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AURINTRICARBOXYLIC ACID, A PUTATIVE INHIBITOR OF APOPTOSIS, IS A POTENT INHIBITOR OF DNA TOPOISOMERASE II *IN VITRO* AND IN CHINESE HAMSTER FIBROSARCOMA CELLS

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Abstract—Aurintricarboxylic acid (ATA) is a polyanionic, polyaromatic compound which has been shown to inhibit apoptotic cell death in various cell types induced by a variety of factors. Since ATA is known to be a general inhibitor of nuclease activities *in vitro* (ID_{50}s ranging from 2 to 50 μM), the *in vivo* effects are usually attributed to inhibition of endogenous endonuclease activities. We show herein that ATA is a potent inhibitor of the nuclear enzyme DNA topoisomerase II. ATA inhibits the catalytic activity of purified yeast topoisomerase II with an ID_{50} of approx. 75 nM as measured by relaxation assays. ATA does not stabilize the covalent DNA–topoisomerase II reaction intermediate ("cleavable complex") as do other inhibitors of this enzyme such as 4′-(9-acridinylamino)-methane sulfon-*m*-anisidide (amsacrine), 4′-demethyl-epipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside) (etoposide) and ellipticines. In contrast, cleavable complex formation induced by amsacrine and etoposide is strongly inhibited in the presence of ATA. ATA also prevents the binding of topoisomerase II to DNA and inhibits topoisomerase II-catalysed ATP hydrolysis. The ability of ATA to interfere with more than one step in the catalytic cycle of DNA topoisomerase II may explain its unusual potency as an inhibitor of this enzyme. ATA reduces the number of amsacrine-induced DNA–protein complexes in intact DC-3F Chinese hamster fibrosarcoma cells and protects these cells from the cytotoxic action of amsacrine. The effects of ATA on DNA–protein complex formation in living cells appear to be due to the direct interaction of the drug with topoisomerase II, since similar results are found when nuclei from untreated DC-3F cells are exposed to amsacrine after a short preincubation with ATA. Cells resistant to 9-hydroxyellipticine, which have been shown to possess altered topoisomerase II activity, are approx. 5-fold more resistant to ATA than the sensitive parental cells as shown by colony formation assays. We conclude that ATA is a potent inhibitor of topoisomerase II and that the drug interacts with topoisomerase II in living cells. Our findings raise the possibility that the protective effects of ATA towards apoptotic cell death might, at least in part, involve DNA topoisomerase II.

Key words: aurintricarboxylic acid; DC-3F Chinese hamster fibrosarcoma cells; DNA topoisomerase II; apoptosis; cytotoxicity

ATA†, (Fig. 1) is a polyanionic, polyaromatic compound derived from aurin, a triphenylmethane dye. ATA has been shown to inhibit the initiation of protein synthesis by preventing the binding of mRNA to the ribosomes [1]. Subsequent studies showed that ATA also inhibits many other nucleic acid-binding proteins including RNA polymerase, replicase, exonuclease III, DNAse I, RNAse A, S1 nuclease and various restriction nucleases at ID_{50}s ranging from 2 to 50 μM [2, 3]. Since ATA is a general inhibitor of nuclease activities, it has been proposed that it may serve as a useful nuclease inhibitor during isolation of cellular nucleic acids [3]. However, the effects of ATA are not limited to nucleic acid-binding proteins, since other enzymes such as glucose-6-phosphate dehydrogenase are also inhibited within the same dose range [4]. Furthermore, ATA does not inhibit all nucleic acid-binding proteins, since at least one, seryl-tRNA synthetase, shows full activity in the presence of high concentrations of ATA [4].

More recently, ATA has been shown to inhibit apoptotic cell death in various cell types induced by a variety of factors including glucocorticoid treatment of thymocytes [5], antigen stimulation of T cell hybridomas [6], removal of trophic factor in sympathetic neurons [7] and treatment of leukemia cells with antitumor agents [8]. The protective effects of ATA are usually attributed to its ability to inhibit the endogenous endonuclease activity involved in fragmentation of DNA into 180 bp oligonucleosome integer fragments (a DNA "ladder") [5, 6, 8, 9]. However, ATA has also been reported to protect cell types where apoptosis is not accompanied by a significant degree of DNA fragmentation [7], suggesting that other enzymes could be involved in the protective effects of ATA.

