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ORIGINAL ARTICLE

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# DA-125, a novel anthracycline derivative showing high-affinity DNA binding and topoisomerase II inhibitory activities, exerts cytotoxicity via c-Jun N-terminal kinase pathway

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**Abstract** *Purpose*: DA-125 [(8*S*,10*S*)-8-(3-Aminopropanoyloxyacetyl)-10-[(2,6-dideoxy-2-fluoro-α-L-talopyranosyl) oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacene-dione hydrochloride] is a novel anthracycline derivative with anticancer activity. In the present study, we compared the cytotoxicity of DA-125 with that of doxorubicin in H4IIE rat hepatoma cells and investigated the mechanistic basis. Because activation of MAP kinases, in particular c-Jun N-terminal kinase (JNK), is implicated in apoptotic cell death, the signaling pathways responsible for DA-125-induced apoptosis were studied. *Methods*: Cytotoxicity and apoptosis were measured in H4IIE cells and cells were stably transfected with a dominant-negative mutant of JNK1 ($JNK1^-$) by MTT and TUNEL assays. Inhibition of topoisomerase II activity was determined in vitro. Drug accumulation and DNA binding affinity were determined by fluorescence spectroscopy. *Results*: The cytotoxicity of DA-125 was greater than that of doxorubicin ($IC_{50}$ 11.5 vs 70 μ*M*). DA-125 induced apoptosis with 30-fold greater potency than doxorubicin. Inhibition of topoisomerase II by DA-125 was fourfold greater. The presence of excess β-alanine, a DA-125 moiety, failed to alter cytotoxicity and accumulation of DA-125, indicating that the improved cytotoxicity of DA-125 did not result from the β-alanine moiety. Greater cellular accumulation of DA-125 correlated with its high-affinity DNA binding. Although neither PD98059 nor SB203580 altered the degree of cytotoxicity induced by DA-125, $JNK1^-$ cells exhibited about a twofold greater viability than control cells. DA-125-induced apoptosis was also decreased in $JNK1^-$-transfected cells. *Conclusions*: DA-125 potently inhibited topoisomerase II activity and induced apoptosis by a high rate of prooxidant production. DA-125 exhibited high-affinity DNA binding with improved cellular drug accumulation. Apoptosis induced by DA-125 involved the pathway of JNK1, but not ERK1/2 or p38 kinase.

**Keywords** DA-125 · Chemotherapy · Topoisomerase · JNK · Apoptosis · DNA binding · Doxorubicin

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## Introduction

DA-125 [(8*S*,10*S*)-8-(3-Aminopropanoyloxyacetyl)-10-[(2,6-dideoxy-2-fluoro-α-L-talopyranosyl) oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride] is a novel anthracycline derivative with anticancer activity [8, 11]. To improve the pharmacological efficacy of anthracycline, a β-alanine moiety and a pyranose ring with fluoride were introduced into the nucleus (Fig. 1). Clinical studies have shown that DA-125 at doses in the range 60–100 mg is tolerable in patients with advanced non-small-cell lung cancer [11]. Other studies of cellular uptake in human cancer cell lines have indicated that DA-125 may achieve earlier and higher levels of intracellular accumulation from the bloodstream into its target tissues than doxorubicin (DX) [15].

The present study was designed to investigate the mechanism involved in the activity of DA-125 and to compare its anticancer pharmacological effect with that of DX. The anticancer effects of anthracyclines result from topoisomerase II poisoning and apoptosis [6]. Anthracycline-induced apoptosis may be mediated by activation of caspases, loss of mitochondrial membrane potential and release of apoptogenic factors [5]. We found that DA-125 potently inhibited topoisomerase II activity and showed high-affinity DNA binding with

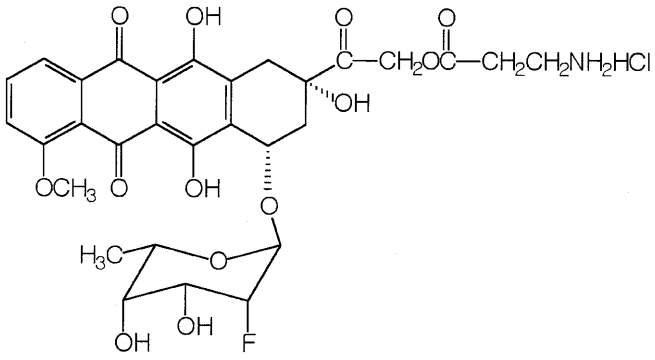

**Fig. 1** Chemical structure of DA-125

enhanced drug accumulation in cells. Anthracycline chemotherapeutic agents generate reactive oxygen species (ROS) including hydroxyl, superoxide free radicals and hydrogen peroxide [13, 17]. We compared the effects of DA-125 and DX on prooxidant production.

Three distinct mammalian mitogen-activated protein (MAP) kinase modules including extracellular signal-regulated kinase (ERK), p38 MAP kinase and c-Jun $NH_2$-terminal kinase (JNK) have been characterized [1]. Apoptosis is a form of programmed cell death that accompanies activation of cellular MAP kinases [2, 18]. It has been shown that DX induces apoptosis of cancer cells in vitro and in vivo [7, 16]. We assessed the effects of DA-125 on cell viability and the extent of apoptotic cell death and showed that DA-125 was an effective apoptosis-inducing cytotoxic agent. We also determined which MAP kinase was responsible for the apoptotic cell death induced by DA-125. Stress-activated protein kinases/JNK (SAPK/JNK) are activated in response to a variety of cellular stresses and are involved in apoptotic signaling [25]. Apoptosis induced by DA-125 appeared to be mediated by the pathway of JNK1, as evidenced by experiments using a cell line stably expressing a dominant negative mutant of JNK1 ($JNK1^-$).

