[CANCER RESEARCH 63, 4527–4532, August 1, 2003]

# Transcription Factor c-Jun Activation Represses *mdr-1* Gene Expression[1]

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## ABSTRACT

**Expression of *mdr-1* is complex and highly regulated. Several lines of evidence indirectly suggest that transcription factor c-Jun may negatively regulate human *mdr-1* gene expression. We recently found that salvicine, a novel topoisomerase II inhibitor, is cytotoxic for multidrug resistance (MDR) tumor cells and down-regulates *mdr-1* expression in MDR K562/A02 cells. Salvicine also stimulates a significant increase in the level of *c-jun* mRNA in HL60 cells. This study investigated the relationship between c-Jun activation and down-regulation of *mdr-1* expression by salvicine in K562/A02 cells. Reverse-transcription PCR and Western blotting analyses revealed that salvicine suppressed *mdr-1* expression in MDR cells and promoted *c-jun* expression in both MDR and parental K562 cells. Moreover, levels of *c-jun* expression were enhanced by salvicine before reduction of *mdr-1* expression in K562/A02 cells. Furthermore, *c-jun* antisense oligodeoxynucleotides prevented salvicine-stimulated enhancement of c-Jun protein and reduction of *mdr-1* gene expression, but did not affect the increase in *c-jun* mRNA levels. Salvicine promoted phosphorylation of c-Jun-N-terminal kinase and c-Jun protein in MDR K562/A02 and parental K562 cells. Electrophoretic mobility shift assay analysis showed that salvicine enhanced DNA binding activity of transcription factor activator protein 1. Additionally, *c-jun* antisense oligodeoxynucleotides also inhibited salvicine-induced apoptosis and cytotoxicity in MDR and parental K562 cells. A possible pathway emerges from these results: salvicine stimulates c-Jun-N-terminal kinase phosphorylation and activation, resulting in c-Jun phosphorylation and activation. Activated c-Jun promotes expression of *c-jun* itself, represses *mdr-1* transcription, and triggers pro-apoptotic signals, resulting in low *mdr-1* expression and cell death. The present results demonstrate that transcription factor c-Jun plays a principal role in down-regulation of *mdr-1* expression and induction of apoptosis in salvicine-treated human MDR K562/A02 cells, providing new insights into the complicated mechanisms regulating *mdr-1* expression. The findings also suggest that c-Jun might be a potential drug target for circumventing tumor MDR.**

## INTRODUCTION

MDR[3] is a major problem in treatment of human cancers with conventional chemotherapeutic drugs. One of the most important mechanisms by which tumor cells resist cytotoxic effects of anticancer agents is overexpression of the *mdr-1* gene and its product P-gp (1). P-gp protein plays roles in inhibition of both drug accumulation and caspase activation in MDR tumor (2). This implies that reduction of *mdr-1*/P-gp expression may circumvent tumor MDR.

Salvicine is a diterpenoid quinone compound synthesized by structural modification of a natural product isolated from the Chinese medicinal plant *Salvia Prionitis Lance* (*Labiatae*; See Ref. 3; Fig. 1). The compound has significant *in vitro* and *in vivo* activity against malignant tumor cells and xenografts, particularly in some human solid tumor models (4). Salvicine is a novel Topo II inhibitor that greatly promotes Topo II-DNA binding and inhibits pre- and post-strand Topo II-mediated DNA religation without interfering with forward cleavage steps (5, 6). One of the major features of salvicine is its activity against MDR tumor cells. It effectively kills MDR cell sublines with $IC_{50}$ values of 1.55 $\mu$M for K562/A02 cells, 4.50 $\mu$M for KB/VCR cells, and 1.40 $\mu$M for MCF-7/ADM cells, close to those for their corresponding parental cell lines: 0.87 $\mu$M for K562 cells, 2.26 $\mu$M for KB cell, and 2.61 $\mu$M for MCF-7 cells. The cytotoxic activity of salvicine is much more potent than that of several classic anticancer drugs, with the average resistance factor for salvicine being 1.42, compared with 344.35, 233.19 and 71.22 for vincristine, doxorubicin, and etoposide, respectively. Salvicine induces similar levels of apoptosis in MDR K562/A02 and parental K562 cells, accompanied by an increased ratio of *bax* to *bcl-2* mRNA (7). A clinical trial involving salvicine is currently underway in China.

Salvicine down-regulates *mdr-1* and P-gp expression in MDR K562/A02 cells (7). Salvicine also stimulated significant increases in *c-jun* mRNA levels in HL60 cells (8). This is intriguing because the transcription factor c-Jun may play a role in regulation of *mdr-1* expression (9). Moreover, there are several lines of evidence indirectly suggesting that c-Jun activation is negatively correlated with human *mdr-1* gene expression (10–13). The present study explored the relationship between activation of the transcription factor c-Jun and down-regulation of *mdr-1* expression by salvicine in MDR K562/A02 cells.

