# Effect of Pyrazoloacridine (NSC 366140) on DNA Topoisomerases I and II[1]

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## ABSTRACT

**Pyrazoloacridine (PA), an acridine congener with an unknown mechanism of action, has shown selective activity against solid tumor cells, cytotoxicity in noncycling and hypoxic cells, and promising antitumor activity in Phase I clinical trials. In the present study, the effect of PA on topoisomerase (topo) activity was evaluated using yeast strains lacking functional topo I or II, mammalian cell nuclear extracts, purified samples of mammalian topo I and topo II, and intact mammalian tissue culture cells. Clonogenic assays revealed that PA cytotoxicity in yeast strains was unaffected by selective loss of topo I or topo II activity. On the other hand, enzyme assays revealed that 2–4 μM PA abolished the catalytic activity of both topo I and topo II *in vitro*. In contrast to topotecan and etoposide, PA did not stabilize covalent topo-DNA complexes. Instead, PA inhibited topotecan-induced stabilization of covalent topo I-DNA complexes and etoposide-induced stabilization of topo II-DNA complexes *in vitro* and in intact cells. Consistent with these results, colony-forming assays indicated that short-term PA exposure inhibited the cytotoxicity of topotecan and etoposide, whereas prolonged PA exposure was itself toxic to these cells. Accumulation studies revealed that PA was concentrated as much as 250-fold in drug-treated cells, resulting in intranuclear concentrations that far exceeded those required to inhibit topo I and topo II. Collectively, these results not only suggest that PA can target both topo I and topo II at clinically achievable concentrations but also indicate that its mechanism is distinct from topo I and topo II poisons presently licensed for clinical use.**

## INTRODUCTION

PA[4] (NSC 366140; Fig. 1*A*), a rationally synthesized acridine derivative, is the first of a new class of compounds to undergo clinical testing as an anticancer agent (1–3). Preclinical studies indicate that this agent has broad-spectrum antitumor activity *in vivo* (4). In addition, PA displays several unique properties, including solid tumor selectivity (1, 4), activity against hypoxic cells (1, 4), and cytotoxicity in noncycling cells (1, 2). Moreover, PA retains full activity against cells that are resistant to other agents on the basis of overexpression of P-glycoprotein (5) or the multidrug resistance protein MRP (6).

Two Phase I clinical trials of PA were completed recently (7, 8). In one of these trials, PA displayed activity against cisplatin- and paclitaxel-resistant ovarian cancer (8). As a result, PA is undergoing Phase II trials in several different solid tumors (9).

In view of this potential clinical activity, the mechanism of cytotoxicity of PA has become a matter of interest. PA has been shown to cause delayed DNA fragmentation in MCF-7 breast cancer cells (10), an observation consistent with induction of apoptosis. Recent studies have indicated that PA can also induce apoptosis in P53-deficient Hep 3B human hepatoma cells (11).

Despite these recent studies, the initial events that lead to PA-induced cytotoxicity are unclear. PA was shown previously to preferentially inhibit RNA synthesis (12). PA also displaced ethidium bromide from DNA with a potency approaching that of doxorubicin (12, 13) and enhanced the viscosity of plasmid DNA (13), suggesting that PA is an intercalating agent. In view of the fact that other intercalating agents target topo I (*e.g.*, actinomycin D; Refs. 14 and 15) or topo II (*e.g.*, doxorubicin and amsacrine; Refs. 16–18), we have examined the effect of PA on topo I and topo II. Results of these studies indicate that clinically achievable concentrations of PA inhibit topo I and topo II *in vitro* and *in situ* by a mechanism that differs from that of other dual topo I/topo II inhibitors such as saintopin (19), intoplicine (20), and the indoloquinolinediones (21). Preliminary accounts of this work have been presented previously (13, 22).

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[4] The abbreviations used are: PA, pyrazoloacridine; topo, topoisomerase; TPT, topotecan; HPLC, high performance liquid chromatography; YPD medium, medium containing yeast extract, peptone, and dextrose (23).

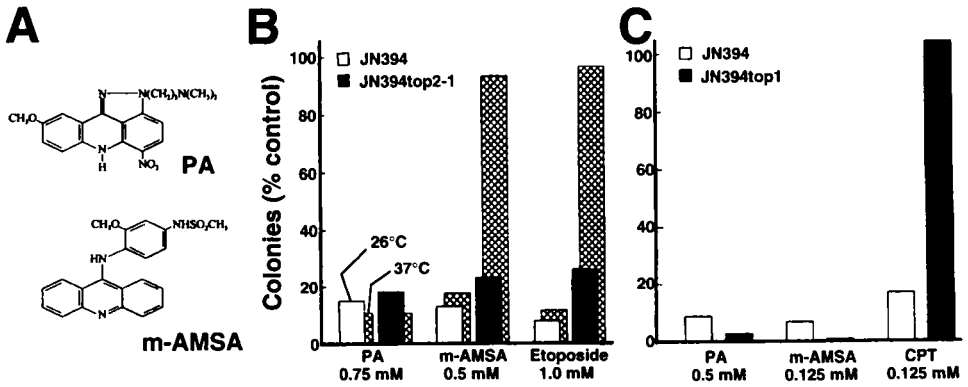


*Fig. 1* *A*, Structures of PA and amsacrine. *B*, effect of mutated topo II on PA sensitivity in *S. cerevisiae*. Parental and temperature-sensitive mutant strains were incubated at 26°C or 37°C for 15 min, maintained at 26°C or 37°C during a subsequent 3-h treatment with drug or diluent, washed, and plated on YPD agar plates. After a 3-day incubation at 26°C, colonies were counted. Data were expressed as a percentage of colony counts in diluent-treated samples. □ and ■, parental JN394 cells and JN394top2-1 cells containing a temperature-sensitive topo II allele, respectively, incubated at 26°C. ▩, the respective strains treated with drug or diluent at 37°C. *C*, effect of topo I gene deletion on PA sensitivity. Yeast cells were incubated at 26°C for 4 h with drug or diluent, washed, and plated on YPD agar plates. Colony formation was assessed 3 days later. □, parental strain JN394; ■, topo I-deficient strain JN394top1. Results are representative of three experiments.