## Materials and methods

### Materials

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was obtained from Life Technologies (Gaithersburg, Md.). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) and other reagents in the molecular studies were supplied by Sigma Chemical Company (St. Louis, Mo.). The $JNK1^-$ mutant vector was a kind gift from Dr. C.H. Lee (Hanyang University Medical School, Seoul, Korea).

### Cell culture

The H4IIE rat hepatoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, Md.) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in humidified atmosphere containing 5% $CO_2$. H4IIE cells were plated at a density of $5 \times 10^6$ per 10-$cm^2$ dish and preincubated for 24 h at 37°C. For all experiments, cells were grown to 80–90% confluency, and were subjected to no more than 20 passages.

### MTT cell viability assay

H4IIE cells were plated at a density of $5 \times 10^4$ cells/well in a 96-well plate to assess cytotoxicity. Cells were exposed to DA-125 at concentrations in the range 0.3–30 μ*M* or DX at concentrations in the range 3–100 μ*M* at 37°C under an atmosphere containing 5% $CO_2$. After 24 h of incubation with either DA-125 or DX, viable adherent cells were stained with MTT (2 mg/ml) for 4 h. The medium was then removed and the formazan crystals produced were dissolved by the addition of 200 μl dimethylsulfoxide. Absorbance was determined at 540 nm using a Titertek Multiskan Automatic ELISA microplate reader (Model MCC/340; Huntsville, Ala.). Cell viability was defined relative to untreated control cells: viability (% control) = (absorbance of treated sample)/(absorbance of control)\times 1000.

### TdT-mediated dUTP nick end-labeling (TUNEL) assay

The TUNEL assay was performed with an in situ cell death detection kit (Roche Diagnostics, Germany). After 24 h incubation with either DA-125 or DX, H4IIE cells were washed with phosphate-buffered saline (PBS) and fixed in paraformaldehyde solution (4% in PBS, pH 7.4) for 1 h at room temperature. The cells on slides were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature, and incubated in a blocking solution of 0.3% $H_2O_2$ in methanol for 1 h at room temperature. The cells were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Cells were washed with PBS, and incubated for 60 min at 37°C after the addition of 50 μl TdT enzyme solution. The reaction mixture was incubated for 30 min at 37°C following the addition of 50 μl antifluorescent antibody (Fab fragment from sheep conjugated with horseradish peroxidase), and further incubated in the presence of 400 μl 3,3′-diaminobenzidine solution for 10 min. Slides were rinsed with PBS, mounted under coverslips, and analyzed under the microscope.

### Cellular accumulation of DA-125

The transport of DA-125 or DX was fluorometrically quantified in H4IIE cells. Cells ($1 \times 10^7$) were incubated with 30 μ*M* DA-125 or DX for 1–20 min, washed three times, scraped and sonicated. Disrupted cells were then placed in the dark at room temperature overnight. To the sample was added 200 μl 40% trichloroacetic acid twice at an interval of 5 min. After centrifugation of the samples at 3000 *g* for 20 min, the fluorescent absorbance of DA-125 or DX in the supernatant was determined at the excitation and emission wavelengths of 485 nm and 530 nm, respectively [14].

### DNA binding assay

The binding of DA-125 or DX to DNA was compared according to the method of DuVernay et al. [3]. The equilibrium binding of the chemotherapeutic agent to DNA was then assessed by Scatchard plot analysis. Briefly, an equilibrium reaction was carried out in a reaction mixture containing 50 m*M* sodium phosphate buffer (pH 6.2), 50 m*M* sodium chloride, 10 m*M* EDTA, and 4–512 μ*M* calf thymus DNA in the presence or absence of 3 μ*M* of each drug at 25°C for 90 min. The concentration of bound drug was fluorometrically determined as described above. The amount of DNA in the reaction mixture was converted to the concentration of nucleotide. The ratio of bound to free drug versus the concentration of bound drug was plotted to obtain the binding affinity and the total number of binding sites.

### Inhibition of topoisomerase II activity

The effects of DA-125 or DX on the activity of topoisomerase II were assessed using a topoisomerase II assay kit (Topogen,

Columbus, Ohio). Briefly, the incubation mixture contained 0.2 μg kinetoplast DNA (kDNA) and 2 U topoisomerase II (human type II) with or without DA-125 (0.1–30 μ*M*) or DX (0.1–100 μ*M*) in buffer containing 50 m*M* Tris-Cl (pH 8.0), 120 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 m*M* ATP, 0.5 m*M* dithiothreitol and 30 μg/ml bovine serum albumin in a total volume of 20 μl. The reaction was continued for 30 min at 37°C and was terminated by the addition of 4 μl gel loading buffer containing 5% sarcosyl, 0.0025% bromophenol blue and 25% glycerol. The relative inhibition of decatenated DNA formation was determined by 1% agarose gel electrophoresis.

