MTT analysis of the effects of the c-Jun phosphorylation inhibitor on oligoamine-induced cell growth inhibition indicated that simultaneous treatment with SP600125 and oligoamine resulted in increased cytotoxicity compared with that seen with either agent alone (Fig. 4B). It is important to note that SP600125 treatment does not affect accumulation of SL-11144 in MDA-MB-435 cells (data not shown).

**Overexpression of TAM67 Sensitizes Tumor Cells to Oligoamine Cytotoxicity**

The finding that SP600125 blocks JNK/c-Jun pathway activation and increases the cytotoxicity of oligoamines suggests that the transcription factor c-Jun may exert a protective role in oligoamine-induced cell death. However, further evidence is needed to support this hypothesis because JNK activation may concurrently regulate a variety of other downstream genes or pathways with diverse functions (27). To further dissect the effect of c-Jun activity on oligoamine-induced cell death, a vector expressing the c-Jun dominant-negative mutant (TAM67), we provide evidence that c-Jun plays a protective role in oligoamine-induced cell death in MDA-MB-435 cells.

**FIGURE 1.** Structures of natural polyamines and oligoamines SL-11144, SL-11159, and SL-11172.
has been deleted, leaving the COOH-terminal DNA binding and dimerization domain intact. Such a mutant c-Jun protein is unable to activate its target genes but still possesses the ability to bind to AP-1 site in promoter regions of target genes and competitively quench the transactivation activity of endogenous wild-type Jun and its dimerization partners (36, 37). Three single clones (TAM67-12, TAM67-19, and TAM67-21) that highly express the ~29-kDa dominant-negative mutant c-Jun protein were identified (Fig. 5A), and these three clones were used for a series of functional experiments. We first examined the effect of TAM67 on the general growth rate of MDA-MB-435 transfectants. Cell growth over 96 h was similar for the parental wild-type, vector, and TAM67 mutant cell lines (Fig. 5B), indicating that the stable expression of either empty vector or TAM67 does not adversely affect the growth of MDA-MB-435 cells.

TAM67 transfected clones were then compared to determine whether the expression of the dominant-negative mutant c-Jun altered the response of tumor cells to oligoamines. The vector control clone and the three independent dominant-negative mutant c-Jun transfectants were treated with increasing concentrations of SL-11144, SL-11159, or SL-11172 (1–20 μM) for 24 h and analyzed by MTT. When compared with the vector control, all three TAM67 clones were significantly more sensitive to treatment with SL-11144 and SL-11159 at all doses studied (P < 0.001). The TAM67 transfectants were also more sensitive to lower concentrations of SL-11172 treatment (1–2.5 μM, P < 0.001; Fig. 6). However, at higher concentrations (5.0 μM or higher) of SL-11172 treatment, there was no obvious difference in cellular sensitivity to SL1-11172 between controls and transfectants.

To confirm the increased sensitivity of TAM67 transfectants to oligoamines, vector and TAM67 transfected cells were subjected to colony formation analysis. Cells were treated with 0.1 μM of each oligoamine for 12 h. The colony-forming efficiency of all three TAM67 transfected cell lines was dramatically diminished after treatment with all three oligoamines compared with the vector transfectant (Fig. 7). These results suggest that specific inhibition of transactivation of endogenous wild-type c-Jun significantly increases the sensitivity of MDA-MB-435 cells to oligoamine cytotoxicity.

**TAM67 Overexpression Enhances Oligoamine-Induced Apoptosis**

In our recent studies, oligoamines have been demonstrated to activate apoptosis-related pathways and induce apoptotic cell death in human breast cancer cells (17). To investigate if overexpression of dominant-negative mutant c-Jun could affect apoptotic response to oligoamines in MDA-MB-435 cells, expression of two key apoptosis effectors, caspase-3 and poly(ADP-ribose) polymerase (PARP), was examined by Western blotting. The vector transfectant and a representative dominant-negative mutant c-Jun transfectant (TAM67-21) were

**FIGURE 2.** Oligoamines inhibit growth of human breast cancer MDA-MB-435 cells in a time- and dose-dependent manner. MDA-MB-435 cells were treated with increasing concentrations of SL-11144, SL-11159, or SL-11172 for 24 or 48 h. MTT assays were performed as described in "Materials and Methods." Points, means of three independent experiments performed in quadruplicate; bars, SD.
treated with SL-11144 for 24 h. SL-11144 treatment led to a greater decrease in the caspase-3 level and more significant cleavage of the PARP protein in TAM67-21 cells compared with the vector control clone (Fig. 8). These results indicate that inhibition of c-Jun activation may enhance apoptotic cell death induced by oligoamines in MDA-MB-435 cells.