## MATERIALS AND METHODS

**Materials.** Salvicine was kindly provided by Prof. Jin-Sheng Zhang (Phytochemistry Department of the Shanghai Institute of *Materia Medica*, Chinese Academy of Sciences). It was dissolved at a concentration of 0.1 M in 100% DMSO as a stock solution, stored at −20°C, and thawed and diluted with complete medium before each experiment. The final DMSO concentration did not exceed 0.1%. Primary antibodies sc-1694, sc-571, sc-1616, sc-822, and sc-6254 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and JSB-1 was purchased from Alexis Biochemicals (San Diego, CA).

**Cell Culture.** The human leukemia K562 parental cell line was obtained from the American Type Culture Collection (Rockville, MD). The doxorubicin-selected MDR K562/A02 (14, 15) subline was obtained from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, People's Republic of China). The MDR cells displayed 310.0-, 146.8-, and 76.1-fold resistance to doxorubicin, etoposide, and vincristine compared with their corresponding parental cells, respectively (7). Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin (Invitrogen, Grand Island, NY) at 37°C in a 5% $CO_2$ humidified atmosphere. MDR K562/A02 cells were routinely maintained in medium containing 200 $\mu$g/L doxorubicin (14) and incubated in drug-free medium for at least one week before use. Cell viability was determined by trypan blue exclusion.

**Oligodeoxynucleotides and Antisense Preparation.** *c-jun* AODs (5′-CGT TTC CAT CTT TGC AGT-3′) and SODs (5′-ACT GCA AAG ATG GAA ACG-3′; phosphorothioate-modified; see Ref. 16) corresponding to the first 18 bases following the AUG sequence of *c-jun* mRNA were synthesized

Received 1/15/03; accepted 5/22/03.

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[1] Supported by grants (No. 30070877, No. KSCX2-SW-202, and No. 2002AA2Z3461) from the National Natural Science Foundation of China (NSFC), the Chinese Academy of Sciences, and the Ministry of Science and Technology of China, respectively; and in part by grants from the Virtual Research Institute of Aging of Nippon Boehringer Ingelheim.

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[3] The abbreviations used are: MDR, multidrug resistance; P-gp, P-glycoprotein; Topo II, topoisomerase II; AOD, antisense oligodeoxynucleotide; SOD, sense oligodeoxynucleotide; RT-PCR, reverse-transcript PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun-N-terminal kinase; EMSA, electrophoretic mobility shift assay; AP1, activator protein 1; MTT, microculture tetrazolium.



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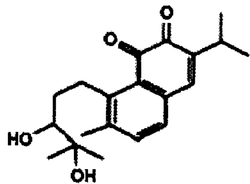


Fig. 1. Chemical structure of salvicine.

by the Shanghai Research Center of Biotechnology, Chinese Academy of Sciences.

**Semiquantitative RT-PCR.** After treatment of cells ($5 \times 10^5$/ml) with salvicine, total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA yield and purity were assessed by spectrophotometric analysis. Total RNA (1 $\mu$g) from each sample was subjected to reverse transcription with random hexamer, deoxynucleoside triphosphates, and Moloney murine leukemia virus reverse transcriptase in a total reaction volume of 20 $\mu$l. PCR was performed on cDNA with the use of *Taq* DNA polymerase, deoxynucleoside triphosphates, and the corresponding primers. The following PCR primers synthesized by the Shanghai Research Center of Biotechnology, Chinese Academy of Sciences, were used: 5′-CCA TGG AGA AGG CTG GGG-3′ (sense) and 5′-CAA AGT TGT CAT GGA TGA CC-3′ (antisense) for the *GAPDH* gene (17); 5′-CCC ATC ATT GCA ATA GCA GG-3′ (sense) and 5′-GTT CAA ACT TCT GCT CCT GA-3′ (antisense) for the *mdr-1* gene (11); and 5′-AAC GAC CTT CTA TGA CGA TGC CCT C-3′ (sense) and 5′-GCG AAC CCC TCC TGC TCA TCT GTC-3′ (antisense) for the *c-jun* gene (8). An aliquot of each reaction mixture was analyzed by electrophoresis on a 1.8% agarose gel, and amplified DNA was visualized by ethidium bromide staining. For quantitation of cDNA, densitometric analysis of a digital image of the agarose gel was performed using a GDS8000 Gel Documentation System (UVP Inc., Upland, CA).