## MATERIALS AND METHODS

**Materials.** PA was synthesized as described previously (1). Additional aliquots of PA as well as amsacrine were obtained from the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). TPT was a gift from SmithKline Beecham (King of Prussia, PA). Reagents were purchased from the following suppliers: etoposide and doxorubicin from Sigma Chemical Co. (St. Louis, MO); and purified human topo I and topo II from Topogen (Columbus, OH). All HPLC solvents were HPLC grade.

**Cytotoxicity of PA in Yeast Strains.** The isogenic yeast strains JN394 (genotype *matA ISE2 rad52::Leu2 Top1 Top2*), JN394top1 containing a topo I disruption (23), and JN394top2-1 containing a temperature-sensitive allele of topo II (24) were kindly provided by Dr. John Nitiss (St. Jude Children's Hospital, Memphis, TN). Yeast were grown overnight at 26°C in YPD medium (23, 24). Late-log phase cells (absorbance = 0.5–1.0, 0.5–1.0 at 600 nm) were adjusted to a concentration of 1–2 × $10^6$ cells/ml in YPD medium and incubated at 26°C or 37°C with drug or diluent. At the indicated times, aliquots were removed, washed three times with water, serially diluted, plated on YPD agar, and incubated at 26°C. Colonies were counted 3 days later. Control plates typically contained 150–300 colonies.

**Cytotoxicity of PA in K562 Myeloid Leukemia Cells.** Logarithmically proliferating K562 cells in RPMI 1640 containing 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine (medium A) were incubated at 37°C with the indicated concentration of PA for 1 h or 24 h, sedimented at 200 × *g* for 5 min, and washed once with medium A. Aliquots containing 1000–5000 cells were plated in 0.3% (w/v) agar as described (25). Colonies containing >50 cells were counted on an inverted phase contrast microscope 10–14 days later. Results were compared with cells treated with diluent ($dH_2O$) alone, which had a cloning efficiency of ~60% in these experiments.

Alternatively, cells were incubated with the indicated concentration of PA or diluent for 15 min, treated with etoposide or TPT for 1 h as indicated, washed, and plated as described above.

**Effect of PA on Topo I and Topo II Activities.** To investigate the effect of PA on topo I catalytic activity (modified from ref. 26), 400 ng of supercoiled plasmid $0^{67}$ was incubated for 30 min at 37°C in a 20-μl reaction mixture containing 2 units of enzyme,[5] the indicated final concentration of PA (added from a concentrated aqueous stock), and reaction buffer consisting of 100 mM NaCl, 50 mM Tris-HCl (pH 7.5 at 21°C), 0.5 mM EDTA, 0.5 mM DTT, and 50 μg/ml BSA. The reaction was terminated by the addition of 1 μl of 10% (w/v) SDS and 2 μl of 10 mg/ml proteinase K, followed by an additional 15-min incubation at 37°C. After extraction with 1:1 phenol:chloroform and with chloroform, samples were applied to 1% (w/v) agarose gels prepared in 36 mM Tris, 30 mM $NaH_2PO_4$, and 1 mM EDTA. Gels were run at 45 V for 4.5 h, stained with 0.5 μg/ml ethidium bromide, and photographed under UV light. The effect of PA on topo II-catalyzed decatenation was investigated using similar techniques, except that 400 ng of catenated trypanosomal kinetoplast DNA was used as a substrate, and reaction buffer was supplemented with 7.5 mM $MgCl_2$ and 1 mM ATP. Identical results were obtained in these assays when experiments were performed using nuclear extracts (prepared as described in Ref. 26) or purified topo I and topo II as sources of topoisomerase activity.

**Effect of PA on Drug-stabilized Topoisomerase-DNA Complexes.** To examine the effect of PA or TPT on topo I-mediated cleavable complex formation *in vitro*, 400 ng of supercoiled plasmid $0^{67}$ was incubated for 30 min at 37°C with 50 units of enzyme in reaction buffer. After termination of the reaction with SDS and proteinase K, samples were extracted with phenol:chloroform and chloroform as described above,

[5] One unit of topo I relaxes 50% of the input plasmid in this assay.

then subjected to electrophoresis in the dark in the presence of 0.5 μg/ml ethidium bromide. Using a similar approach, the effect of PA or etoposide on topo II-mediated cleavable complex formation *in vitro* was examined by incubating 400 ng of supercoiled plasmid 0[67] for 30 min at 37°C with 4 units of purified topo II[6] in reaction buffer supplemented with 7.5 mM $MgCl_2$ and 1 mM ATP, stopping the reaction with SDS and proteinase K, extracting the samples with phenol:chloroform and chloroform, and performing electrophoresis in the presence of 0.5 μg/ml ethidium bromide as described above.

**Band Depletion Assay.** The formation of covalent topoisomerase-DNA adducts in intact cells was examined using a band depletion assay (reviewed in Ref. 27). In brief, logarithmically growing K562 cells were harvested by sedimentation, washed once with medium B [RPMI 1640 containing 10 mM HEPES (pH 7.4 at 20°C)] and resuspended at a concentration of 1–2 × $10^6$ cells/ml in medium B. One-ml aliquots were added to 1.5-ml microfuge tubes containing varying concentrations of PA or diluent and incubated for 15 min at 37°C. TPT or etoposide was then added as indicated. Cells were incubated for an additional 45 min at 37°C, sedimented at 3200 × *g* for 1 min, immediately solubilized in 6 M guanidine hydrochloride under reducing conditions, and prepared for electrophoresis as described (27). Immunoblotting was performed (27) using mouse monoclonal antibodies that recognize topo I (kindly provided by Dr. Y-C. Cheng, Yale University Medical School) or topo IIα (kindly provided by Dr. Udo Kellner, University of Kiel).