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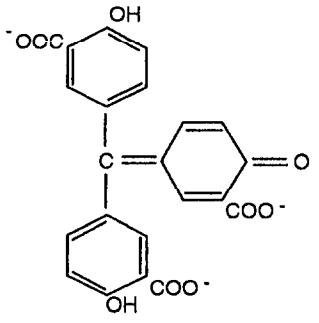
† Abbreviations: ATA, aurintricarboxylic acid; amsacrine, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; 9-OHE, 9-hydroxyellipticine; etoposide, 4′-demethyl-epipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside).



Aurintricarboxylic acid

Fig. 1. Structure of ATA.

In this study, we demonstrate that ATA is a potent inhibitor of DNA topoisomerase II *in vitro* where it inhibits two different steps in the catalytic cycle. We also show that ATA reduces the number of amsacrine-induced protein-associated DNA strand breaks in living DC-3F Chinese hamster fibrosarcoma cells and protects these cells from the cytotoxic action of amsacrine. The effects of ATA on DNA–protein complex formation in living cells appear to be due to direct interaction of the drug with topoisomerase II since similar results are found when nuclei from untreated DC-3F cells are exposed to amsacrine after a short preincubation with ATA.

MATERIALS AND METHODS

Drugs and chemicals. ATA was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Amsacrine and etoposide (VP-16–213) were gifts from Jerzy Konopa (Technical University of Gdansk, Gdansk, Poland) and WT Bradner (Bristol-Myers Squibb, Syracuse, NY, U.S.A.), respectively. [^{14}C]-amsacrine (>99% pure; 20.8 mCi/mmol; 1 mCi = 37 MBq) was a gift from the Drug Synthesis and Chemistry Branch (National Cancer Institute, Bethesda, MD, U.S.A.). All other chemicals were of reagent grade.

DNA substrates. Supercoiled plasmid pBR322 DNA (>95% form I) was purchased from Boehringer Mannheim (Germany). Highly catenated kinetoplast DNA was purified from *Trypanosoma cruzi* (kindly provided by Guy Riou, Villejuif, France) after DNA extraction and sucrose sedimentation [10].

Enzymes. DNA topoisomerase II was obtained from *Saccharomyces cerevisiae* as described [11, 12]. Briefly, DNA topoisomerase II was overexpressed in yeast from a multicopy expression plasmid kindly provided by James Wang (Harvard University, Boston, MA, U.S.A.) and purified by a four-step procedure consisting of yeast disruption, elution from a polyethyleneimine/celite column, ammonium sulfate precipitation and phosphocellulose chromatography [11, 12]. The purified enzyme preparation contained no detectable DNA topoisomerase I activity.

Relaxation assay. The reaction mixture contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 165 mM KCl, 1 mM ATP and 150 ng of pBR322 DNA. The reaction was initiated by the addition of DNA topoisomerase II and allowed to proceed at 30° for 10 min. Reactions were terminated by addition of a loading buffer containing SDS, bromophenol blue, and sucrose (1%, 0.05% and 10% final concentrations, respectively). The samples were electrophoresed in 1% agarose gels at 2 V/cm for 18 hr in Tris/borate/EDTA buffer at pH 8. Photographic negatives of ethidium bromide-stained agarose gels were scanned with a Joyce-Loebl Chromoscan 3 densitometer and the peak areas of supercoiled DNA determined. All topoisomerase II assays were done at least twice with two different enzyme preparations.

Decatenation assay. Reaction conditions were as described above except that 150 ng of kinetoplast DNA was used as a substrate instead of pBR322, and the incubation was for 15 min. Electrophoresis was in 1.2% agarose gels at 5 V/cm for 4 hr. Liberated minicircles were quantified by densitometric scanning of photographic negatives.

Binding of topoisomerase II to DNA. Assays were carried out by an electrophoretic mobility shift procedure [13]. The reaction mixture was as described for the relaxation assay except that 50-fold more DNA topoisomerase II was used and the assay buffer contained no ATP. Binding occurred over 10 min at 30° and was stopped by the addition of loading buffer. Enzyme-bound DNA was separated from free substrate by agarose gel electrophoresis [13].