### Assay of intracellular peroxides

Production of intracellular peroxides was monitored spectrofluorometically using DCFH-DA as a fluorescent dye [12]. Oxidation of DCFH by peroxides yields dichlorofluorescein (DCF) a fluorescent derivative. H4IIE cells were suspended 20 min after incubation in medium with either DA-125 or DX, and DCFH-DA dissolved in ethanol was then added to a final concentration of 10 μ*M*. The dye-loaded cells were incubated at 37°C for 5–35 min following measurements of the initial fluorescence. Production of fluorescent DCF was monitored at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence plate reader (Tecan, Tecan US, Research Triangle Park, N.C.). Data are expressed as the relative change from the initial fluorescence.

### Stable transfection of a JNK1$^-$ mutant

Cells were transfected using Transfectam according to the manufacturer's instructions (Promega, Madison, Wis.). H4IIE cells were replated 24 h before transfection at a density of $2 \times 10^6$ cells in a 10-$cm^2$ plastic dish. For JNK1 transfection, 20 μl Transfectam was mixed with 10 μg of a JNK1$^-$ mutant plasmid (KmJNK1) in 2.5 ml minimal essential medium (MEM). Cells were transfected by the addition of MEM containing each plasmid and Transfectam, and then incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ for 6 h. After the addition of 6.25 ml MEM with 10% fetal calf serum, cells were incubated for 48 h. To establish a stable JNK1$^-$-transfected H4IIE cell line, viable cells were subcultured at least five times in medium containing 50 μg geneticin (Gibco-BRL Life Technologies, Gaithersburg, Md.). Cells stably transfected with JNK1$^-$ were subjected to MTT or TUNEL assay.

### Expression of JNK1/2 and its activity in JNK1$^-$ stable cells

JNK expression in JNK1$^-$ stable cells was determined as described previously (Son et al., submitted for publication). Cells were lysed in buffer containing 20 m*M* Tris-Cl (pH 7.5), 1% Triton X-100, 137 m*M* sodium chloride, 10% glycerol, 2 m*M* EDTA, 1 m*M* sodium orthovanadate, 25 m*M* β-glycerophosphate, 2 m*M* sodium pyrophosphate, 1 m*M* phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin. Lysates were centrifuged at 10,000 *g* at 4°C for 10 min. Anti-JNK antibody was added to the supernatant containing 500 μg of lysate, and the reaction mixture was incubated with gentle agitation at 4°C for 2 h. The immune complex was allowed to bind protein A-agarose for 2 h and precipitated by centrifugation. The immune complex-protein A-agarose was washed twice with lysis buffer and once with kinase buffer containing 25 m*M* Tris-Cl (pH 7.4), 25 m*M* β-glycerophosphate, 25 m*M* magnesium chloride, 1 m*M* dithiothreitol and 0.1 m*M* sodium orthovanadate. The immune complex was precipitated by centrifugation at 10,000 *g* for 2 min, and resuspended in 25 μl kinase buffer. The reaction was initiated by the addition of 2 μg GST-c-Jun (1–79) and 5 μCi [$\gamma$-$^{32}$P]ATP to the reaction mixture, continued at 30°C for 30 min, and terminated by the addition of 25 μl 2 × SDS-PAGE sample dilution buffer. Proteins were separated on 12% gel, which were autoradiographed after fixing and drying. Unphosphorylated JNK1/2 in each sample was determined using anti-JNK antibody as a positive control.

### Data analysis

One-way analysis of variance (ANOVA) was used to assess the significance of differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $P < 0.05$ or $P < 0.01$.

## Results

### Cell viability and apoptosis

The MTT assay was carried out to determine the extent of cell death caused by the presence of DA-125. The presence of DA-125 (0.3–30 μ*M*) induced 80–90% cell death in a concentration-dependent manner (Fig. 2A). The $EC_{50}$ value for DA-125 was 11.5 μ*M*, whereas that for DX was 70 μ*M*. The potency of DA-125 was about sixfold greater than that of DX.

Whether DA-125 induced apoptosis was investigated to establish the mechanistic basis of its cytotoxicity. DA-125 induced apoptosis with 50% of apoptotic cell death being observed at a concentration of about 3 μ*M* (Fig. 2B). Treatment of cells with 0.3–3 μ*M* DA-125 for 24 h caused 5–50% of cells to be apoptotic (Fig. 2B). DX resulted in a similar degree of apoptosis at concentrations in the range 10–30 μ*M*. The potency of DA-125 in inducing apoptosis was 30-fold greater than that of DX. The greater potency of DA-125 was consistent with the results of the MTT assay.

### Cellular drug accumulation

Cells were incubated with DA-125 or DX at 30 μ*M* for 1–20 min to determine the relative cellular drug accumulation. The concentrations of DA-125 or DX in cells were determined by fluorescent spectroscopy using cell lysates. DA-125 had accumulated in cells by 20 min to a greater extent than DX, and DA-125 had accumulated by 1 min (Fig. 3A). The intracellular concentration of DA-125 was 12.7 μmol/$10^7$ cells at 20 min, whereas the concentration of DX was 5.4 μmol/$10^7$ cells. The accumulation of DA-125 in cells was 2.4-fold greater than that of DX.