**Measurement of PA Accumulation and Subcellular Distribution.** Log-phase K562 cells were sedimented at 200 × *g* for 5 min and resuspended at a concentration of 2–3 × $10^6$/ml in medium A. Cells were treated with 0–10 μM PA for 60 min at 37°C, sedimented at 3200 × *g* for 1 min, washed three times with ice-cold PBS, and lysed by vigorous agitation in 1 ml of −20°C methanol. After a 5-min incubation on ice, samples were sedimented at 12000 × *g* for 5 min to remove macromolecules. Supernatants were stored at −20°C until analyzed. Concentrations of PA in these supernatants were calculated from standard curves (0–10 μM PA) prepared by adding known quantities of PA to methanolic extracts prepared from untreated K562 cells.

Reverse-phase HPLC separations of standard and unknown supernatants were accomplished on a Lichrosorb RP-8 (E. M. Sciences) analytical column (250-mm × 4-mm inside diameter, 10-μm particles) fitted with a Brownlee RP2 (Chromtech) guard column (15-mm × 3.2-mm inside diameter, 7-μm particles). The mobile phase consisted of 20:5:80 acetonitrile:tetrahydrofuran:100 mM potassium phosphate (adjusted to an apparent pH of 4.0 with 1 M KOH after mixing the solvents) delivered at a flow rate of 1.0 ml/min. Absorbance of the column effluent was monitored at 450 nm.

The mean volume of K562 cells (1500 fl) was determined on a Coulter counter that was equipped with a channel analyzer and calibrated using polystyrene beads of defined diameter (Becton Dickinson, Mountainview, CA). From the concentration of PA in the cell extracts, the volume of the K562 cells, and the number of cells present in the sample, the mean cellular concentration of PA in the cells was calculated.

To examine the subcellular distribution of PA, K562 cells in medium A were incubated for 60 min at 37°C with 10 μM PA, washed three times with ice-cold PBS, resuspended in PBS, and examined on a Zeiss LSM 310 confocal microscope using the 488-nm band from an argon-krypton laser for excitation and a 590-nm long-pass emission filter. Images were acquired under conditions where autofluorescence of the cells yielded no discernible signal. Quantitative estimates of the subcellular distribution of PA were derived from the two-dimensional images by comparing the integrated signal intensities (area × intensity) of nuclei and the corresponding whole cells using NIH Image 1.61 software and then performing calculations based on the assumption that nuclei and cells were spherical.

## RESULTS

**Cytotoxicity of PA in *Saccharomyces cerevisiae*.** In the present study, a number of complementary techniques were used to examine the possibility that PA might alter the functions of DNA topoisomerases. The possibility that PA might target these enzymes was suggested by previous observations that some intercalating agents alter topoisomerase function (15–18, 28, 29) and by the structural similarity between PA and amsacrine, a well-characterized topo II poison (Fig. 1*A*). Studies in yeast strains that contain temperature-sensitive alleles of topo II have provided strong genetic evidence that amsacrine kills cells by stabilizing topo II-DNA complexes (24). To determine whether PA kills cells by an identical mechanism, we compared the effects of PA, etoposide, and amsacrine on clonogenic survival of the JN394top2-1 strain following incubation with drugs under conditions where topo II was present (26°C) or essentially absent (37°C). As reported previously by Nitiss *et al.* (24), the cytotoxicity of amsacrine or etoposide in the JN394top2-1 strain was abrogated by incubation at 37°C (Fig. 1*B*). In contrast, PA remained cytotoxic under conditions of diminished topo II activity (Fig. 1*B*). This result clearly distinguishes the mechanism of cytotoxicity of PA from that of amsacrine.

The possibility that PA kills cells solely by stabilizing topo I-DNA complexes was studied by comparing the effect of this agent in yeast cells that differ in topo I content. As reported previously (23), the cytotoxicity of the topo I poison camptothecin was markedly diminished in JN394top1 cells, which contain a disrupted topo I gene, compared with wild-type JN394 cells (Fig. 1*C*).[7] In contrast, PA was at least as toxic in JN394top1 cells as in parental cells (Fig. 1*C*). This result, coupled with the result in Fig. 1*B*, indicates that PA differs from the classical topoisomerase poisons in its mechanism of cytotoxicity. These observations prompted us to examine the effect of PA on topoisomerases in greater detail.

[6] One unit of topo II decatenates 200 ng of kinetoplast DNA in 30 min at 37°C.

[7] The enhanced cytotoxicity of amsacrine and etoposide in yeast cells after topo I disruption (Fig. 1*C*) has been noted previously (42, 43) and has been attributed to increased reliance on topo II during replication in these cells (44).

