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DA-125, a New Antitumor Agent, Inhibits Topoisomerase II as Topoisomerase Poison and DNA Intercalator Simultaneously

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DA-125, a novel derivative of adriamycin, is known for its anti-cancer activity. In this study, the inhibitory mechanism of DA-125 on topoisomerase was investigated in the simian virus 40 (SV40) replicating CV-1 cell by studying the SV40 DNA replication intermediates and DNA-topoisomerase complexes. DNA-protein complexes that were formed in the drug-treated cells were quantitated by using a glass filter assay. SV40 DNA replication intermediates that were accumulated in the drug-treated CV-1 cell were analyzed in a high resolution gel. DA-125 did not accumulate B-dimers of SV40 DNA replication intermediates which were found in the adriamycin-treated CV-1 cells. DA-125 induced a dose-dependent formation of the DNA-protein complexes, while adriamycin did not. When adriamycin and etoposide (VP16) were added to the SV40-infected cells at the same time, adriamycin blocked the formation of the DNA-protein complexes induced by VP16 in a dose-dependent manner. However, DA-125 blocked the formation of the DNA-protein complexes induced by VP16 up to the maximum level of the DNA-protein complexes that were induced by DA-125 alone. Adriamycin and DA-125 did not inhibit the formation of the DNA-protein complexes that were caused by camptothecin, a known topoisomerase I poison. DA-125 is bifunctional in inhibiting topoisomerase II because it simultaneously has the properties of the topoisomerase II poison and the DNA intercalator. As a topoisomerase II poison, DA-125 alone induced dose-dependent formation of the DNA-protein complexes. However, as a DNA intercalator, it quantitatively inhibited the formation of the DNA-protein complexes induced by a strong topoisomerase II poison VP16. Furthermore considering that the levels of the DNA-protein complex induced by VP16 were decreased by DA-125 in terms of the topoisomerase II poison, we suggest that DA-125 has a higher affinity to the drug-binding sites of DNA than VP16 has.

Key words: DA-125, Topoisomerase II, DNA intercalator, Adriamycin, DNA-protein complex

INTRODUCTION

Adriamycin, a representative anti-tumor agent of the anthracycline antibiotic, is one of the most widely used cancer chemotherapeutic drugs. However, its clinical use is limited due to its severe cardiotoxicity (Donehower *et al.*, 1979; Fantel *et al.*, 1985). Anthracycline antibiotics were known to generate free radicals that damage the cardiac muscle *via* lipid peroxidation (Myers *et al.*, 1977). Recently, DA-125, a derivative of adriamycin (Fig. 1), was reported to have less cardiotoxicity and other side effects (Chung *et al.*, 1995). Several studies showed that DA-125 has strong antineoplastic activity on gastric and pulmonary tumors (Baik *et al.*, 1993; Hong *et al.*, 1997: Kang *et al.*, 1999). The anti-tumor activity of anthracycline antibiotics, including adriamycin and DA-125, is based on the fact that the drugs block DNA replication and synthesis. In particular, adriamycin is known to inhibit topoisomerase that is essential for DNA replication (Kim *et al.*, 2001).

Topoisomerases are enzymes that catalyze the interconversion of different topological forms of DNA (Wang, 1985). Topoisomerase I changes the DNA structure by introducing transient single-strand DNA breaks through which another DNA strand can be passed. Topoisomerase II changes the DNA structure by introducing transient double-strand DNA breaks through which a double-strand

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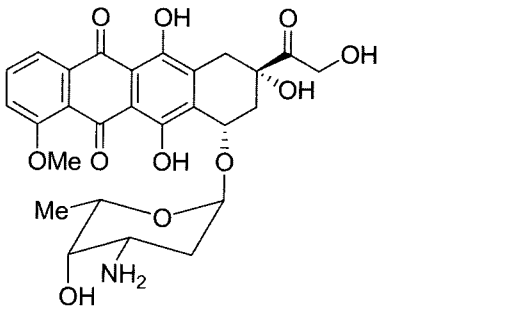
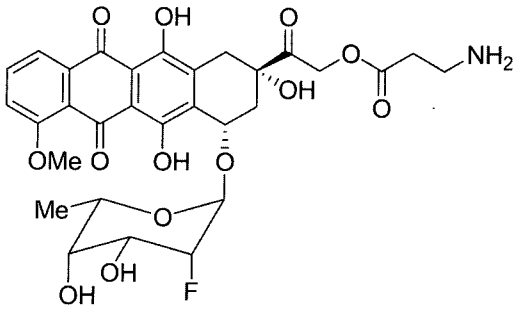