A DNA binding experiment was performed to determine whether greater accumulation of DA-125 in cells was associated with its high-affinity binding to DNA. DA-125 exhibited more than twofold greater DNA binding affinity than DX. Scatchard plot analysis showed that the DNA binding affinities of DA-125 and DX were 0.216 and 0.530 nmol/nucleotide, respectively (Fig. 3B). Total numbers of DNA binding sites were identical between the two drugs. High-affinity binding of DA-125 to DNA would be associated with cellular accumulation.

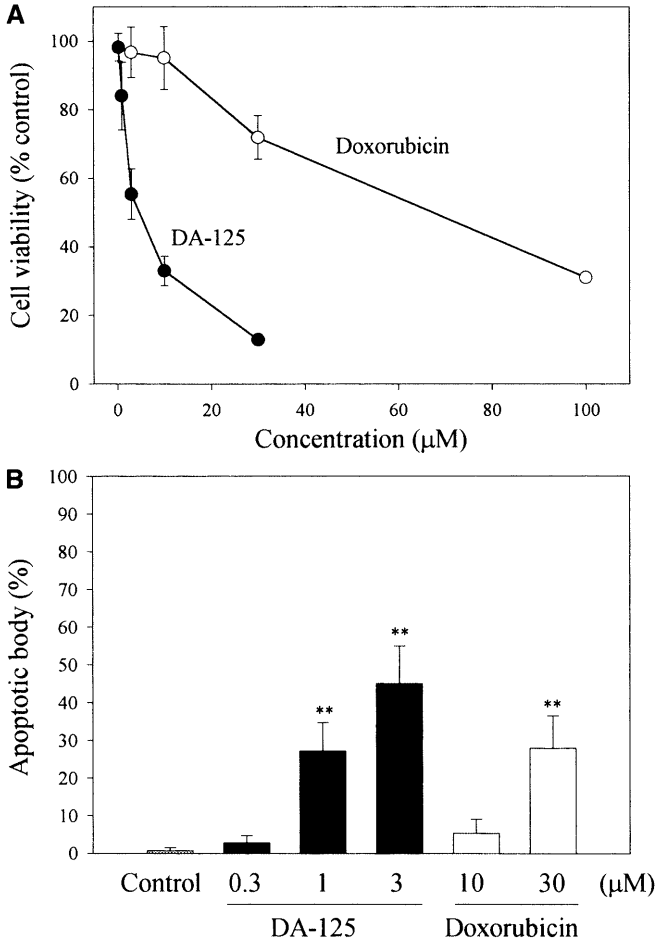


**Fig. 2A, B** The effects of DA-125 and DX on the viability and apoptosis of H4IIE hepatoma cells. **A** MTT cell viability assay. H4IIE cells were cultured with various concentrations of DA-125 or DX for 24 h. The MTT assay was performed to determine the viability of the cells after treatment with each chemotherapeutic agent for 24 h. Data are the means ± SD from four separate experiments. **B** The relative extent of apoptosis as determined by the TUNEL assay. Apoptosis was assessed in cells cultured with 0.3–3 μ*M* DA-125 or with 10–30 μ*M* DX for 24 h. The apoptotic index was calculated as the number of apoptotic cells per total number of cells × 100. Data are the means ± SEM from five independent experiments (**$P < 0.01$ vs control)

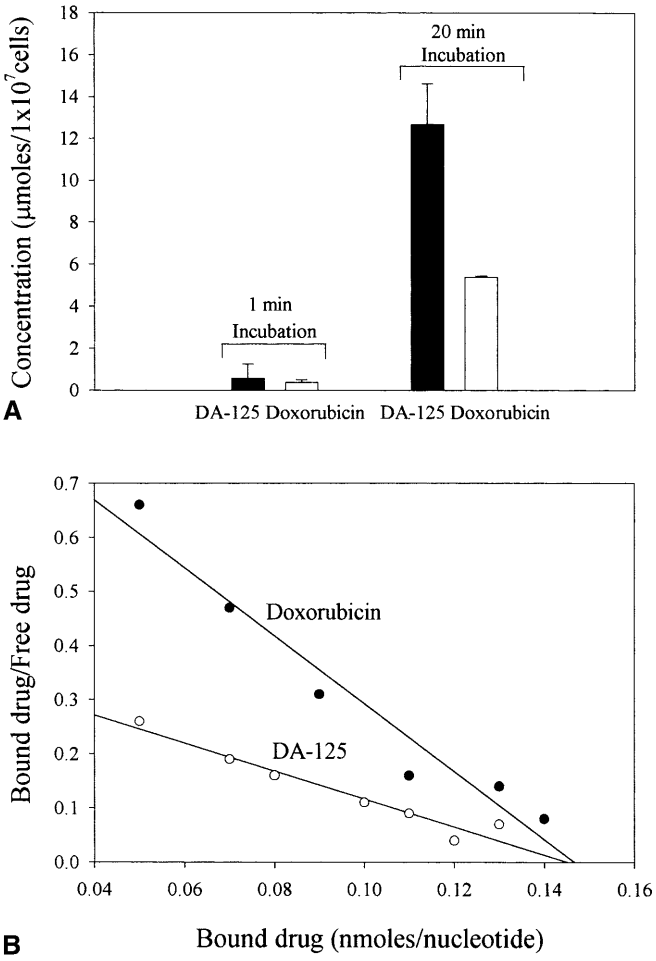


**Fig. 3A, B** Enhanced accumulation of DA-125 in cells. **A** Cells were incubated with each chemotherapeutic agent for 1 or 20 min at 37°C. Drug concentrations in disrupted cells were measured by fluorometric analysis. Data are the means ± SD from four separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman-Keuls test (**$P < 0.01$ vs control; control mRNA level = 1). **B** Scatchard plots for equilibrium drug binding. The interaction of DA-125 or DX with calf thymus DNA was studied in an equilibrium experiment