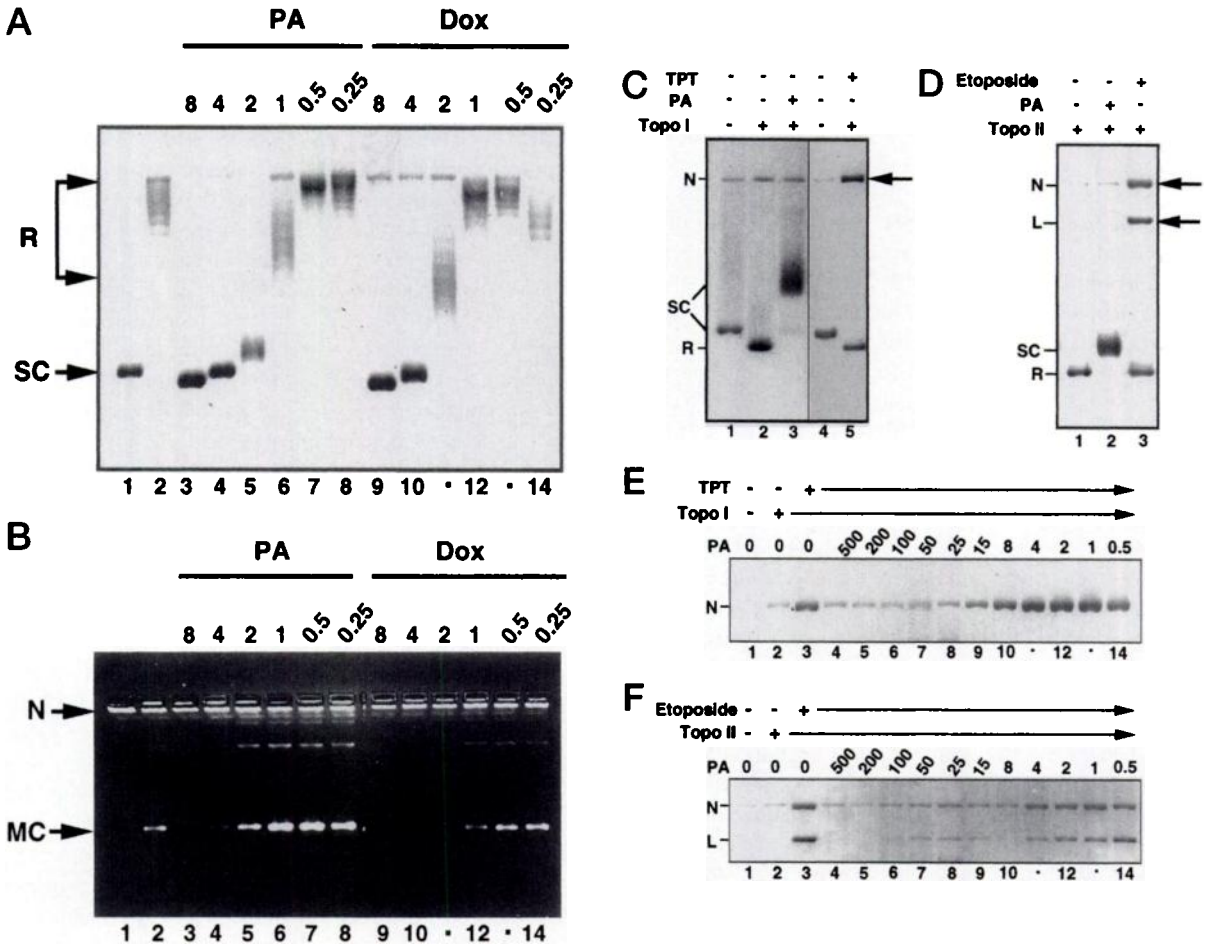


*Fig. 2* Effect of PA on topoisomerase catalytic activity and formation of topoisomerase-DNA covalent complexes. *A*, inhibition of topo I activity. Supercoiled plasmid was incubated with 2 units of topo I in the absence (*Lane 2*) or presence of PA (*Lanes 3–8*) or doxorubicin (*Lanes 9–14*) at a final concentration of 0.25–8 μM as indicated. *Lane 1*, substrate DNA incubated without enzyme. *SC*, supercoiled DNA; *R*, relaxed DNA. *B*, inhibition of topo II activity. Kinetoplast DNA was incubated with 2 units of topo II in the absence (*Lane 2*) or presence of PA (*Lanes 3–8*) or doxorubicin (*Lanes 9–14*) at a final concentration of 0.25–8 μM as indicated. *Lane 1*, substrate DNA incubated without enzyme. *N*, kinetoplast network DNA. *MC*, released minicircle DNA. Results are representative of four experiments. *C*, stabilization of topo I-DNA complexes by TPT but not PA. Aliquots of supercoiled plasmid $O^{67}$ were incubated for 30 min at 37°C with nuclear extract containing 50 units of topo I in the absence or presence of TPT (1.56 μM) or PA (200 μM) as indicated. After treatment with SDS and proteinase K, samples were separated on 1.0% agarose gels in the presence of 0.5 μg/ml ethidium bromide to better separate nicked (*N*) from relaxed (*R*) DNA. Note that TPT stabilizes topoisomerase I-DNA complexes (as indicated by the increased amount of nicked DNA), whereas PA does not. *D*, stabilization of topo II-DNA complexes by etoposide but not PA. Aliquots of supercoiled plasmid $O^{67}$ were incubated for 30 min at 37°C with purified topo II in the absence or presence of etoposide (400 μM) or PA (500 μM) as indicated, then treated with SDS/proteinase K and subjected to electrophoresis as described for *C*. Note that etoposide stabilizes topoisomerase-DNA complexes (as indicated by the increased amounts of nicked and linear DNA), whereas PA does not. *E* and *F*, PA inhibits stabilization of topoisomerase-DNA complexes. Aliquots of supercoiled plasmid $O^{67}$ were incubated for 30 min at 37°C with the indicated concentration of PA along with nuclear extract containing 50 units of topo I in the presence of 6.25 μM TPT (*E*) or 4 units of purified topo II in the presence of 400 μM etoposide (*F*). After treatment with SDS and proteinase K, samples were separated on 1.0% agarose gels in the presence of 0.5 μg/ml ethidium bromide. The portions of gels containing nicked (*N*) and linear (*L*) plasmid are shown.

**PA Inhibits Topoisomerases under Cell-free Conditions.** In a complementary series of experiments, the effect of PA on topoisomerase activity was examined under cell-free conditions. When topo I catalytic activity was assayed by following conversion of a supercoiled plasmid to relaxed circular DNA (Fig. 2*A*, *Lanes 1* and *2*, respectively), PA inhibited this activity at concentrations as low as 2 μM (Fig. 2*A*, *Lane 5*). Doxorubicin, an intercalating agent that binds to DNA with affinity similar to that of PA (13), likewise inhibited topo I activity at 2–4 μM (Fig. 2*A*, *Lanes 10* and *11*). When topo II catalytic activity was assayed by monitoring release of free DNA minicircles from a network of kinetoplast DNA (Fig. 2*B*, *Lanes 1* and *2*), >80% inhibition was observed at 4 μM PA (Fig. 2*B*, *Lane 4*). Doxorubicin similarly inhibited topo II-catalyzed decatenation at concentrations of 2 μM or more (Fig. 2*B*, *Lane 11*). These observations indicate that PA inhibits both topo I and topo II catalytic activity.