Effects of Oligoamine on Intracellular Polyamines Metabolism in TAM67 and Vector Control Transfectants

To address whether the overexpression of TAM67 alters the effects of oligoamines on the polyamine metabolic pathway, we evaluated the intracellular polyamine pools and the activities of ODC, a key polyamine biosynthetic enzyme, and SSAT, a key polyamine catabolic enzyme, in empty vector and TAM67 transfected cells. The TAM67-21 transfectant was again selected as representative for these studies. Treatment of both vector controls and TAM67-21 transfectants with 10 μM SL-11144 for 12 h was associated with a similar decrease in natural polyamine levels, increased SSAT activity, and diminished ODC activity in both cell lines (Table 1).

Discussion

The antiproliferative mechanisms of various polyamine analogues are being elucidated. Possible mechanisms include the fact that polyamine analogues can bind to nucleic acids, leading to the distortion of nucleic acid structure and impairment of normal function (38). Another potential...
mechanism is the rapid depletion of the natural polyamine pool associated with the superinduction of SSAT by polyamine analogues in some cell lines (19, 20, 22, 39). The oligoamines have longer chains than natural mammalian cellular polyamine molecules, which allow the analogues to condense and collapse DNA at much lower concentrations (15, 16).

In this work, we demonstrate that the decamines, SL-11144 and SL-11159, and the dodecamine, SL-11172, significantly inhibit the in vitro growth of human breast cancer MDA-MB-435 cells and increase c-Jun and c-Fos expression and c-Jun phosphorylation (Fig. 3). Cotreatment with a JNK selective inhibitor, SP600125, sensitizes tumor cells to the oligoamines. This result implicates a possible protective role of the JNK/Jun pathway in oligoamine-treated MDA-MB-435 cells. AP-1 family members, particularly c-Jun and c-Fos, play critical roles in cellular proliferation, differentiation, transformation, and apoptosis, likely through their effects on several important AP-1 target genes, including cyclin D1, p16, p19Arf, p53, p21Cip1/Waf1, and FasL (40–43). Due to the complexity of regulatory signaling impinging on AP-1/c-Jun, the net effect of AP-1 on the balance between cell survival and death varies greatly, depending on cell context, nature of the extracellular stimuli, and the signaling pathways that are simultaneously activated. For example, studies in neuronal cells demonstrated that inhibition of c-Jun activity by dominant-negative mutant
forms protects cells from apoptosis induced by withdrawal of nerve growth factor, and expression of a phosphorylation-deficient c-Jun mutant blocked phosphorylation of c-Jun by JNK and inhibited apoptosis (44–48). However, other lines of evidence suggest that JNK/Jun activation may protect other cells from stress-induced death. For example, in vitro studies demonstrated that JNK1 plays a protective role in Fas-induced apoptosis (49), and cells overexpressing mutant c-Jun are more vulnerable to apoptosis triggered by UV irradiation (50). Other in vivo studies suggested that c-Jun-deficient embryos exhibited massive apoptosis in liver cells (51). Taken together, these results suggest that AP-1 may have divergent functions in regulating stress-mediated cell death in different cell contexts.

To study the exact role of c-Jun in oligoamine-induced cell growth inhibition and death, clones of the MDA-MB-435 cell line that stably express a mutant transactivation domain deletion c-Jun (TAM67) were established. All three transfectants displayed normal growth, suggesting that dominant-negative mutant c-Jun protein is well tolerated in untreated cells. However, MTT proliferation assays show that mutant c-Jun transfectants were significantly more sensitive to the dodecamine SL-11144 treatment (<5 μM). Values represent the means of duplicated determinations. ND, not detected.