Formation of cleavable complex. The experimental conditions were the same as for the relaxation except that approx. 50-fold more DNA topoisomerase II was used. For the assays containing both ATA and amsacrine or etoposide, pBR322 DNA was added first, followed by amsacrine/etoposide and then ATA; the reaction was initiated with the addition of enzyme. After 20 min at 30° the reactions were terminated by addition of 1% SDS and 0.1% proteinase K followed by incubation at 50° for 30 min. Electrophoresis in 1% agarose gels containing ethidium bromide (0.5 μg/mL) was carried out at 2 V/cm for 18 hr in Tris/borate/EDTA buffer (pH 8) with ethidium bromide (0.5 μg/mL).

Hydrolysis of ATP by topoisomerase II. Topoisomerase II-catalysed ATPase assay was carried out according to Ref. 13. The reaction mixture was as described for the relaxation assay except that 50-fold more DNA topoisomerase II was used and the assay mixture contained 0.5 mM γ-[^{32}P]ATP [3000 Ci/mmol] and 6 μg of pBR322. Samples were incubated at 30° for 10 min and immediately analysed by thin-layer chromatography [13]. Radioactive areas corresponding to inorganic phosphate released by ATP hydrolysis and areas corresponding to remaining ATP were cut out of the chromatograms and quantified by liquid scintillation counting.

Cells and culture medium. The Chinese hamster fibrosarcoma cell line DC-3F and the 9-hyd-

roxyellipticine resistant subline DC-3F/9-OHE have been described [14] as have the media and growth conditions [14]. To exclude a possible involvement of the P-glycoprotein, which might result in a reduced uptake of ATA, DC-3F/9-OHE-U a subline which does not express the MDR-phenotype was used [15].

Formation of covalent topoisomerase II–DNA complexes in intact DC-3F cells and isolated nuclei. This assay was carried out essentially as described previously [16]. Briefly, cellular DNA and protein were metabolically labeled by incubating approx. 2×10^5 DC-3F cells with both [^{14}C]leucine (0.2 μCi/mL) and [^{3}H]thymidine (0.6 μCi/mL) overnight. To study amsacrine-mediated cleavable complex formation, cells were treated with amsacrine at the indicated concentrations for 3 hr. To study the effect of ATA on amsacrine-mediated cleavable complex formation, cells were treated with 5 μM ATA for 18 hr before addition of amsacrine. After incubation with drugs, each sample was divided into 0.5 mL aliquots, and reactions were stopped by adding 0.5 mL of a stop solution containing 2.5% SDS, 10 mM EDTA (pH 8) and 0.8 mg/mL salmon sperm DNA. Cell lysates were passed 10 times through a 23-gauge needle and then heated at 65° for 15 min. KCl (250 mM final concentration) was added to each tube. The tubes were vortexed for 10 sec, put on ice for 5 min, and then centrifuged at 10,000 *g* for 5 min at 4°. Each pellet was washed three times with 1 mL of a solution containing 10 mM Tris–HCl (pH 8), 100 mM KCl, 1 mM EDTA, pH 8 and 1 mg/mL salmon sperm DNA. The pellets were then dissolved in 0.5 mL water at 65° for 15 min, and 0.5 mL was transferred to a vial for scintillation counting. Results are expressed as the ratio of [^{3}H]DNA/[^{14}C]protein, with the cpm of protein precipitated as the internal control for all samples.

In another series of experiments, DC-3F cells were exposed to [^{14}C]leucine (0.2 μCi/mL) and [^{3}H]-thymidine (0.6 μCi/mL) for 18 hours to label the DNA and proteins. Nuclei were isolated [17], preincubated with 1 μM ATA for 10 min and exposed to various concentrations of amsacrine for 20 min at 37°. The drug-induced DNA–protein complex formation was determined as described for whole cells. All experiments, either with whole cells or isolated nuclei, were done at least three times with each point in triplicate.

Drug accumulation assay. Exponentially growing DC-3F cells were exposed to [^{14}C]amsacrine (final specific activity 20.8 mCi/mmol). After 0.5, 1 or 3 hr incubation the cells were washed twice with ice-cold 0.154 M NaCl and detached with trypsin. Cell suspensions were harvested and counted in 15 mL of scintillation fluid. The experiment was done twice with each point in triplicate.