## Role of the β-alanine moiety of DA-125

We were interested in whether the β-alanine moiety present in DA-125 was responsible for its transport into cells. Cells were incubated with each drug for 24 h in the presence or absence of 1 m*M* β-alanine, and cell viability was assessed (Table 1). The presence of β-alanine (1 m*M*) failed to alter cell viability. We also determined whether excess β-alanine interfered with accumulation of DA-125. The competition experiment showed that the extent of drug accumulation was not affected by excess β-alanine in cells incubated with DA-125 for 20 min (Table 1). These results indicate that the β-alanine moiety of DA-125 is not responsible for the improved cellular drug accumulation.

## Inhibition of topoisomerase II activity

Inhibition of topoisomerase II activity by DA-125 or DX was determined in an in vitro assay. DA-125 inhibited topoisomerase II activity in a concentration-dependent manner with the $IC_{50}$ value being 3.4 ± 1.0 μ*M*, whereas DX exerted its effect at a concentrations about fourfold greater ($IC_{50}$ 11.2 ± 1.3 μ*M*, $P < 0.01$) (Fig. 4). These findings show that the improved cytotoxicity of DA-125 also resulted from the high-affinity inhibition of topoisomerase II activity.

## Prooxidant production

The rate of peroxide production was assayed in dye-loaded cells in the presence or absence of DA-125. DA-

**Table 1** Viability of cells treated with DA-125 or DX in combination with β-alanine. Cell viability was determined by MTT assay following treatment of H4IIE cells with each chemotherapeutic agent in the presence or absence of 1 m*M* β-alanine for 24 h. Drug accumulation in the cells was measured 20 min after treatment. Data are the means ± SD from four separate experiments (*ND* not determined)

| Treatment | Cell viability (% control) | | Drug accumulation (μmoles/1 × $10^7$ cells) | |
|---|---|---|---|---|
| | No β-alanine | β-alanine | No β-alanine | β-alanine |
| Control | 100 ± 11 | 102 ± 10 | ND | ND |
| + DA-125 (10 μ*M* ) | 26 ± 2 | 28 ± 2 | 12.7 ± 2.0 | 12.4 ± 0.9 |
| + DX (100 μ*M*) | 43 ± 2 | 45 ± 3 | 5.4 ± 0.1 | 5.4 ± 0.2 |

125 at 1 μ*M* increased oxidation of DCFH to DCF in a time-dependent manner, whereas DA-125 at 0.3 μ*M* was less effective (Fig. 5A). DX at the same concentrations increased prooxidant production minimally (Fig. 5B). Hence, DA-125 at micro- or submicromolar concentrations actively produces ROS.

## Role of MAP kinases

MAP kinases constitute important cellular signaling pathways, which convert various stress signals into intracellular responses. To determine whether the pathways of MAP kinases including ERK1/2, p38 MAP kinase and JNK were involved in DA-125-induced cytotoxicity, H4IIE cells were incubated with 10 μ*M* of DA-125 in the presence or absence of a specific MAP kinase inhibitor. Both PD98059 (50 μ*M*), an ERK inhibitor, and SB203580 (10 μ*M*), a p38 kinase inhibitor,

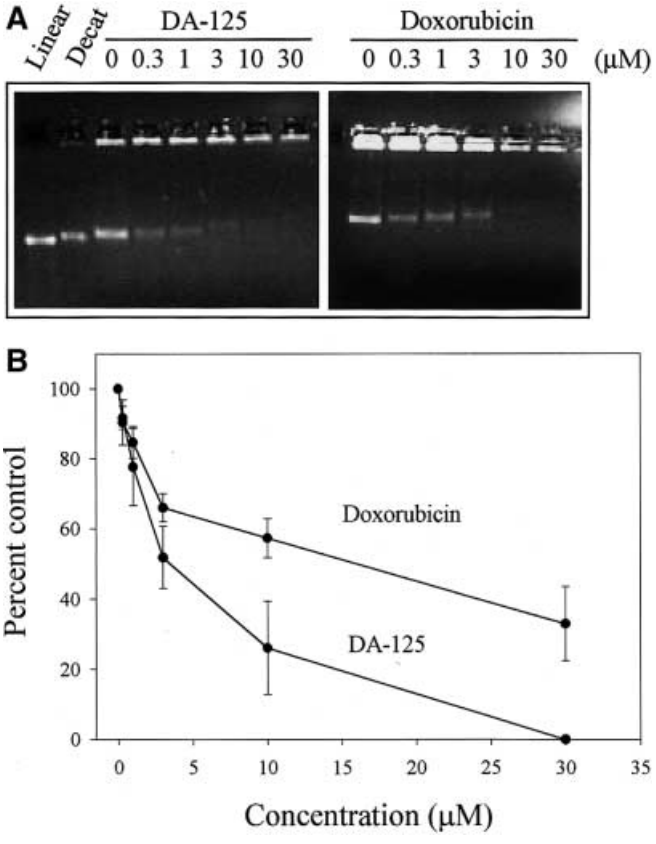


**Fig. 4A, B** Inhibition of topoisomerase II activity. **A** Representative photographs for the inhibition of topoisomerase II activity by various concentrations of DA-125 or DX (*Linear* linear kDNA marker, *Decat* topo II decatenated kDNA marker). **B** Percent control of topoisomerase II activity determined by scanning densitometry of the band intensities. Data are the means ± SD from three determinations