To examine the possibility that PA nonspecifically inhibits all DNA-targeting enzymes, the effect of PA on three restriction endonucleases (*Eco*RI, *Not*I, and *Sfi*I) and viral DNA ligase was

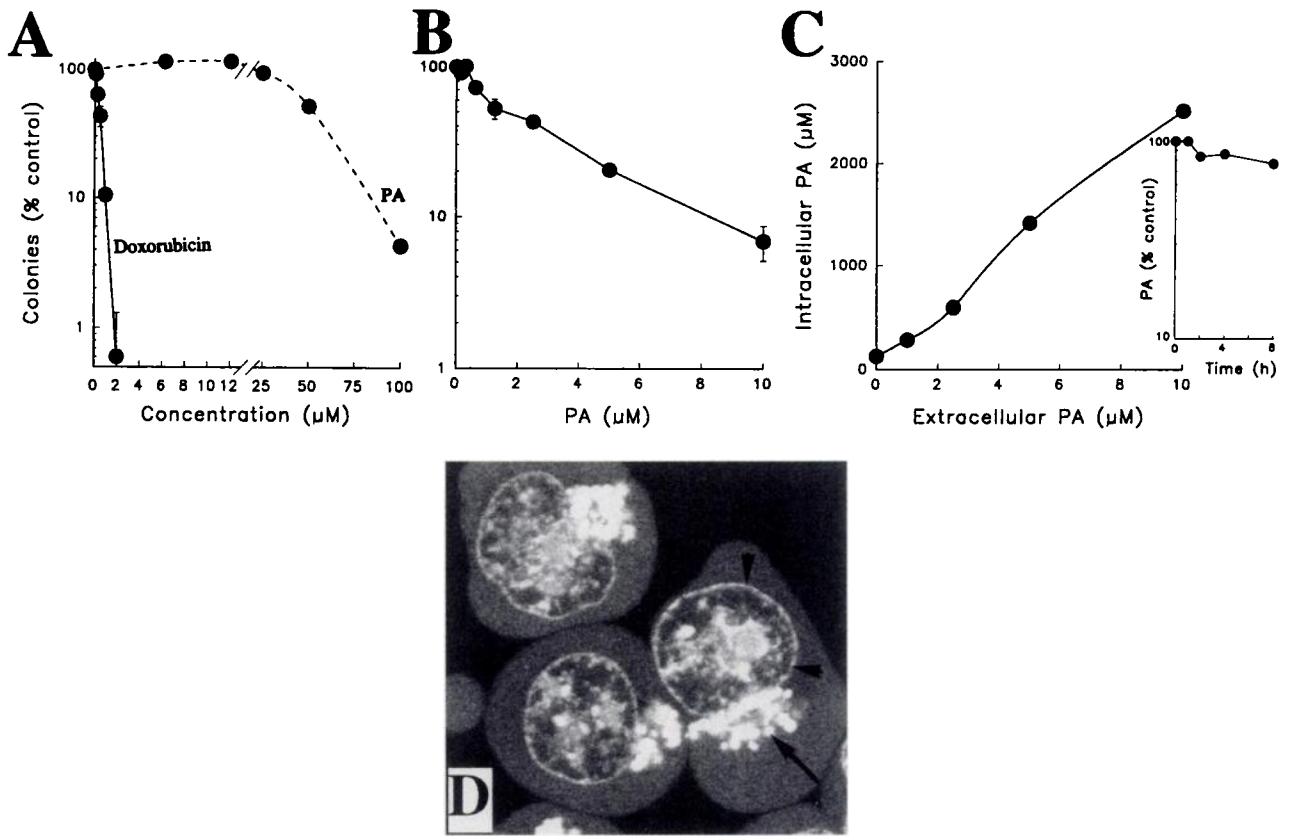


*Fig. 3* Assessment of PA cytotoxicity and accumulation in K562 cells. In *A*, K562 cells were treated for 1 h with the indicated concentration of PA or doxorubicin, then washed and plated in drug-free agar to determine clonogenic survival. In *B*, K562 cells were treated for 24 h with the indicated concentration of PA, then washed and plated in drug-free agar to determine clonogenic survival. *Bars*, SD. In *C*, K562 cells were incubated with the indicated concentration of PA, then washed and extracted so that average cellular PA concentrations could be determined as described in "Materials and Methods." *Inset*, retention of PA in K562 cells after removal of PA from the extracellular medium. *D*, subcellular distribution of PA as assessed by confocal microscopy. Results are representative of three experiments.

investigated. In assays performed under conditions where a 50% decrease in restriction enzyme activity would be readily detectable, concentrations of PA up to 50 μM had little effect on *Sfi*I and no effect on *Eco*RI or *Not*I. Likewise, concentrations of PA up to 10 μM had no effect on DNA ligase (data not shown). In short, PA inhibited topo I and topo II at concentrations below those that affected other DNA-targeting enzymes.

**PA Does Not Stabilize Covalent Topoisomerase-DNA Complexes.** Additional experiments were performed to determine whether the inhibition of topoisomerase catalytic activity results from stabilization of covalent topoisomerase-DNA complexes. To examine the ability of PA to stabilize topo I-DNA covalent complexes, supercoiled plasmid was incubated with topo I in the absence and presence of PA, treated with SDS and proteinase K, and subjected to electrophoresis under conditions that separate nicked and supercoiled plasmid (Fig. 2*C*). The topo I poison TPT markedly increased the amount of nicked plasmid detected in this assay (Fig. 2*C*, compare *Lanes 4* and *5*), reflecting interruption of the phosphodiester backbone when covalent topo I-DNA complexes are formed. In contrast, PA failed to increase the amount of nicked plasmid (Fig. 2*C*, *Lane 3*).[8] Identical results were obtained using 10 PA concentrations between 0.5 and 500 μM.