**Western Blotting Analysis.** K562/A02 cells ($5 \times 10^5$/ml) were exposed to different concentrations of salvicine at 37°C for the indicated times. Harvested cell pellets were suspended in suspension buffer [0.1 M NaCl, 0.01 M Tris-HCl (pH 7.6), 1.0 mM EDTA (pH 8.0), 1.0 $\mu$g/ml aprotinin, 100 $\mu$g/ml phenylmethylsulfonylfluoride] and then lysed in lysis buffer [100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromphenol blue, 20% glycerol]. Equal amounts of cell lysate were separated on 7.5% Tris-glycine-SDS polyacrylamide gels and proteins electroblotted onto nitrocellulose membranes. Proteins were identified with the use of polyclonal antibody sc-1694 (1:1000) for c-Jun, sc-571 (1:1000) for JNK, sc-1616 (1:1000) for $\beta$-actin or monoclonal antibody sc-822 (1:1000) for p-c-Jun, sc-6254 (1:1000) for p-JNK, and JSB-1 (1:500) for P-gp. Detection was performed using horseradish peroxidase-conjugated secondary antibody and SuperSignal West Pico Chemiluminescent Substrate (Pierce Inc, Rockford, IL) according to the manufacturer's instructions.

**DNA Agarose Gel Electrophoresis (7).** The fragmentation of total cellular DNA was determined by electrophoresis of purified DNA on 1.8% agarose gels stained with ethidium bromide (0.5 $\mu$g/ml) to facilitate visualization under UV light. Standard molecular-weight markers (pUC Mix Marker, 8; MBI Fermentas, Inc., Burlington, Ontario, Canada) were electrophoresed on each gel.

**EMSA.** Nuclear extracts prepared as described previously (18) were assayed for DNA binding proteins using the Gelshift kit (Geneka Biotechnology, Inc., Montréal, Québec, Canada) according to the manufacturer's instructions. Briefly, the AP1 wild-type double-strand oligonucleotide probe was 5′-end [$\gamma$-$^{32}$P] dATP-labeled using T4 polynucleotide kinase (Amersham Biosciences, Uppsala, Sweden) and purified from unincorporated nucleotides by centrifugation through a MicroSpin G-25 column (Geneka Biotechnology, Inc.). The sequences of AP1 double-strand oligonucleotides used in this experiment were: 5′-CGC TTG ATG AGT CAG CCG GAA-3′ for wild-type and 5′-CGC TTG ATG ACC CAG CCG GAA-3′ for mutant-type, respectively. The gel shift reaction was conducted as experimental procedure C1 in the protocol. DNA-protein complexes were electrophoresed on 5% polyacrylamide (38:2) gels in 0.25× Tris-boric acid electrophoresis buffer. Gels were dried on 3 $\mu$M Whatman paper and visualized by autoradiography.

**MTT Assay.** After pretreatment with 50 $\mu$g/ml *c-jun* AODs or SODs for 1 h, both MDR and parental K562 cells ($5 \times 10^5$/ml) were exposed to 20 $\mu$M salvicine for 24 h in 96-well plates. At the end of incubation, cytotoxicity was measured by adding 20 $\mu$l 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co.; see Refs. 19, 20] to each well and the plates incubated at 37°C for 4 h. "Triplex" solution (10% SDS, 5% isobutanol, 12 mM HCl) was then added and the plates were incubated at 37°C for 12–20 h. Media and DMSO control wells, in which salvicine was absent, were included in all experiments. Absorbance values at $A_{570}$ nm were determined using a VERSAmax tunable microplate reader (Molecular Devices). The growth inhibition rate was calculated by the equation: growth inhibition rate $= [1-(A_{570\ \mathrm{treated}}/A_{570\ \mathrm{control}})] \times 100\%$.

## RESULTS

**Salvicine Increases c-*jun* mRNA Levels before Reduction of *mdr-1* mRNA Levels in MDR K562/A02 Cells.** We investigated the effects of salvicine on levels of *c-jun* and *mdr-1* mRNA in MDR