Adriamycin



DA-125

Fig. 1. The chemical structures of adriamycin and DA-125

DNA helix can be passed. These processes mediated by topoisomerases are absolutely necessary for proper DNA replication and transcription. Therefore, topoisomerase activities are found in most cells, and these activities are especially very high in tumor cells. For this reason, many anticancer drugs were developed to target topoisomerases (Liu, 1989).

Antitopoisomerase agents that work as anticancer drugs can be classified into two groups depending on their action mechanism. The first type of agents, called topoisomerase poison, stabilizes the covalent DNA-topoisomerase reaction intermediate and prevents turnover or cycling of the enzyme, which results in the inhibition of strand transfer reactions and DNA replication (Tewey *et al.*, 1984a). Camptothecin, VP16 (etoposide), and VM26 (teniposide) are examples of the topoisomerase poison. The agent of the second type, called the DNA intercalator, are interlaid between DNA bases and inhibit topoisomerase activity by blocking the access of topoisomerase to DNA (Douc-Rasy *et al.*, 1984). By preventing the formation of the covalent DNA-topoisomerase reaction intermediate, the second type of agents also interfere with the formation of the DNA-topoisomerase complexes that are mediated by topoisomerase poisons (Shin *et al.*, 1990). Proflavine, 9-aminoacridine, and adriamycin are typical DNA intercalators. In order to study the inhibitory mechanism of DA-125 on topoisomerases, we analyzed DNA-replication intermediates and DNA-protein complexes that were formed in the SV40-infected CV-1 cells.

MATERIALS AND METHODS

Materials

Adriamycin and DA-125 were obtained from Donga Pharmaceutical Co. (South Korea). Camptothecin and VP16 were from Sigma Chemical Co (St. Louis, MO, USA). Adriamycin was dissolved in deionized water. DA-125, camptothecin, and VP16 were dissolved in dimethyl sulfoxide.

Cell culture and virus infection

African green monkey kidney cells (CV-1) were grown in Dulbeccos modified Eagles medium (Gibco Laboratories) containing 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were infected with the SV40 strain 777 at a multiplicity of 10 PFU/cell, and the experiments were carried out 36 h after infection (Snapka, 1986).

Labeling and drug treatment

At 36 h post-infection at the peak of the SV40 DNA replication, old media was removed, and the serum-free medium containing [*methyl*-^{3}H]thymidine (100 μCi/mL) was added. The drug was added to labeling media 15 min after the start of the label. Hirt extraction was carried out after 30 min of labeling (Hirt, 1967). If necessary, the Hirt supernatant was digested with proteinase K (1 mg/mL) at 37°C overnight. DNA was first extracted with chloroform-isopropanol (24:1), precipitated with ethanol, and then taken up in an electrophoresis loading buffer.

High resolution gel electrophoresis

Pulse labeled SV40 DNA replication intermediates were analyzed in high resolution gel electrophoresis (Snapka *et al.*, 1991). High resolution of SV40 DNA replication intermediates was performed in 0.8% agarose at neutral pH and low voltage. Under these conditions viral DNA replication intermediates are well separated on the basis of compactness. Electrophoresis was carried out for 36 h at 0.8 V/cm.

Gel fluorography

The gel was removed from the electrophoresis tank and was directly placed in a solution of PPO (2,5-diphenyl-oxazole) in glacial acetic acid (20 g/L). The gel was allowed to equilibrate overnight, and the gel was placed under slowly running tap water also overnight. When the gel attained an even, chalky white color, it was dried on a vacuum gel dryer. The dried gel was placed in an X-ray cassette with a preflashed Kodak XAR-5 film, and the cassette was placed at 80°C overnight (Snapka *et al.*, 1991).