Cytotoxicity. All experiments were carried out with exponentially growing cells. Briefly, cells were exposed to various drug concentrations for 3 hr at 37°. Following drug treatment, the cells were washed with ice-cold 0.9% NaCl solution and 200–300 cells were plated in triplicate in 60 mm diameter Petri dishes. Alternatively, cell survival was measured by colony formation in the continuous presence of drug-containing media. Each experiment was done at least twice with each point in triplicate.

RESULTS

ATA inhibits the catalytic activity of DNA topoisomerase II

The effect of ATA on the catalytic activity of DNA topoisomerase II was measured by relaxation of supercoiled plasmid DNA as shown in Fig. 2. The relaxation is inhibited by ATA starting at 50 nM (lane 5). At 100 nM (lane 7) the reaction is totally inhibited. In comparison, under similar experimental conditions, 20 μM suramin (lane 12) or 50 μM amsacrine is required for total inhibition of the catalytic activity of DNA topoisomerase II (results not shown). In the absence of topoisomerase II, even higher concentrations of ATA had no effect on the migration of DNA (lane 2), indicating weak, if any, interactions between DNA and ATA. This is consistent with two previous studies which report that ATA inhibits nucleic acid-binding enzymes by binding to the protein, but not to the nucleic acid [2, 18]. The effect of ATA on the catalytic activity of DNA topoisomerase II was also measured by decatenation as shown in Fig. 3. In the absence of drug, decatenated minicircles (mc) are formed when kinetoplast DNA (cat) is incubated with topoisomerase II. The decatenation reaction is inhibited by ATA starting at 100 nM (lane 8). At 200 nM (lane 10) the reaction is totally inhibited. Therefore, as determined by two independent assay systems, ATA is a potent inhibitor of the catalytic strand passage activity of topoisomerase II from *Saccharomyces cerevisiae*.

Effect of ATA on the catalytic cycle of DNA topoisomerase II

In order to characterize the mechanism by which ATA inhibits the overall catalytic activity of topoisomerase II, the effect of ATA on the individual reactions that comprise the catalytic cycle of the enzyme was determined. The catalytic cycle of topoisomerase II can be broken into the following steps [19, 20]: binding of topoisomerase II to its DNA substrate, the formation of double-stranded protein-associated DNA strand breaks which permit double-stranded DNA passage; closure of protein-associated DNA breaks; and ATP hydrolysis and enzyme turnover (i.e. reinitiation of a new round of catalysis).

The effect of ATA on topoisomerase II-DNA binding

The effect of ATA on the affinity of topoisomerase II for negatively supercoiled pBR322 plasmid DNA was determined by an electrophoretic mobility shift assay [13]. In this assay, the binding of topoisomerase II to DNA retards its electrophoretic migration in an agarose gel (Fig. 4, compare lanes 1 and 2). The presence of ATA affects the affinity of the enzyme for its DNA substrate starting at 20 μM (lane 5). At 100 μM (lane 7) ATA totally prevents the binding of topoisomerase II to DNA.

Effect of ATA on cleavable complex formation

Topoisomerase II-mediated DNA strand passage requires breakage and rejoining of double stranded DNA. During this process, the enzyme becomes covalently linked to the 5′ phosphate of both DNA

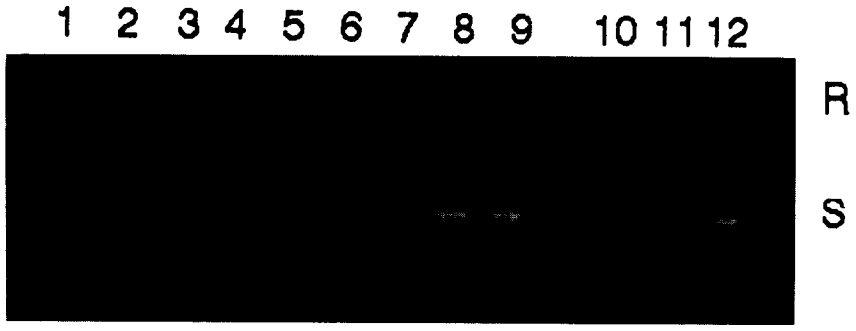


Fig. 2. Inhibition of the catalytic activity of purified yeast DNA topoisomerase II by ATA as measured by relaxation assay. Supercoiled pBR322 plasmid DNA (lane 1) was relaxed by topoisomerase II in the absence (lanes 3 and 10) or presence of 20, 50, 75, 100, 150 and 200 nM ATA (lanes 4–9) or 10 and 20 μM suramin (lanes 11 and 12). Plasmid pBR322 DNA was also incubated with 200 nM ATA in the absence of topoisomerase II (lane 2). S, supercoiled; R, relaxed. Data shown are typical of three independent experiments.