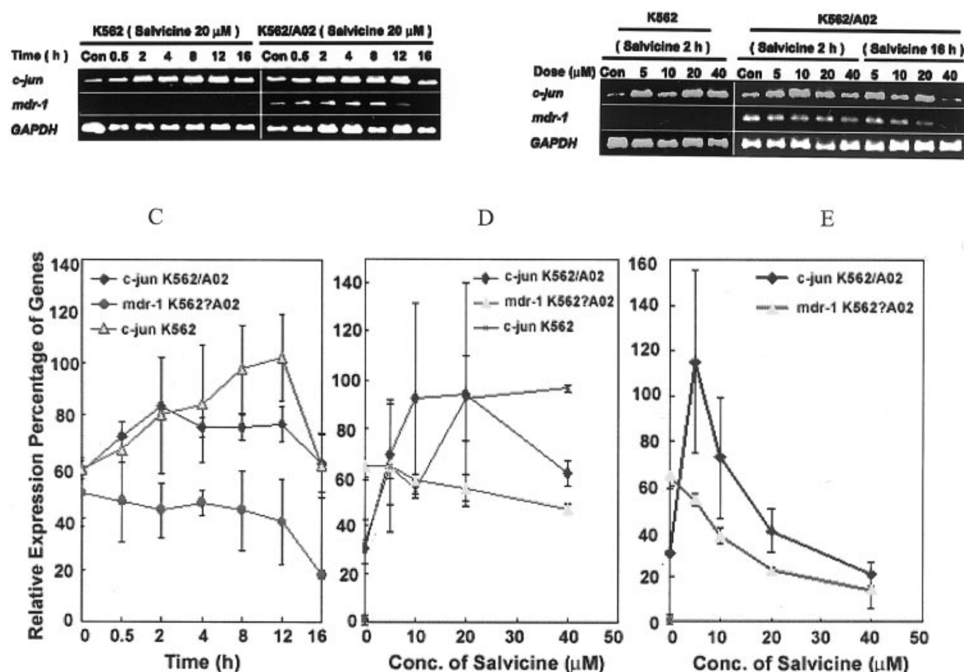


Fig. 2. Effect of salvicine on expression of *c-jun* and *mdr-1* mRNA in MDR K562/A02 cells. RT-PCR was performed to detect *c-jun* and *mdr-1* mRNA in MDR K562/A02 cells ($5 \times 10^5$/ml) treated with 20 $\mu$M salvicine for 0.5–16 h (*A*) or 5–40 $\mu$M salvicine for 2 or 16 h (*B*). *C–E*, semiquantitation of *c-jun* and *mdr-1* cDNA in both MDR and parental K562 cells in *A* and *B* by densitometric analysis of a digital image, respectively. Relative expression percentage of genes (*Y* axis) represented the intensities of *c-jun* or *mdr-1* expression relative to those of *GAPDH* expression (taken as 100%). Data are expressed as mean ± SD, $n = 3$. *Con*, control group.



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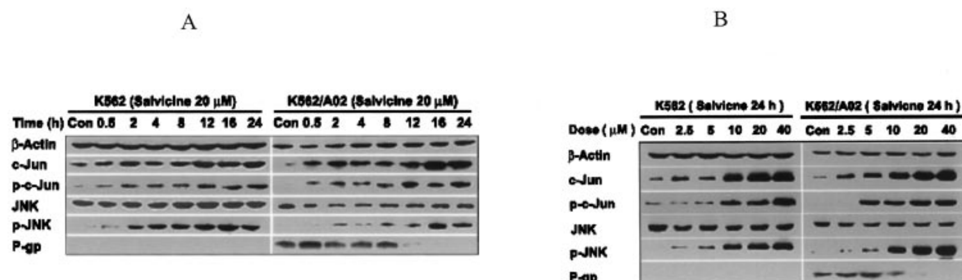


Fig. 3. Effect of salvicine on c-Jun, p-c-Jun, JNK, p-JNK, and P-gp protein levels in MDR K562/A02 cells. Cells ($5 \times 10^5$/ml) were treated as indicated and lysates electrophoresed on 7.5% SDS-polyacrylamide gels. Proteins were electrotransferred to nitrocellulose membranes, which were probed with antibodies appropriate for detecting the indicated proteins (see "Materials and Methods"). *A,* time effect; *B*, concentration effect. Experiments were repeated 3 times. *Con*, control group.

K562/A02 cells using RT-PCR. After treatment of MDR K562/A02 and parental K562 cells with 20 $\mu$M salvicine, *c-jun* mRNA levels first rose then decreased in both cell lines (Fig. 2, *A* and *C*). In MDR cells, *c-jun* mRNA levels rose up to 1.24 times the basal value at 0.5 h, reached their maximum of about 1.44-times the basal at 2 h, and remained high before beginning to decrease at 12 h, and returned to near control levels at 16 h. A similar phenomenon was observed in parental K562 cells, in which *c-jun* mRNA levels reached a maximum of 1.73 times the basal at 12 h. On the other hand, levels of *mdr-1* mRNA changed little before 8 h, declined after 12 h, and went down to 36.7% of the basal in K562/A02 cells at 16 h. In parental K562 cells, no *mdr-1* mRNA was detectable.