A similar strategy was used to examine the ability of PA to stabilize topo II-DNA complexes (Fig. 2*D*). When supercoiled plasmid was incubated with purified topo II in the absence and presence of drug, treated with SDS and proteinase K, and subjected to electrophoresis, etoposide markedly increased the amounts of nicked and linear plasmid (Fig. 2*D*, *Lane 3*), reflecting the stabilization of covalent topo II-DNA complexes at one or both active sites of the topo II dimer, respectively. In contrast, PA failed to increase the nicked and linear species (Fig. 2*D*, *Lane 2*). Identical results were again obtained using 10 PA concentrations between 0.5 and 500 μM. Collectively, the results

[8] The altered migration of the supercoiled DNA in *Lane 3* reflects the topo I-mediated relaxation of DNA in the presence of the intercalating agent PA. Similar results have been described previously with other intercalating agents (38).

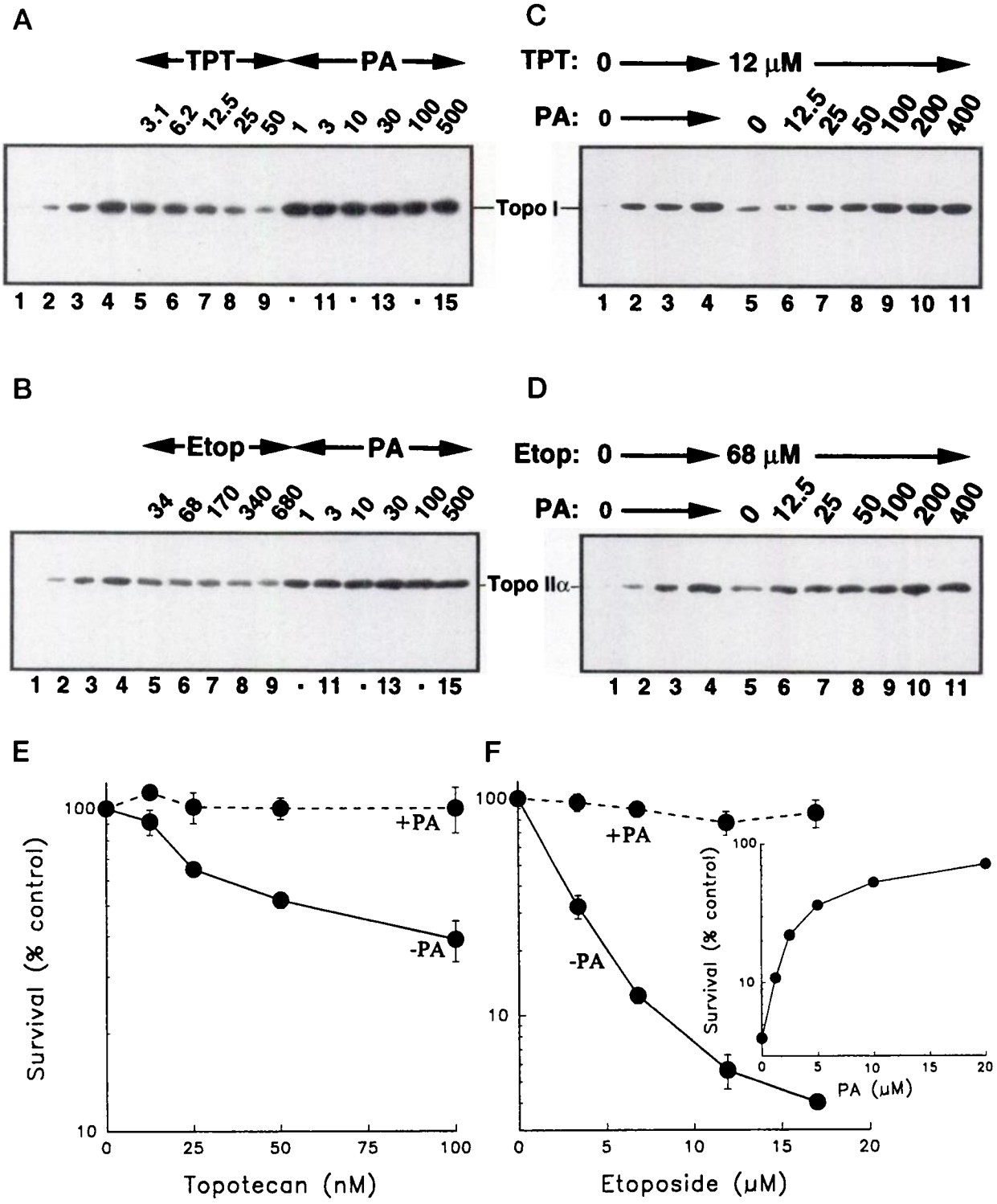


*Fig. 4* Effect of PA on cleavable complex stabilization and cytotoxicity of etoposide and TPT. *A*, effect of PA on topo I-DNA complexes in intact K562 cells. Cells were treated with 0.5% DMSO (*Lanes 1–4*), 3.1–50 μM TPT (*Lanes 5–9*), or 1–500 μM PA (*Lanes 10–15*) for 45 min, then lysed under denaturing conditions and prepared for SDS-PAGE. Gels were loaded with polypeptides from $0.3 \times 10^5$ cells (*Lane 1*) $0.75 \times 10^5$ (*Lane 2*), $1.5 \times 10^5$ (*Lane 3*), or $3.0 \times 10^5$ cells (*Lanes 4–15*). After electrophoresis, samples were transferred to nitrocellulose and probed with anti-topo I. *B*, band depletion assay for topo II. Cells were treated with 1% DMSO (*Lanes 1–4*), 34–680 μM etoposide (*Lanes 5–9*), or 1–500 μM PA (*Lanes 10–15*) for 45 min, then lysed under denaturing conditions and prepared for SDS-PAGE. Gels were loaded with polypeptides from $0.3 \times 10^5$ cells (*Lane 1*), $0.75 \times 10^5$ cells (*Lane 2*), $1.5 \times 10^5$ cells (*Lane 3*), or $3.0 \times 10^5$ cells (*Lanes 4–15*). After electrophoresis, samples were transferred to nitrocellulose and probed with an antiserum that recognizes topo IIα. Similar results (band disappearance with etoposide but not PA) were obtained when blots were probed with an antiserum that recognizes topo IIα and topo IIβ. *C*, effect of PA on TPT-induced stabilization of topo I-DNA covalent

presented in Fig. 2, *C* and *D*, indicate that PA does not stabilize covalent topoisomerase-DNA complexes.