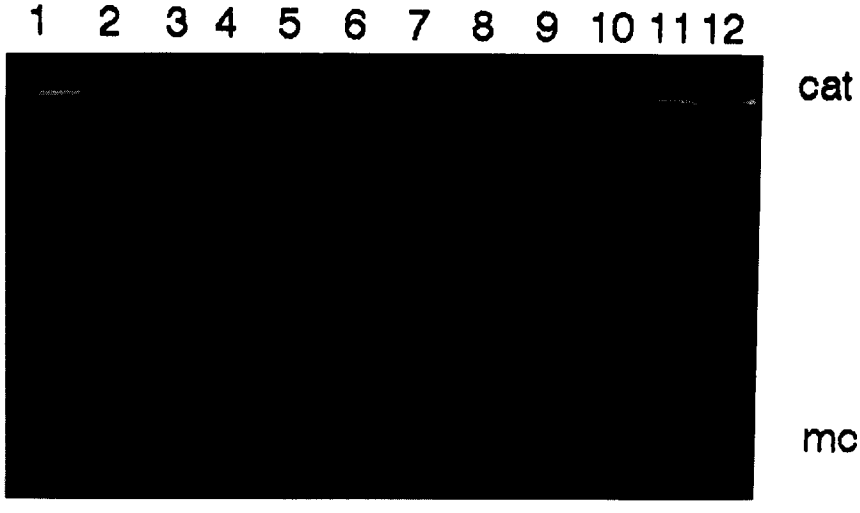


Fig. 3. Inhibition of the catalytic activity of purified yeast DNA topoisomerase II by ATA as measured by decatenation. Kinetoplast DNA (lane 1) was decatenated by topoisomerase II in the absence (lane 2) or presence of 5, 10, 20, 50, 75, 100, 150, 200, 250 and 500 nM ATA (lanes 3–12). cat, catenated kinetoplast DNA; mc, decatenated minicircles. Data shown are typical of two independent experiments.

strands via a tyrosine–DNA phosphodiester linkage [21, 22]. The covalent reaction intermediate is called the cleavable complex and can be demonstrated experimentally by the enzyme-dependent formation of linear DNA from supercoiled DNA after treatment with SDS and proteinase K [23]. Clinically used DNA topoisomerase II inhibitors such as amsacrine and etoposide act by stabilizing the cleavable complex [24–26]. ATA at concentrations up to 50 μM does not induce DNA cleavage (Fig. 5, lanes 4–6). In contrast, 20 μM ATA completely inhibits endogenous cleavable complex formation (compare lane 5 with lane 3). It should be noted that the disappearance of endogenous cleavable complexes ("L", lanes 3–6) closely follows the inhibition of relaxation (the change in migration of the supercoiled "S" DNA). The apparent discrepancy between the dose of ATA needed to inhibit relaxation in this assay ($\approx$5–20 μM) and that observed for the relaxation assay ($\approx$100 nM) is due to the different amounts of enzyme employed in the two assays. Since relaxation is a catalytic event where one topoisomerase II molecule can relax many DNA molecules whereas cleavable complex formation is a stoichiometric reaction where the formation of linear DNA molecules is a result of the binding of one topoisomerase II molecule to one DNA molecule, more enzyme (here $\approx$50 fold) is needed for the cleavable complex assay than for the relaxation assay.

We also examined whether ATA is able to inhibit amsacrine or etoposide-induced cleavable complex formation. As little as 5 μM ATA has an effect on amsacrine-induced DNA cleavage (compare lanes 7