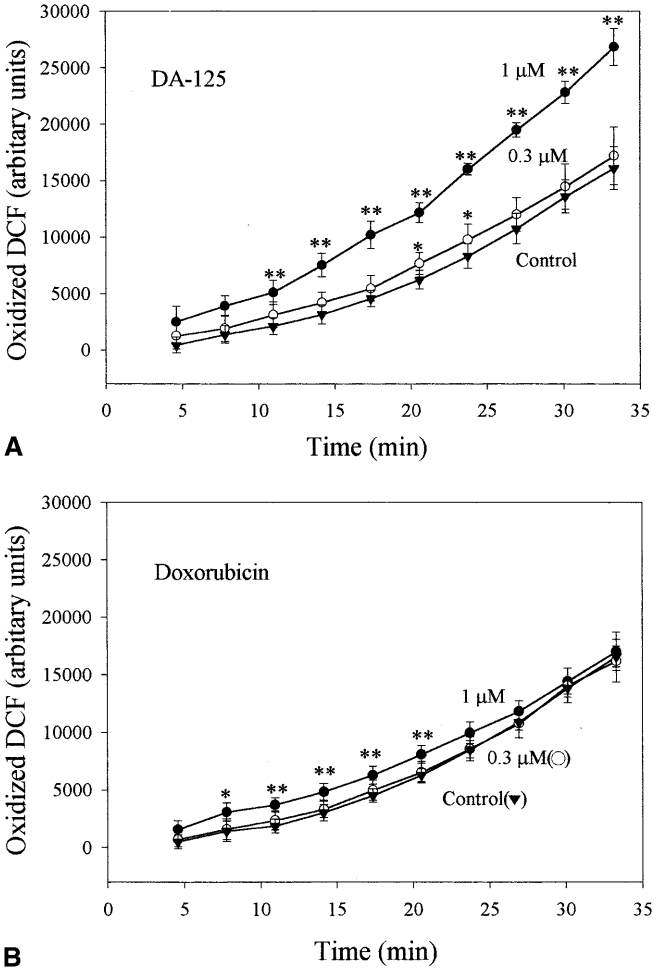


**Fig. 5A, B** Oxidized DCF fluorescence in H4IIE cells. H4IIE cells loaded with DCFH-DA were incubated with 0.3–1 μ*M* DA-125 (**A**) or DX (**B**), and the fluorescence of oxidized DCF was monitored at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence plate reader. Data are the means ± SD from five separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman-Keuls test (**$P < 0.01$ vs respective control)

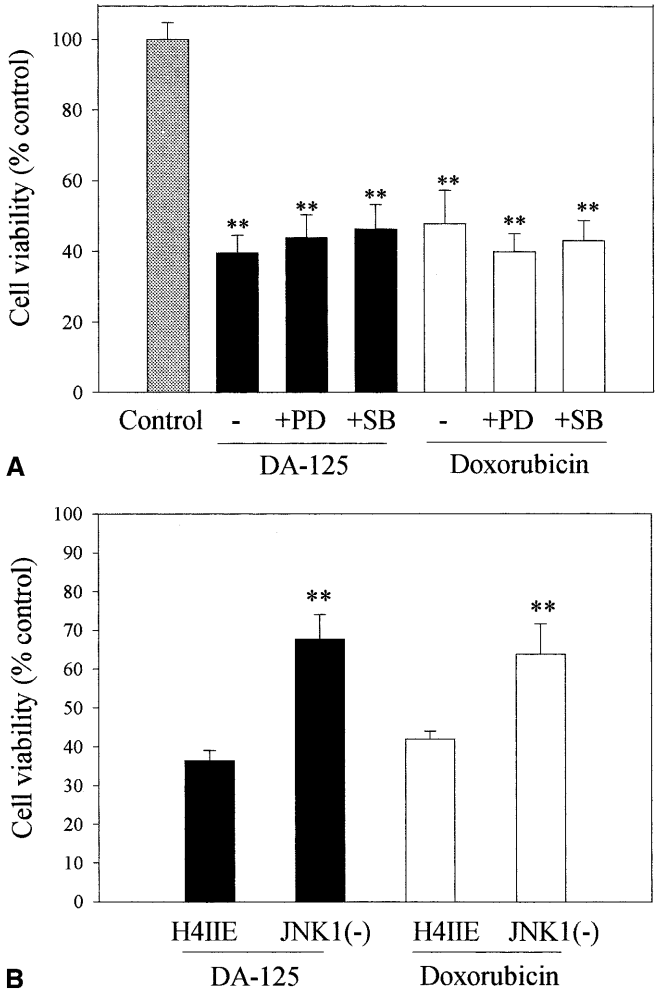


**Fig. 6A, B** The role of MAP kinases in the cell death induced by DA-125 and DX. **A** The effects of PD98059 (*PD*) or SB203580 (*SB*) on the cytotoxicity of DA-125 or DX in H4IIE cells. **B** Effects of DA-125 and DX on the viability of JNK1$^-$ stably transfected cells. The viability of the cells was determined 24 h after treatment with 10 μ*M* DA-125 or 100 μ*M* DX. Data are the means ± SD from five separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman-Keuls test (**$P < 0.01$ vs respective control)