Glass filter assay

The assay for the DNA-protein complexes was based on the different binding properties of DNA and protein to glass filter (Shin *et al.*, 1990). Under very high salt conditions, such as 4 M guanidinium HCl (GuHCl), both protein and DNA bind quantitatively to the filter. Under lower salt conditions, only protein and protein-DNA complexes bind to the filter. To determine the total labeled DNA, 20 μL of aliquot of Hirt supernatant was added to 1.0 mL of 4.0 M GuHCl and was filtered through a GF/C glass filter (Whatman) prewetted with 4.0 M GuHCl. The filter was first rinsed with 4 mL of 4.0 M GuHCl, followed by 5 mL of ice-cold ethanol, and then dried. Labeled DNA retained on the filters was quantitated by liquid scintillation counting.

To determine the DNA-protein complexes, filter binding was carried in 0.4 M GuHCl. 20 μL of aliquot of the sample identical with that used for determining total DNA was added to 1 mL of 0.4 M GuHCl and was filtered through a GF/C glass filter prewetted with 0.4 M GuHCl. The filter was first rinsed with 4 mL of 0.4 M GuHCl, then with 5 mL of ice-cold ethanol, and dried. The radioactivity retained on the filter was quantitated as above. The amount of the protein-DNA complexes was reported as a percentage of the total DNA.

RESULTS

Patterns of SV40 DNA replication intermediates induced by DA-125 are different from those induced by adriamycin

In order to study the effect of DA-125 on the formation of SV40 DNA replication intermediates, known topoismerase inhibitors, such as camptothecin, VP16, adriamycin, and DA-125, were added to the SV40-infected cell, respectively, and the SV40 DNA replication intermediates were analyzed in a high resolution gel. As one cycle of SV40 DNA replication is complete within 15 min, labeling of the infected cells with [methy-^{3}H] thymidine for 30 min was enough to show all the replication intermediates including the forms I, II, III, and late Cairns (LC) structures as shown in Fig. 2 (Sundin and Varshavsky 1980). Camptothecin, a known topoisomerase I inhibitor, induced broken LC (LC'), which is a typical aberrant replication intermediate found in the camptothecin-treated, SV40 replicating cells (Fig. 2, lanes 3 and 4) (Shin and Snapka, 1990). VP16, a topoisomerase II poison, accumulated LC structures (Fig. 2, lanes 5 and 6). Adriamycin, which is a representative topoisomerase II inhibitor as a DNA intercalator, showed accumulation of B-dimers (Fig. 2, lanes 7 and 8) while DA-125 did not induce the formation of B-dimers (Fig. 2, lanes 9-12). Accumulation of B-dimers is very characteristic in the adriamycin-treated, SV40-replicating cells (Snapka *et al.*, 1988). The pattern of SV40 DNA replication intermediates induced by DA-125 was very similar to that induced by VP16.