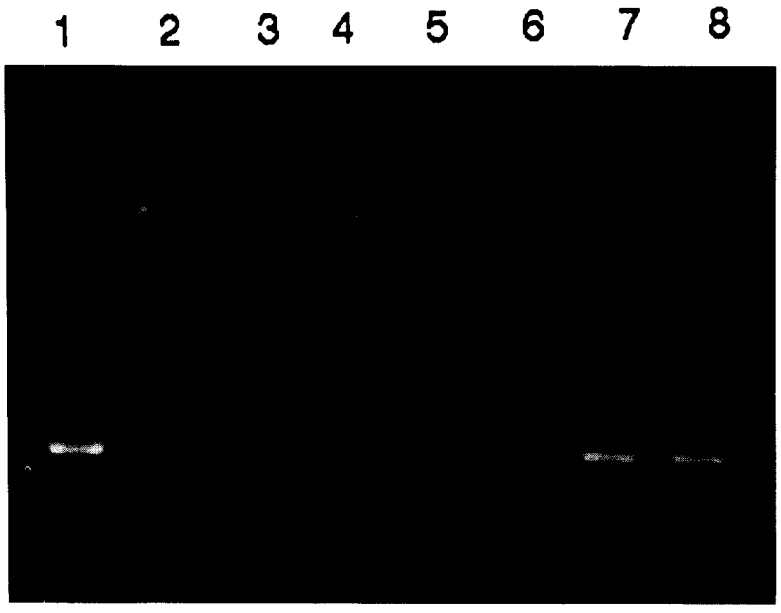


Fig. 4. Inhibition of the binding of purified yeast DNA topoisomerase II to supercoiled pBR322 plasmid DNA by an electrophoretic mobility shift assay. Supercoiled pBR322 plasmid DNA (lane 1) was incubated with purified topoisomerase II in the absence (lane 2) or presence of 1, 10, 20, 50, 100 and 200 μM ATA (lanes 3–8). Data shown are typical of two independent experiments.

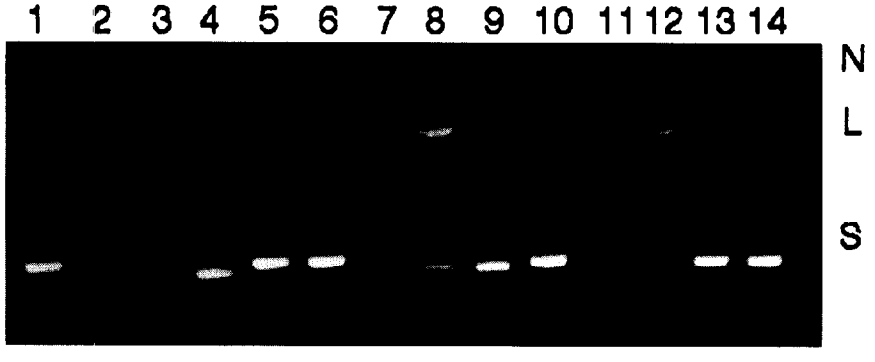


Fig. 5. Effect of ATA on DNA cleavage mediated by purified yeast DNA topoisomerase II. Topoisomerase II was incubated with supercoiled pBR322 plasmid DNA in the presence of ATA, amsacrine or etoposide. Lane 1, supercoiled pBR322 plasmid DNA; lane 2, linear pBR322 plasmid DNA; lanes 3–6, 0, 5, 20 and 50 μM ATA; lanes 7–10, 25 μM amsacrine with 0, 5, 20 and 50 μM ATA; lanes 11–14, 25 μM etoposide with 0, 5, 20 and 50 μM ATA. S, supercoiled; N, nicked; L, linear. Data shown are typical of two independent experiments.

and 8) and at 20 μM (lane 9) ATA totally inhibits the DNA cleavage induced by 25 μM amsacrine. A similar effect of ATA is observed on etoposide-stabilized cleavable complexes since DNA cleavage induced by 25 μM etoposide is inhibited by ATA in a concentration-dependent manner (lanes 11–14).

The effect of ATA on ATP hydrolysis

The ATPase activity of DNA topoisomerase II is stimulated in the presence of negatively supercoiled DNA [27, 28]. Since the results presented above show that ATA prevents the binding of topoisomerase II to DNA, the ATPase assays were carried out both in the presence and absence of DNA. Figure 6 shows that ATA inhibits topoisomerase II-catalysed ATP hydrolysis with an ED_{50} of approx. 20 μM both in the presence and absence of DNA. Interestingly, the DNA-dependent enhancing effects of the enzyme's ATPase activity is maintained even at 50 μM ATA. The reason is that ATPase activity is more sensitive to ATA than is DNA binding. Therefore, at 50 μM ATA ATP hydrolysis is practically totally inhibited whereas some topoisomerase II molecules still bind to the DNA (Fig. 4).