The same trend was observed when cells were exposed to gradient concentrations of salvicine for fixed durations (Fig. 2, *B*, *D*, and *E*). In the range of 5 to 40 $\mu$M, salvicine elevated *c-jun* mRNA levels in K562/A02 and K562 cells, but did not significantly affect *mdr-1* mRNA at 2 h. At 16 h, 5 $\mu$M salvicine enhanced *c-jun* mRNA levels in MDR cells, and at higher concentrations, *c-jun* mRNA levels reduced gradually, whereas *mdr-1* mRNA levels decreased progressively with increasing concentrations. These results indicate that salvicine-stimulated decreases in *mdr-1* mRNA levels occur subsequent to increases in *c-jun* mRNA levels in MDR cells. It is worth noting that the nature of the biphasic change in *c-jun* mRNA levels elicited by salvicine is identical to the rapid mRNA turnover of immediate early genes (21). Unlike the regular changes of *c-jun* and *mdr-1* mRNA levels, levels of *GAPDH* mRNA, used as the internal control, did not alter significantly in both cell lines (Fig. 2, *A* and *B*).

**Salvicine Raises c-Jun, and Lowers P-gp, Protein Expression in MDR K562/A02 Cells.** We sought to confirm the above-mentioned mRNA findings by analyzing protein expression using Western blotting. After treatment of both MDR and parental cells with 20 $\mu$M salvicine, c-Jun protein levels began to rise as early as 0.5 h (Fig. 3*A*). In contrast to the changes at the mRNA level, c-Jun protein reached a plateau after 12 h and remained high to 24 h. A decline in P-gp protein levels was observed, consistent with that of *mdr-1* mRNA levels in salvicine-treated K562/A02 cells. A similar phenomenon appeared in experiments exploring concentration-response relationship (Fig. 3*B*). Levels of c-Jun increased concentration-dependently in both cell lines treated with salvicine for 24 h, but levels of P-gp decreased progressively at concentrations higher than 10 $\mu$M in MDR cells. As was the case for mRNA levels, enhancement of c-Jun protein levels occurred earlier than the decline in P-gp protein levels in MDR cells.

The above-mentioned mRNA and protein results indicate the existence of a possible cause-and-effect relationship between activation of c-Jun and down-regulation of *mdr-1* expression in salvicine-treated K562/A02 cells.

**Salvicine Promotes Phosphorylation of JNK Kinase and c-Jun Protein in MDR K562/A02 and Parental K562 Cells.** Activation of c-Jun protein, performed by JNK kinase, which also must be phosphorylated to be active, requires phosphorylation on serines 63 and 73, (22, 23). We investigated whether salvicine-induced c-Jun protein was in its active, phosphorylated form (p-c-Jun) and whether this process involved phosphorylated JNK (p-JNK). Treatment of either

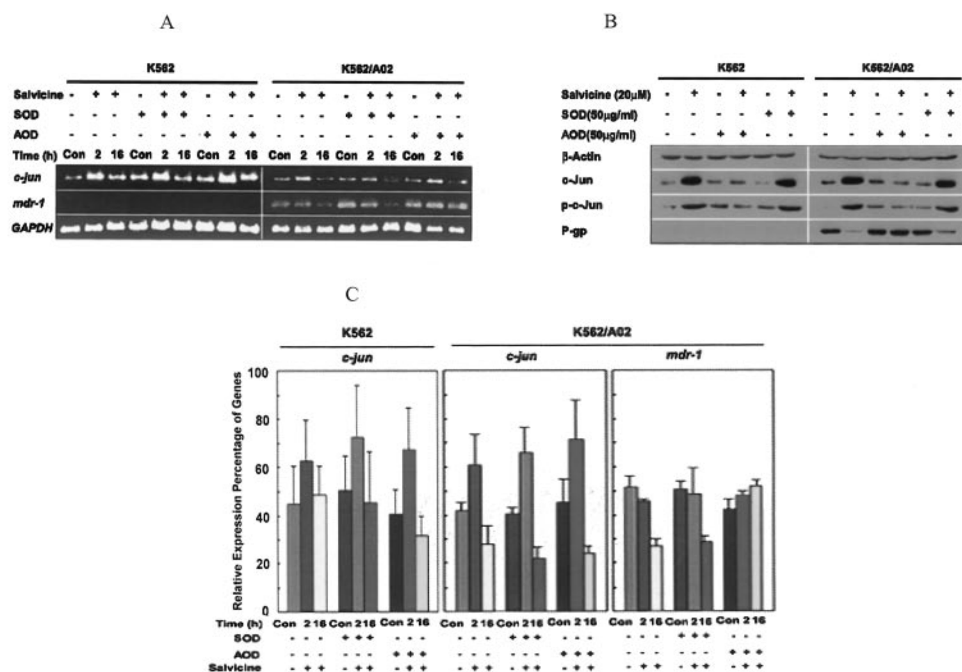


Fig. 4. Effect of *c-jun* AODs on expression of *c-jun* and *mdr-1* genes in MDR K562/A02 cells. After pretreatment with 50 $\mu$g/ml *c-jun* AODs or *c-jun* SODs for 1 h, both MDR and parental K562 cells ($5 \times 10^5$/ml) were exposed to 20 $\mu$M salvicine for 2 h or 16 h (*A*) or 24 h (*B*). *A*, the results of RT-PCR analysis. *B*, the results of Western blot analysis. *C*, semiquantitation of *c-jun* cDNA and *mdr-1* mRNA levels shown in *A* by densitometric analysis of a digital image. Relative expression percentage of genes (*Y* axis) represented the intensities of *c-jun* or *mdr-1* expression relative to those of *GAPDH* expression (taken as 100%). Data are expressed as mean ± SD. Experiments were repeated 3 times. *Con*, control group.