Instead, PA actually inhibited the stabilization of covalent topoisomerase-DNA complexes by other agents. For example, when nuclear extracts were incubated with supercoiled plasmid and TPT in the presence of increasing PA concentrations, PA treatment resulted in a dose-dependent decrease in the amount of TPT-stabilized nicks (Figs. 2*E*, *Lanes 4–14*). Likewise, when purified topo II was incubated with supercoiled plasmid and etoposide in the presence of increasing PA concentrations, PA treatment resulted in a dose-dependent decrease in etoposide-induced formation of nicked and linear species (Fig. 2*F*, *Lanes 4–14*). These observations again indicate that the effects of PA are different from those of the topoisomerase poisons presently licensed for clinical use.

**Effect of PA on K562 Human Leukemia Cells.** Subsequent experiments were designed to address the following questions in intact cells: (*a*) At extracellular concentrations of PA that result in cytotoxicity, are intracellular PA concentrations in the range that inhibit topoisomerases *in vitro*? (*b*) Does PA affect stabilization of topoisomerase-DNA complexes *in situ*? To address these issues, the accumulation and cytotoxicity of PA were examined in K562 human leukemia cells. This cell line was chosen because of its high cloning efficiency in soft agar (~60%) and its ease of manipulation for subsequent band depletion assays.

When K562 cells were incubated with PA for 1 h and then plated in soft agar, an $IC_{50}$ of ~50 μM was observed (Fig. 3*A*). In contrast, when cells were incubated for 24 h with PA, the $IC_{50}$ was 1.25 μM (Fig. 3*B*). This latter value is close to the 1 μM PA serum concentration that is sustained in patients for >24 h after i.v. administration of this agent (8). These observations provide the range of concentrations that were explored in further biochemical experiments.

To determine the intracellular PA levels that accompany these extracellular concentrations, K562 cells were incubated for 1 h with 1–10 μM extracellular PA, extensively washed, and extracted so that cellular PA content could be determined by HPLC. Results of these experiments (Fig. 3*C*) indicated that PA was concentrated approximately 250-fold by K562 cells. After removal of extracellular PA from the medium, the half-time for loss of PA from cells exceeded 8 h (Fig. 3*C*, *inset*), indicating that PA was stably bound within cells. Because PA is intrinsically fluorescent, the subcellular distribution of the cell-associated drug could be determined by confocal microscopy. These experiments indicated that PA was distributed throughout the cell but was concentrated in perinuclear vesicles and in nuclei (Fig. 3*D*). Estimates derived from these confocal images suggested that 23 ± 5% of the total cellular PA was detectable in nuclei, which occupied 27 ± 5% of the intracellular volume. Based on these observations, an extracellular PA concentration of 1 μM would be expected to result in an average intranuclear PA concentration of ~200 μM. Collectively, the experiments in Fig. 3 indicate that cytotoxic extracellular concentrations of PA result in intranuclear PA concentrations that are well within the range shown to inhibit topo I and topo II under cell-free conditions.

**PA Alters the Stabilization of Covalent Topoisomerase-DNA Complexes in Intact Cells.** To determine whether PA was capable of interacting with topo I and topo II in intact cells, topoisomerase band depletion assays (27) and colony-forming assays were performed after treatment of cells with TPT or etoposide in the absence and presence of PA.

Treatment of intact cells with TPT results in a dose-dependent decrease in the topo I signal at $M_r$ ~100,000 (Fig. 4*A*, *Lanes 5–9*). This loss of signal at $M_r$ ~100,000, which is specific for topo I and is reversed by brief heating to 48°C (30), reflects the altered mobility of increasing numbers of topo I molecules as they become covalently bound to DNA. Likewise, treatment with etoposide results in a dose-dependent decrease in the signal for topo IIα at $M_r$ ~170,000 (Fig. 4*B*, *Lanes 5–9*), reflecting the altered mobility of topo IIα molecules as they become covalently bound to DNA. In contrast to TPT and etoposide, PA at a wide range of concentrations failed to cause detectable decreases in the signals for topo I or topo II (Fig. 4, *A* and *B*, *Lanes 10–15*). Coupled with previous alkaline elution results (10, 13), these observations suggest that PA fails to stabilize covalent topoisomerase-DNA complexes in intact cells.

Instead, PA inhibited the ability of TPT and etoposide to stabilize covalent topoisomerase-DNA complexes in intact cells just as it had under cell-free conditions. Band depletion assays revealed that the TPT-induced stabilization of covalent topo I-DNA complexes was diminished in a dose-dependent fashion by the presence of PA (Fig. 4*C*, *Lanes 5–11*). Likewise, the etoposide-induced stabilization of covalent topo II-DNA adducts was diminished in a dose-dependent fashion in the present of PA (Fig. 4*D*, *Lanes 5–11*). Consistent with these observations, PA also diminished the cytotoxicity of TPT (Fig. 4*E*) and etoposide (Fig. 4*F*) in these cells. This inhibitory effect was observed at a PA concentration as low as 1.25 μM (Fig. 4*F*, *inset*), a concentration that is readily surpassed in patients receiving PA (8). Collectively, the results in Fig. 4, *C–F*, indicate that PA is capable of altering the action of topo I and topo II in intact mammalian cells in a manner similar to its effect on topoisomerases under cell-free conditions.