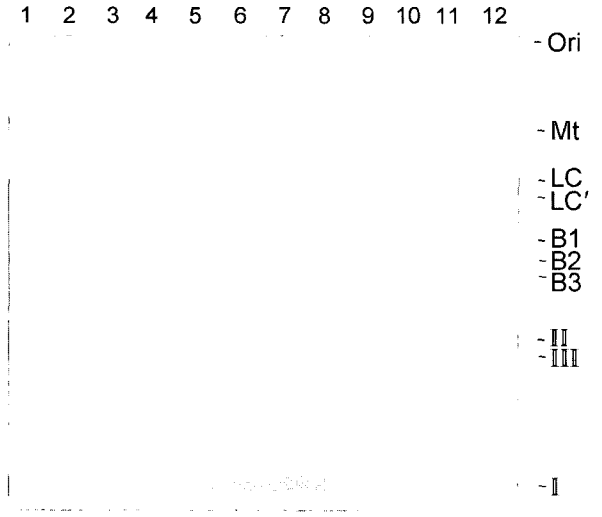


Fig. 2. Patterns of aberrant SV40 DNA replication intermediates after exposure of infected cells to topoisomerase inhibitors. Each lane represents the Hirt-extracted SV40 DNA from one 35-mm-diameter plate of infected CV-1 cells. At 36 hrs postinfection, replicating SV40 DNA was labeled for 30 min by adding [methyl-^{3}H] thymidine to the cells. Concentration of the drug indicated below as a final concentration was added to the labeling medium at 15 min after the start of labeling. The labeling medium was then drawn off, and the viral DNA was extracted by the Hirt lysis method. Lanes 1 and 2, 5% DMSO as a solvent control; lanes 3 and 4, 100 μM camptothecin; lanes 5 and 6, 250 μM VP16; lanes 7 and 8, 500 μM adriamycin; lanes 9 and 10, 500 μM DA-125; lanes 11 and 12, 250 μM DA-125; Ori, origin of electrophoresis; Mt, mitochondrial DNA; LC, late Cairns structures; LC', family of altered late Cairns structures with one broken replication fork; I, form I (superhelical) SV40 DNA monomer; II, form II (relaxed circle) SV40 DNA monomer; III, form III (linear) SV40 DNA monomer; B1-B3, catenated dimers with one member of each dimer relaxed and the other superhelical.

DA-125 induces the formation of the DNA-protein complexes like other topoisomerase poisons

Hirt lysis of the infected cell converted covalent DNA-topoismerase complexes that were temporarily present in the cells into a covalently linked complex to be remained irreversibly *in vitro*. The complexes could be quantitated by a filter assay (Shin *et al.*, 1990) or a K-SDS precipitation method (Woryrianoski *et al*, 1988). Because DA-125 showed patterns of SV40 DNA replication intermediates distinguishable from those of adriamycin, DNA-protein complexes induced by DA-125 were quantitated in order to compare them with those induced by other topoisomerase inhibitors. The typical topoisomerase poisons, camptothecin (100 μM) and VP-16 (250 μM), at the concentrations which were previously reported to form near maximum levels of the DNA-protein complex induced 60.8 and 21.6%, respectively, while DA-125 (500 μM) and adriamycin

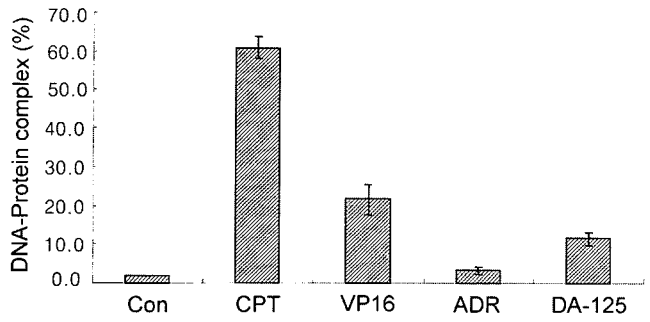


Fig. 3. DNA-protein complexes induced by topoisomerase inhibitors. Infected cells were labeled for 30 min and treated with drugs indicated for 15 min before extraction. A 20 μL of Hirt extract supernatant was added to 1 mL of 0.4 M GuHCl or 4.0 M GuHCl solution, respectively, and then filtered through a GF/C glass filter. Labeled DNA that was retained on the filter was quantitated in a liquid scintillation counter. The amounts of DNA-protein complexes are estimated as a ratio of the radioactivity measured in the 0.4 M GuHCl to the radioactivity measured in the 4.0 M GuHCl. Con, control (5% DMSO); ADR, 500 μM adriamycin; DA-125, 500 μM DA-125; CPT, 100 μM camptothecin; VP16, 250 μM VP16. Each point represents the mean ± S.E.M. (n = 3-8).

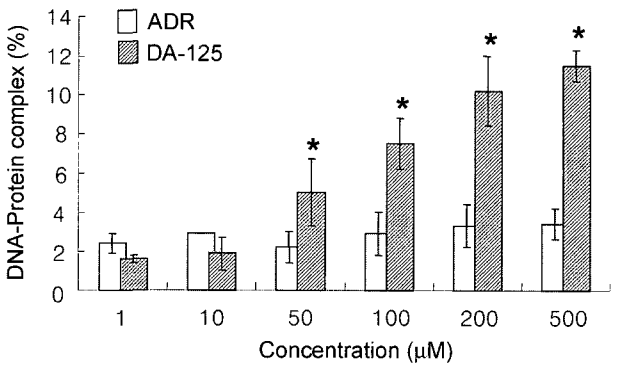


Fig. 4. Dose-dependent formation of the DNA-protein complexes induced by DA-125 as a topoisomerase poison. Infected cells were treated with DA-125 or adriamycin (ADR) at the concentrations indicated above. DNA-protein complexes were measured as described in Fig. 3. Each point represents the mean ± S.E.M. (n = 3-8). *$P<0.05$ compared with adriamycin-treated groups.