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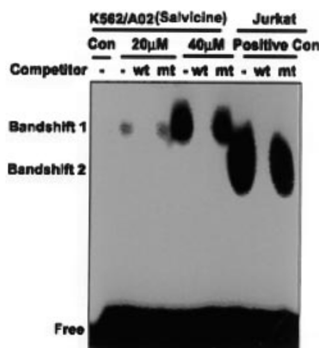


Fig. 5. Effect of salvicine on DNA binding activity of AP1 in MDR K562/A02 cells. MDR K562/A02 cells ($5 \times 10^5$/ml) were treated with 20 or 40 $\mu$M salvicine for 24 h, and nuclear extracts were prepared and assayed for DNA binding proteins using the Gelshift kit. Concurrently, a competition assay was conducted using up to 100-fold molar excess of wild-type (*wt*) or mutant-type (*mt*) distrand oligonucleotide. Experiments were repeated 3 times. *Con*, control group.

MDR or parental cells with salvicine led to increased levels of p-JNK and p-c-Jun, the altered amounts of which were reflected incubation time (Fig. 3*A*) and salvicine concentration (Fig. 3*B*). Basically, these changes paralleled those in the levels of c-Jun. In contrast, the total amount of JNK did not change (Fig. 3). The results indicate that salvicine stimulated phosphorylation of c-Jun at serines 63 and 73 by activating JNK kinase in a time- and concentration-dependent manner.

**c-*jun* AODs Rescue Reduction of *mdr-1* Gene Expression after Activation of c-Jun by Salvicine.** AODs, with their high specificity, versatility, and cost-effect ratio, are invaluable reagents for specific regulation of gene expression. They are able to sequence-specifically promote mRNA degradation and/or block translation to protein (20, 21). To confirm whether c-Jun activation is a prerequisite for reduction of *mdr-1* mRNA levels in salvicine-treated K562/A02 cells, we synthesized a sequence of *c-jun* AODs, which has previously been shown to block heat shock- and ceramide-induced apoptosis (14, 24). Pretreatment of cells with 50 $\mu$g/ml *c-jun* AODs for 1 h did not change the response pattern of *c-jun* mRNA to salvicine, but altered the levels of c-Jun protein, *mdr-1* mRNA, and P-gp protein (Fig. 4). In both *c-jun* AODs-treated and -untreated groups, *c-jun* mRNA levels still rose at 2 h and declined approximately to control values at 16 h in salvicine-treated K562/A02 and K562 cells. Contrarily, *c-jun* AODs completely reversed the reduction of *mdr-1* mRNA by salvicine in MDR cells (Fig. 4*A*). At the protein level, *c-jun* AODs prevented up-regulation of c-Jun and p-c-Jun and the down-regulation of P-gp by salvicine (Fig. 4*B*). In contrast, the internal controls, *GAPDH* mRNA and $\beta$-actin protein expression, did not change significantly (Fig. 4, *A* and *B*). These data further demonstrate that enhancement of *c-jun* expression might lead to down-regulation of *mdr-1* expression in salvicine-treated MDR cells. The results also reveal that *c-jun* AODs mainly affect translation, not stability, of *c-jun* mRNA. As expected, *c-jun* SODs did not alter the effects of salvicine (Fig. 4).

**Salvicine Enhances the DNA Binding Activity of AP1.** There is an AP1 DNA binding element in the promoter region of the human *mdr-1* gene, which can be bound by the AP1 transcription factor containing c-Jun (7). Because induction of *c-jun* expression does not always mean activation of AP1 (23), we studied the effect of salvicine on AP1 DNA binding activity using EMSA. The nuclear extract derived from phorbol 12-myristate 13-acetate (TPA)-treated human T lymphocyte leukemia Jurkat cells was used as a positive control. We observed that nuclear extracts from K562/A02 cells treated with 20 and 40 $\mu$M salvicine for 24 h contained a binding activity to the AP1 DNA binding consensus sequence, whereas those from untreated K562/A02 cells did not (Fig. 5). The results were consistent with increased levels of c-Jun and p-c-Jun and decreased levels of *mdr*-1 mRNA and P-gp protein. Interestingly, it appears that the components of AP1 in salvicine-treated K562/A02 cells might be different from those in phorbol 12-myristate 13-acetate-treated Jurkat cells because bandshift 1 lagged significantly behind bandshift 2 (Fig. 5).