Our studies also investigated the effects of oligoamine on the polyamine metabolic pathway in vector and TAM67 transfected cells (Table 1). ODC is a key regulatory enzyme in polyamine biosynthesis. The significant decrease of ODC activity in SL-11144-treated MDA-MB-435 cells suggests that ODC might be one of the targets for oligoamines. However, it should be noted that the effects of SL-11144 on ODC activity appear to be indirect. When cellular extracts of MDA-MB-435 cells containing ODC enzyme are exposed to concentrations of SL-11144 up to 1000 μM, no change in ODC activity is observed. This in contrast to results observed with the specific ODC inhibitor, 2-difluoromethylornithine, which produces nearly complete inhibition of ODC activity in cell extracts at concentrations > 100 μM (data not shown). Likely, the cellular effects of SL-11144 resulting in decreased ODC activity are not a result of direct enzyme inhibition but are a result of regulatory mechanisms including the increased production of ODC antizyme as demonstrated by Mitchell et al. (52).

In conclusion, we present evidence that treatment with a JNK inhibitor or specific blockade of AP-1 by a dominant-negative mutant c-Jun sensitizes MDA-MB-435 cells to cell death induced by three novel polyamine analogues. The results strongly suggest that the AP-1 proteins play a protective role in this cell line in response to stress and cell death signals. Our work provides new insights into the molecular mechanisms of polyamine analogues in cancer cells. It also suggests that AP-1 may be a useful target for improving the therapeutic efficacy of polyamine analogues in human breast cancer.

### Materials and Methods

#### Compounds, Cell Line, and Culture Conditions

The polyamine analogues SL-11144, SL-1159, and SL-11172 (Fig. 1) were synthesized as reported previously (15, 16). A concentrated stock solution (10 mM in double-distilled water) was diluted with the medium to the desired concentrations for...
specific experiments. The selective JNK1, JNK2, and JNK3 inhibitor SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA) was prepared as a stock solution of 20 mM in 100% DMSO. The MDA-MB-435 human carcinoma cell line was originally isolated from the pleural effusion of a patient with breast carcinoma (28, 29). It should be noted that one recent report, using microarray analysis, indicated that MDA-MB-435 cells expressed certain markers associated with melanoma (53). However, this report has not been confirmed and is not consistent with the clinical course of the patient from whom the cells were derived. Cells were maintained in improved MEM supplemented with 5% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin and incubated at 37°C in a 5% CO₂ atmosphere.

Construction of Expression Plasmid and Stable Transfection of TAM67

The cDNA of mutant c-Jun (TAM67) that lacks amino acids 3–122 of the transactivation domain was kindly provided by Dr. Steve N. Georas (Johns Hopkins) and subcloned into the pcDNA3.1 mammalian expression vector (Invitrogen, Carlsbad, CA). Transfections were performed by Lipofect-AMINE Plus reagent (Invitrogen) as recommended by the manufacturer. Stable transfectants were selected by incubating the cells in the medium containing 500 μg/ml geneticin (G418). Cells from individual colonies were examined for TAM67 expression by Western blot analysis with an antibody that recognizes the COOH terminus of c-Jun (sc-44; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Growth Inhibition and Colony Formation Assays

Growth inhibition was assessed by MTT assays as described previously (54). Briefly, 2000–5000 cells were plated in 96-well dishes and treated with the various concentrations of drug regimes for the indicated times. All of the experiments were plated in quadruplicate and were carried out at least thrice. The results of assays were presented as means ± SD. Colony formation assay was performed as published previously (55). Colonies that contained >50 cells were scored. Relative clonogenic efficiency was assessed as numbers of colonies in treated group/numbers of colonies in control group. All experiments were plated in triplicate and were carried out at least thrice. The results were expressed as means ± SD.

Western Blotting

Cells were treated with indicated drug concentrations and times, harvested by trypsinization, and washed with PBS. Cellular protein was isolated using a protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS. Protein concentrations were determined using Micro Protein Assay Kit (Pierce Chemical Co., Rockford, IL). Equal amounts of protein (50 μg/lane) were fractionated on 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies against c-Jun, c-Fos, caspase-3 (1:2000; Santa Cruz Biotechnology), and PARP (1:2000; Calbiochem, San Diego, CA). After washing with PBS, the membranes were incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:3000; DAKO Corp., Carpinteria, CA) followed by enhanced chemiluminescent staining using the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). Actin was used to normalize for protein loading. All the experiments were performed at least twice with similar results.

Analysis of Intracellular Polyamine Pools, SSAT Activity, and ODC Activity

The intracellular polyamine content of treated and untreated cells was determined by precolumn dansylation and reversed phase high-performance liquid chromatography (56). SSAT and ODC activities were measured using cellular extracts as described previously (57, 58). Protein concentrations were determined according to the method of Bradford (59).

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References


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