failed to alter the cytotoxicity of DA-125 (Fig. 6A). PD98059 at 50 μ*M* and SB203580 at 10 μ*M* effectively inhibit activation by oxidative stress of ERK1/2 and p38 MAP kinase induced by sulfur amino acid deprivation in H4IIE cells (Son et al., submitted for publication).

To further study the role of JNK1 in DA-125-induced cytotoxicity, the viability of cells stably transfected with JNK1$^-$ was assessed. These cells expressed JNK2, but not JNK1 (Table 2). The viability of untransfected H4IIE cells was decreased to 36% and 41% in the presence of DA-125 at 10 μ*M* and DX at 100 μ*M*, respectively, but the viability of cells stably expressing JNK1$^-$ increased to 68% and 64%, respectively (Fig. 6B). The TUNEL assay confirmed a significant reduction in DA-125-inducible apoptosis in JNK1$^-$-transfected H4IIE cells, as compared to control cells (26% ± 12% vs 80% ± 10%, $P < 0.01$; Fig. 7). These findings provide evidence that activation of JNK1, but not ERK1/2 and p38 kinase, is responsible for the cytotoxicity of DA-125.

## Discussion

A previous study has shown that DA-125 exerts antitumor activity in human stomach, colon and liver cell lines and reaches higher intracellular levels earlier than the prototype drug DX [11]. In the present study, we first characterized the action of DA-125 on the cytotoxicity of H4IIE rat hepatoma cells and investigated the mechanistic basis for the improved cytotoxicity. DA-125 induced cell death more potently than DX with an $IC_{50}$ value of 11.5 μ*M* (i.e. a sixfold greater potency). Introduction of β-alanine and a fluoropyranose ring to the nucleus of anthracycline markedly enhances its binding affinity to DNA. We initially proposed the hypothesis that the β-alanine moiety might be responsible for the enhanced accumulation of DA-125 in cells. However, the competition experiment with DA-125 and excess β-alanine showed no alteration in cytotoxicity or in drug accumulation. The results support the notion that an increase in cellular accumulation of DA-125 is not associated with the β-alanine moiety.

We also determined whether the improved accumulation of DA-125 resulted from its high-affinity binding to DNA. Equilibrium DNA-binding experiments revealed that the binding affinity of DA-125 to DNA was twofold greater than that of DX. The higher DNA binding of DA-125 would accelerate drug accumulation in cells. This was confirmed by a 2.4-fold greater accumulation of DA-125 in cells, which would increase the concentration of the drug in the locality of the DNA.

**Table 2** JNK1/2 expression and JNK activity in cells stably transfected with JNK1$^-$. The levels of unphosphorylated JNK were determined in control and JNK1$^-$ cells. Cells stably transfected with JNK1$^-$ showed no expression of JNK1. The activity of JNK was determined by phosphorylation of GST-c-Jun for 3 h in cells exposed to oxidative stress induced by sulfur amino acid deprivation (Son et al., submitted for publication). Results were confirmed in repeated experiments (*ND* not determined)

| Cell lines | Expression of JNK1/2 (% control) | | JNK activity (% control) | |
|---|---|---|---|---|
| | JNK1 | JNK2 | Unactivated | Activated |
| H4IIE cells | 100 | 100 | 100 | 221 |
| JNK1$^-$ H4IIE cells | 0 | 95 | ND | 83 |