To determine the specificity of the binding activity, EMSA binding reactions were also performed in the presence of up to 100-fold molar excess of unlabeled probe. Results showed that the labeled probe was competed away from the shifted complexes, whereas a 100-fold molar excess of a mutant oligonucleotide did not compete with the labeled probe (Fig. 5). Taken together, the data demonstrate that salvicine-stimulated AP1 complexes recognize and bind the AP1 DNA binding consensus sequence. These results provide further evidence supporting the hypothesis that activation of transcription factor c-Jun down-regulates *mdr-1* gene expression in salvicine-treated MDR K562/A02 cells.

**c-*jun* AODs Inhibit Salvicine-induced Apoptosis and Cytotoxicity in MDR K562/A02 and Parental K562 Cells.** We examined the effects of *c-jun* AODs on salvicine-induced apoptosis in MDR K562/A02 and parental K562 cells to further confirm the role of transcription factor c-Jun in regulation of MDR. After 1 h preincubation with 50 $\mu$g/ml *c-jun* SODs or AODs, cells were treated with 20 $\mu$M salvicine for 24 h. DNA agarose gel electrophoresis showed that salvicine-induced apoptosis was clearly inhibited by *c-jun* AODs, but not by SODs (Fig. 6). Consistent with this, pretreatment with *c-jun* AODs also dramatically decreased the salvicine cytotoxicity against both cell lines (Fig. 6). Pretreatment with *c-jun* AODs caused the growth inhibitory rate elicited by salvicine to decrease from 64.64 to 9.53% in MDR K562/A02 cells and from 67.48% to 23.82% in parental cells. It is worth noting that the degree of growth inhibition of resistant cells was significantly greater than that of parental cells. These results demonstrate that alteration of *c-jun* expression is a critical feature of salvicine-stimulated apoptosis and cytotoxicity.

## DISCUSSION

Most strategies developed to reverse the MDR phenotype involve the use of resistance modulators. These have in common the ability to

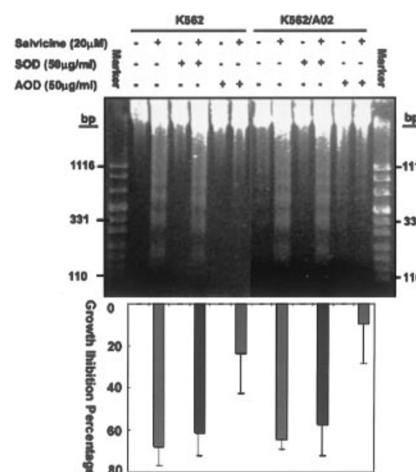


Fig. 6. Effect of c-*jun* AODs on salvicine-induced apoptosis and cytotoxicity in MDR K562/A02 cells. After pretreatment with 50 $\mu$g/ml *c-jun* AODs or *c-jun* SODs for 1 h, MDR and parental K562 cells ($5 \times 10^5$/ml) were exposed to 20 $\mu$M salvicine for 24 h. For apoptosis measurement, DNA was extracted and electrophoresed on a 1.8% agarose gel with ethidium bromide staining to facilitate visualization under UV light. For cytotoxicity measurement, the MTT assay was conducted as described in "Materials and Methods." The data were expressed as mean ± SD, $n = 3$.



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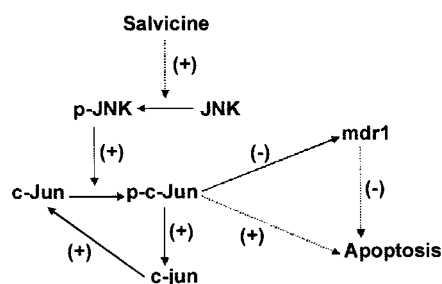


Fig. 7. Pathways involved in the activation of c-Jun by salvicine.

reverse the phenotype through inhibition of MDR transporter function (1). A more efficient strategy to circumvent MDR would be to down-regulate expression of genes coding for the transporters. This requires a profound understanding of the molecular mechanisms and signal-transduction pathways involved in regulation of MDR-related genes. The regulation of *mdr-1* gene expression is highly complex. Such complexity is embodied in multiple transcription-regulatory elements contained in the 5′ and 3′ flanking sequences of the *mdr-1* gene, and numerous protein factors involved in transcription-regulatory processes in cell-type- and stimulus-dependent manners. The mechanisms regulating *mdr-1* expression are not yet fully understood.