(500 μM) showed 11.5 and 3.3%, respectively (Fig. 3). The results showed that DA-125 induced the DNA-protein complex of significant amounts when compared to the prototype compound adriamycin, indicating that the inhibitory mechanism of DA-125 is different from that of adriamycin.

DA-125 as a topoisomerase poison induces dose-dependent formation of the DNA-protein complexes

To compare the patterns of formation of DNA-protein complexes induced by DA-125 and adriamycin, we quantitated the complexes that were induced by the two drugs of various doses (Fig. 4). DA-125 showed dose-dependent formation of the complex with 1.6, 1.9, 5.0, 7.5, 10.2, and 11.5% at 1, 10, 50, 100, 200, and 500 μM, respectively. However, adriamycin did not induce the formation of the complex at any of the tested doses. The differences between the two drugs in inducing the formation of the DNA-protein complexes indicate that the inhibitory mechanism of DA-125 on DNA replication and topoisomerases is very different from that of adriamycin. A dose-dependent increase of the complex provides the evidence that DA-125 worked as a topoisomerase poison like VP16.

DA-125 as a DNA intercalator inhibits the formation of the DNA-protein complexes mediated by VP16

The DNA intercalators such as proflavine, 9-aminoacridine, and adriamycin have been known to inhibit topoisomerase activities by preventing topoisomerase from accessing DNA, and by repressing the formation of the DNA-protein complex induced by topoisomease poisons (Douc-Rasy *et al.*, 1984; Tewey *et al.*, 1984b). In order to investigate if DA-125 has DNA intercalator properties like adriamycin, DA-125 and a known topoisomerase poison, VP16 or camptothecin, were simultaneously added to the SV40-infected cells, and the amounts of the DNA-protein complexes induced by VP16 were analyzed (Fig. 5). VP16 is a strong topoisomerase II poison (Tewey *et al.*, 1984a). 250 μM VP16 alone showed a 22% formation of the DNA-protein complexes, which was then dose-dependently decreased by adding adriamycin together (Fig. 5A). However, the decrease of VP16-induced DNA-protein complex by DA-125 was maximized at 50 μM DA-125, and then, the levels of the complex were increased in a dose-dependent manner of DA-125. This result indicates that DA-125 has a higher affinity to DNA than VP16 does to DNA, and DA-125 predominately occupied the drug-binding sites of DNA as its concentration was increased. At 50 μM DA-125, SV40 DNA was completely saturated with DA-125 so that the drug-binding sites available for VP16 were minimized, and the formation of the DNA-protein complex was also minimized by DA-125's properties as a DNA intercalator. However, over 50 μM DA-125, levels of the DNA-protein complexes increased due to the properties of DA-125 as a topoisomerase poison. Therefore, DA-125 can be seen as having both the properties of a topoisomerase poison and a DNA intercalator.

Camptothecin is a strong topoisomerase I poison. Camptothecin of 100 μM alone induced approximately 60% DNA-protein complexes. The levels of the DNA-protein complexes induced by camptothecin were not decreased by the presence of DA-125 or adriamycin up to 500 μM (Fig. 5B), which suggests that DA-125 as a DNA intercalator is topoisomerase II-specific.