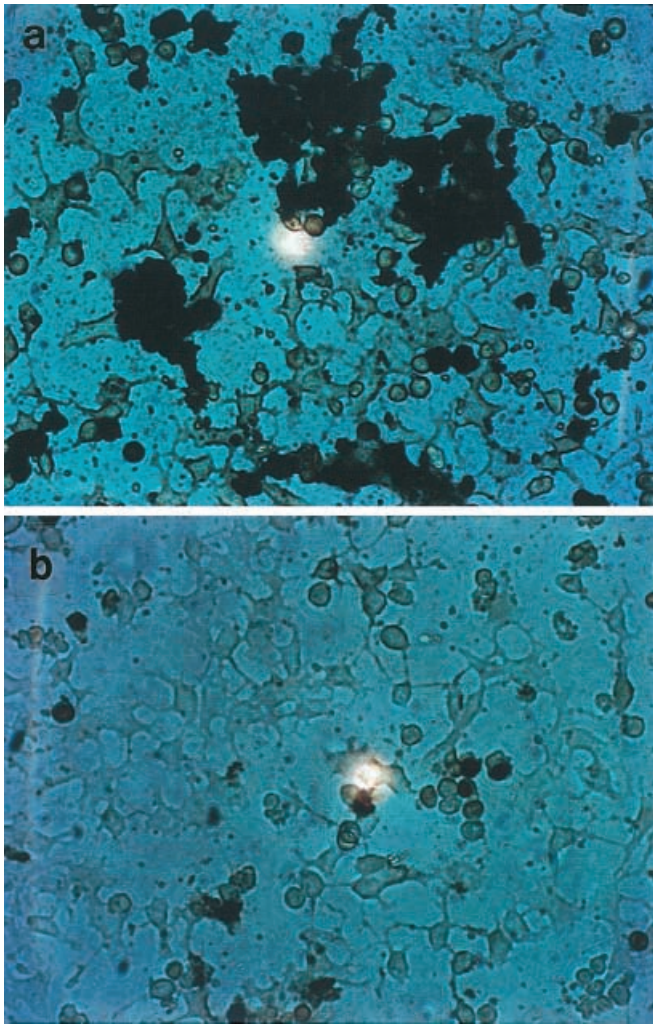


**Fig. 7A, B** TUNEL assay of apoptosis in JNK1$^-$ cells. Marked apoptosis is apparent in H4IIE cells cultured with 10 μ*M* DA-125 for 24 h (**A**), but apoptosis is decreased in JNK1$^-$ H4IIE cells (**B**). The results were confirmed in multiple experiments (×200)

Furthermore, we revealed that DA-125 potently inhibits topoisomerase II activity in vitro and that the $IC_{50}$ value of DA-125 for topoisomerase II inhibition was much smaller than that of DX. Hence, the effective cytotoxicity of DA-125 may result from its potent topoisomerase II inhibition as well as from its high-affinity DNA binding and improved drug accumulation in cells.

Studies were extended to determine whether the cytotoxicity of DA-125 resulted from apoptosis. The TUNEL assay revealed that DA-125 induced apoptotic cell death at relatively low concentrations (0.3–3 μ*M*), whereas DX-induced apoptosis was observed at concentrations of 30 μ*M* or greater. A number of cytotoxic agents including anthracycline chemotherapeutic agents increase oxidative stress in cells, the extent of which correlates with the cytotoxicity of the agent. Oxidative stress was measured by oxidation of DCFH in H4IIE cells incubated with DA-125. DA-125 at concentrations in the range 0.3–1 μ*M* was effective in oxidizing DCFH, whereas DX in the same concentration range was minimally active. These results clearly demonstrate that DA-125 at low concentrations effectively produces ROS. This may result from interruption of energy metabolism. The extent of prooxidant production by DA-125 is in agreement with enhanced cellular accumulation of the anticancer agent. Production of prooxidant species by DA-125 together with its high-affinity binding to DNA may be associated with its high apoptotic potency.

Studies have shown that activation of MAP kinases including ERK1/2, p38 MAP kinase and JNK is associated with apoptotic pathway(s) for cytotoxic chemicals [10, 19]. Because the MAP kinase family have been implicated in regulating cell survival and cell death, we further determined whether DA-125 activated the three MAP kinases. Preliminary studies showed that DA-125 activated ERK1/2 and JNK at early times (e.g. 5–10 min), whereas p38 kinase was not activated during the first 6 h after DA-125 treatment. Activation of ERK has been explained as a cell survival or proliferation signal. Extensive studies support the role of ERK activation as an antiapoptotic signal [24]. In contrast, ERK activation may also be involved in signaling cell death. Recently, we have shown that potentiation of the toxicity of cadmium in sulfur amino acid deficiency is mediated by the activation of MAP kinases (Son et al., submitted for publication).

In the present study, PD98059, a specific inhibitor of ERK, failed to affect cytotoxicity induced by DA-125, demonstrating that ERK activation is not related to DA-125-induced cytotoxicity. Activation of p38 kinase or JNK is an early response of cells to exposure to a variety of stresses including DNA-damaging agents [4, 22, 23, 25]. Activation of p38 MAP kinase or JNK precedes the induction of apoptosis. We determined whether suppression of p38 MAP kinase or JNK1 would prevent the extent of apoptosis inducible by DA-125. A specific inhibitor of p38 MAP kinase, SB203580, did not prevent DA-125- or DX-induced cytotoxicity.

Although a variety of stressful stimuli concomitantly activate p38 MAP kinase and JNK, JNK represents a distinct stress-activated pathway. JNK-induced phosphorylation of c-Jun activates AP-1 and activation of AP-1 is considered an apoptotic signal [9, 20]. Moreover, an antisense oligonucleotide of JNK reduces apoptosis through downregulation of SAPK/JNK when cells are treated with the anticancer agents etoposide or camptothecin [21]. We carried out experiments with cells expressing JNK1$^-$. These cells reduced DA-125-induced cell death. However, JNK1 inhibition failed to completely prevent DA-125-induced cytotoxicity, which might have been because of a contribution from other forms of JNK. The TUNEL assay also revealed that the extent of DA-125-induced apoptosis was markedly reduced in JNK1$^-$ cells. This result confirms that DA-125 induces apoptotic cell death via a pathway involving JNK1.

In summary, DA-125 exerted potent cytotoxicity via high-affinity DNA binding and topoisomerase II inhi-

bitory activities and induced apoptotic cell death with prooxidant production, and the apoptosis induced by DA-125 was mediated via a pathway involving c-Jun N-terminal kinase 1, but not ERK1/2 or p38 kinase.

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