## DISCUSSION

Despite the promising preclinical activity displayed by the synthetic acridine derivative PA (see "Introduction"), the mech-

complexes. K562 cells were treated with 12 μM TPT in the presence of 0–400 μM PA (*Lanes 5–11*) for 45 min, then lysed under denaturing conditions and prepared for SDS-PAGE followed by immunoblotting with anti-topo I. *Lanes 4–11* were loaded with polypeptides from $3.0 \times 10^5$ cells. *Lanes 1–3* contained a serial dilution of diluent-treated cells as in *A*. *D*, effect of PA on etoposide-induced stabilization of topo II-DNA covalent complexes. K562 cells were treated with 68 μM etoposide in the presence of 0–400 μM PA (*Lanes 5–11*) for 45 min, then lysed under denaturing conditions and analyzed as in *C*. The blot was probed with anti-topo IIα. *E* and *F*, clonogenic assay comparing effect of a 1-h treatment with TPT (*E*) or etoposide (*F*) in K562 cells that had been preincubated for 15 min in the absence (—) or presence (---) of 25 μM PA. Note that PA abolishes the cytotoxicity of etoposide and TPT without having any toxicity under these conditions. *Inset*, clonogenic assay comparing survival when cells were preincubated for 15 min in the presence of the indicated concentration of PA before 17 μM etoposide was added for an additional 60 min. *Bars*, SD.

anism of cytotoxicity of this agent has remained uncertain. In the present work, we have demonstrated that: (*a*) PA inhibits both topo I and topo II; (*b*) this inhibition occurs without stabilization of topoisomerase-DNA complexes; and (*c*) the effect of PA on topoisomerases results in abrogation of the effects of the topo I poison TPT or the topo II poison etoposide *in vitro* and in intact cells. These observations provide the broad outline of a potential cytotoxic mechanism for this compound.

The effect of PA on topo I and topo II appears to be different from that of topoisomerase-directed drugs presently in clinical use. The studies performed in yeast strains containing altered topoisomerases (Fig. 1) rule out the possibility that PA has the same cytotoxic mechanism as amsacrine or camptothecin. These yeast studies could not, however, rule out the possibility that PA might stabilize covalent topo I-DNA complexes and topo II-DNA complexes simultaneously. Previous reports have identified agents that stabilize both types of complexes, including saintopin (19), intoplicine (20), the indoloquinolinediones (21), and possibly actinomycin D (14, 16). In contrast to these agents, PA does not appear to stabilize topoisomerase-DNA complexes *in vitro* (Fig. 2) or in intact cells (Fig. 4; Refs. 10 and 13). Instead, PA inhibits the catalytic activity of topo I (Fig. 2*A*) and topo II (Fig. 2*B*) by a process that does not involve stabilization of cleavage complexes. Although other agents that inhibit topoisomerase catalytic activity without stabilizing covalent enzyme-DNA intermediates have been described, including the topo II-targeted agents merbarone (31) and ICRF 193 (32) as well as the topo I-directed agent β-lapachone (Ref. 33; for alternative view, see Ref. 34), PA differs from these agents as well. The ability to inhibit both classes of topoisomerases *in vitro* without stabilizing covalent topoisomerase-DNA complexes appears to distinguish PA from all of the previously cited agents.

Several observations suggest that the catalytic inhibition of topo I and topo II might also contribute to the cytotoxicity of PA in intact cells. First, the ability of PA to decrease TPT- and etoposide-stabilized topoisomerase-DNA complexes in intact cells (Fig. 4, *C* and *D*) indicates that PA is capable of affecting these enzymes *in situ*. Second, the time course for PA-induced toxicity is also consistent with a mechanism that does not involve stabilization of covalent topoisomerase-DNA complexes. In contrast to drugs such as TPT (Fig. 4*E*), etoposide (Fig. 4*F*), and doxorubicin (Fig. 3*A*), PA is relatively nontoxic when incubated with cells for 1 h (Fig. 3*A*). This relative lack of toxicity of a 1-h exposure is not explained by poor PA accumulation. Studies of drug accumulation and subcellular distribution (Fig. 3, *C* and *D*) indicate that PA is rapidly concentrated in nuclei to levels that readily inhibit topo I and topo II *in vitro* (Fig. 2, *A* and *B*) and affect these enzymes *in situ* (Fig. 4, *C* and *D*). Based on these results, it appears that K562 cells are able to survive for some period of time with diminished topoisomerase activity, although prolonged inhibition of these enzymes is obviously associated with toxicity (Fig. 3*B*), just as it is in lower eukaryotes such as yeast (35).

The results obtained with PA are to be compared and contrasted with those obtained with doxorubicin. These two agents not only bind to DNA with similar affinity (13) but also inhibit topo I and topo II catalytic activity *in vitro* with similar potencies (Fig. 2, *A* and *B*). These observations raise the possibility that PA, like doxorubicin, might be inhibiting topo I and topo II as a result of intercalation into DNA (15). The relatively large footprints of topo I and topo II enzymes on DNA (20 and 28 nucleotides, respectively; Refs. 36 and 37) might make these enzymes particularly sensitive to the effects of intercalating agents (38, 39). Nonetheless, the cytotoxicity of doxorubicin clearly differs from that of PA (Fig. 3*A*). Doxorubicin has been shown to have other effects that contribute to its cytotoxicity, including stabilization of topo II-DNA complexes (16), formation of free radicals (40), and possibly effects at the level of the plasma membrane (41). These other mechanisms presumably contribute to the extensive cell killing that is observed upon brief exposure to submicromolar concentrations of doxorubicin (Fig. 3*A*). In contrast, the paucity of cytotoxicity observed after a 1-h exposure to low micromolar concentrations of PA (Fig. 3*A*) is consistent with the view that PA is not acting through these other, relatively rapid mechanisms of inducing lethal damage.[9]

It is noteworthy that the 1–10 μM extracellular PA concentrations used in most of the present experiments are within the range of concentrations observed in patients receiving PA (8). Although we cannot rule out the possibility that PA might also inhibit other enzymes involved in DNA metabolism, the present observations raise the possibility that inhibition of topo I and topo II activity without stabilization of cleavable complexes might be highly pertinent to the cytotoxic actions of prolonged PA exposure *in vitro* and *in vivo*.

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[9] As indicated in Fig. 3*A*, extremely high concentrations of PA (>25 μM) are associated with cytotoxicity, even with exposures as short as 1 h. The present studies have not addressed the mechanism of this more acute toxicity and cannot rule out the possibility that PA acts through a different target at these very high concentrations.

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