The data in the present study revealed that salvicine stimulated *c-jun* gene expression and inhibited *mdr-1* gene expression in MDR K562/A02 cells. Moreover, elevation of c-Jun mRNA and protein levels occurred before reduction of *mdr-1* mRNA and P-gp protein levels. Salvicine also enhanced levels of the active forms of JNK and c-Jun, and raised the DNA-binding activity of AP1. Using *c-jun* AODs, we confirmed that c-Jun activation is a prerequisite for reduction of *mdr-1* mRNA and P-gp protein levels. The specific *c-jun* AODs disrupted enhancement of c-Jun and p-c-Jun by salvicine and simultaneously prevented the reduction of *mdr-1* mRNA and P-gp protein levels. Most importantly, *c-jun* AODs inhibited apoptosis and cytotoxicity induced by salvicine in both MDR and parental K562 cells. Integrating these results, we suggest a possible pathway describing down-regulation of *mdr-1* expression by salvicine (Fig. 7). Salvicine stimulates JNK phosphorylation, and activated JNK phosphorylates serines 63 and 73 of c-Jun, resulting in increased transcription activation activity. Phosphorylated c-Jun promotes expression of *c-jun* itself, thus increasing c-Jun levels (22). On the other hand, the transcription-factor complex containing c-Jun binds to the consensus AP1 target element in the *mdr-1* gene promoter and represses transcription, resulting in reduction of *mdr-1* mRNA and P-gp expression in MDR K562/A02 cells. Although this pathway indicates how c-Jun activation represses *mdr-1* expression, some molecular links remain to be clarified, such as how salvicine stimulates JNK protein phosphorylation and the connection between c-Jun activation and apoptosis.

Several previous studies suggest that c-Jun can inhibit expression of *mdr-1* in human MDR cells. Firstly, MDR FM3A/M cells overexpressing P-gp have significantly lower basal and drug-stimulated JNK activity than parental FM3A/M cells, and are resistant to anticancer drugs. After JNK gene transfection, MDR FM3A/M cells recover the basal and drug-stimulated activities of JNK and the susceptibility to anticancer drugs (10). Secondly, reactive oxygen species down-regulate P-gp expression and activate JNK in multicellular prostate tumor spheroids (11). Thirdly, tumor necrosis factor $\alpha$ can suppress *mdr-1* expression in MDR cells (25, 26) and can promote *c-jun* expression (27, 28). Finally, in mouse hepatoma cell lines, a canonical AP1 binding sequence in the promoter of *mdr3/mdr-1a* negatively regulates gene expression (13). In this study, our data directly demonstrate for the first time that c-Jun activation down-regulates *mdr-1* gene expression in a human MDR cell line.

It has been shown that JNK kinase and transcription factor c-Jun are required for apoptosis induced by various stimuli (29, 30). Using *c-jun* AODs, we come to the same conclusion in salvicine-treated MDR K562/A02 and parental K562 cells (Fig. 6). On the one hand, MDR tumor cells are generally resistant to apoptosis induction (31, 32). Functional P-gp inhibits activation of caspase-3 and -8 by some apoptotic stimuli, resulting in apoptosis resistance in MDR tumor cells (33, 34). We have shown that salvicine-induced MDR and parental K562 cell apoptosis is caspase-3-dependent (7). Our previous work (7) and the present study show that salvicine can induce apoptosis and cytotoxicity in both MDR and parental K562 cells. The activation of c-Jun and antagonism by *c-jun* AODs indicate that *c-jun* is principally responsible for the effects of salvicine and suggest that c-Jun activated by salvicine probably triggers a pro-apoptotic signal that leads to cell death in both MDR and parental cell lines. On the other hand, P-gp is expressed in MDR cells but not in parental cells (7, 14, 15), which challenges the notion that its down-regulation via c-Jun activation contributes to the effects of salvicine in MDR cells. However, inhibition of salvicine cytotoxicity by *c-jun* AODs is significantly greater in MDR cells than in parental cells (Fig. 6). An explanation may be that *c-jun* AODs, in addition to decreasing pro-apoptotic signals, restore P-gp inhibition of death in MDR K562/A02 cells. Therefore, we could reasonably infer that transcription factor c-Jun mediates the negative regulation of *mdr-1* gene expression in salvicine-treated MDR K562/A02 cells. At the same time, our results also show the complex versatility of c-Jun.

In summary, we demonstrate that transcription factor c-Jun is a principal determinant in down-regulation of *mdr-1* gene expression and induction of apoptosis by salvicine in human MDR K562/A02 cells, providing new insights into the complicated regulatory mechanisms of *mdr-1* gene expression. The findings suggest c-Jun might be a potential drug target for circumventing tumor MDR. In addition, the present results provide a biochemical basis for possible clinical application of salvicine alone, or in combination with conventional antineoplastic agents in treating MDR tumors.

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