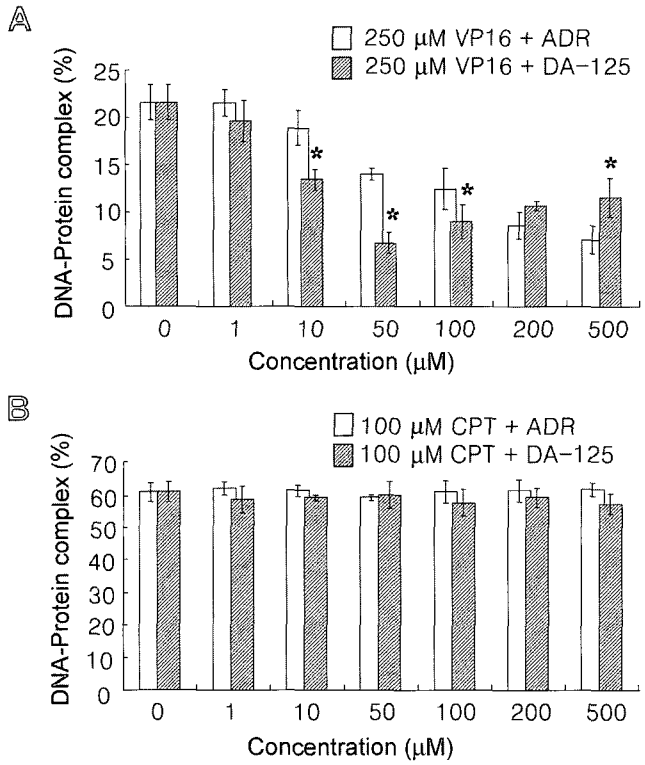


Fig. 5. DA-125 and adriamycin as DNA intercalators to block the formation of the DNA-protein complexes. **A**: Inhibition of DA-125 and adriamycin on the formation of the DNA-protein complexes induced by a strong topoisomerase II poison VP16. **B**: Failure of inhibition of DA-125 and adriamycin on the formation of the DNA-protein complexes induced by a strong topoisomerase I poison camptothecin. Infected cells were simultaneously treated for 15 min with DA-125 or adriamycin (ADR) of the concentrations indicated above in the presence of 250 μM VP16 or 100 μM camptothecin (CPT). DNA-protein complexes were measured as described in Fig. 3. Each point represents the mean ± S.E.M. (n = 3-8). * $P<0.05$ compared with adriamycin-treated groups.

DISCUSSION

Several studies have reported that DA-125 has anti-tumor activity in human lung and gastric cancers (Hong *et al.*, 1997; Kang *et al.*, 1999). DA-125 was previously suggested to exert anti-tumor activity by inhibiting topoisomerase II, which is highly active in many cancer cells (Kim *et al.*, 2001). In the present study, we first investigated the mechanism of DA-125's action on topoisomerase II by comparing the ability of DA-125 to inhibit topoisomerase in SV40-infected cells with that of adriamycin, a prototype compound. Although DA-125 is a derivative of adriamycin, its inhibitory mechanism was distinguished from that of adriamycin. Since no remarkable formation of the DNA-protein complexes was observed in the adriamycin-treated cells, it is clear that adriamycin inhibited topoisomerase II as a DNA intercalator (Douc-Rasy *et al.*, 1984; Tewey *et al.*, 1984b). Contrary to expectation, DA-125 inhibited topoisomerase II by two different mechanisms. First, it acted as a DNA intercalator like adriamycin so that it blocked the formation of the DNA-protein complexes that were induced by VP16 (Fig. 5A). Second, it acted like a topoisomerase poison and induced dose-dependent formation of the stabilized DNA-topoisomerase II complex, which is a characteristics of topoisomerase poisons, such as VP16 and VM26. Furthermore, in the analysis of SV40 DNA replication intermediates, DA-125 did not induce the accumulation of B-dimers of SV40 DNA replication intermediates that were found in the SV40-infected, adriamycin-treated cells (Snapka *et al.*, 1988). The overall distribution of SV40 DNA replication intermediates in the DA-125-treated cells was similar to the distribution in the VP16-treated cells but different than that in the adriamycin-treated cells (Fig. 2). Moreover, because DA-125 was not able to block the formation of the DNA-protein complexes induced by campothecin which is a well-known topoisomerase I poison, it was proposed that DA-125 inhibits only topoisomerase II. Therefore, these evidences suggest conclusively that DA-125 inhibits topoisomerase II by using both mechanisms of a DNA intercalator and a topoisomerase poison. In addition, considering that 50 μM DA-125 markedly inhibited the formation of VP16-induced DNA-protein complexes as a DNA intercalator, DA-125 might be much more active in inhibiting topoismerase II as a DNA intercalator rather than as a topoisomerase poison.

The DNA tumor virus SV40 has been widely used for understanding the roles of topoisomerase in eukaryotic DNA replication. SV40 is considered as a model for the mammalian replicon because of its extensive use with cellular DNA replication machinery and chromosomal proteins (DePhamphilis *et al.*, 1983). For this reason, our results that were observed in the SV40-infected cells provide strong evidence that DA-125 effectively inhibits topoisomerase II *in vivo* by the two mechanisms of a topoisomerase poison and a DNA intercalator.

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