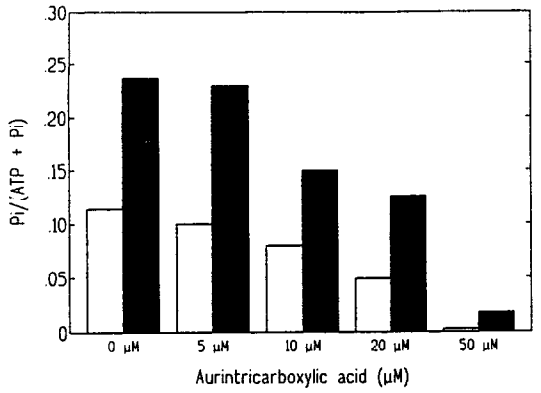


Fig. 6. Effect of ATA on topoisomerase II-catalysed ATP hydrolysis. The results compare the activity of topoisomerase II in the presence (■) and absence (□) of DNA. Data shown are typical of three independent experiments.

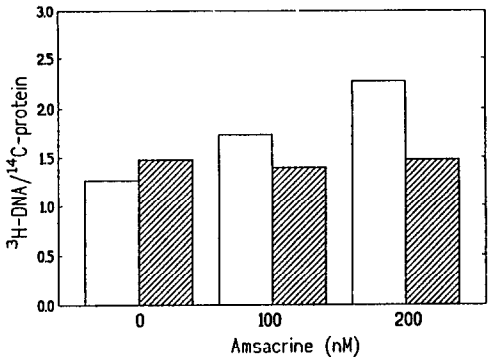


Fig. 8. Effect of ATA on amsacrine-stabilized DNA–protein complex formation in isolated nuclei from untreated DC-3F cells. ^{3}H/^{14}C-labeled nuclei were incubated in the absence (□) or presence (▨) of ATA (1 μM) for 10 min and then treated with amsacrine (100 or 200 nM) for 20 min. Data shown are typical of three independent experiments.

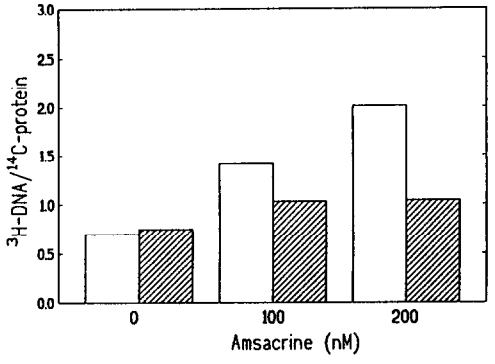


Fig. 7. Effect of ATA on amsacrine-stabilized DNA–protein complex formation in intact DC-3F Chinese hamster fibrosarcoma cells. ^{3}H/^{14}C-labeled cells were grown in the absence (□) or presence (▨) of ATA (5 μM) for 18 hr and then treated with amsacrine (100 or 200 nM) for 3 hr. Data shown are typical of four independent experiments.

ATA inhibits the formation of covalent DNA-topoisomerase II complexes in intact DC-3F Chinese hamster fibrosarcoma cells

The SDS–KCl precipitation assay was used to determine the occurrence of covalent DNA–topoisomerase II complexes in intact DC-3F cells treated with ATA for 18 hr at doses up to 5 μM. The 18 hr incubation period was chosen because previous experiments in our laboratory have shown that polyanionic, polyaromatic compounds need a long time to enter the cells [29]. No DNA–protein complex formation is induced even at 5 μM of ATA (results not shown). Since ATA strongly inhibits the cleavable complex formation induced by amsacrine *in vitro* (Fig. 5), the occurrence of cleavable complexes induced by amsacrine in the absence and presence of ATA was also studied (Fig. 7). ATA (5 μM) inhibits the amsacrine-induced formation of DNA–protein complex formation. At this dose ATA has no detectable effects on cell growth. The decrease in DNA–protein complexes is not due to a decrease in amsacrine uptake in ATA-treated cells since the drug uptake is similar for control and ATA-treated cells (results not shown).

ATA inhibits the formation of covalent DNA–topoisomerase II complexes in nuclei from DC-3F cells

To eliminate the possibility that the long (18 hr) preincubation period with ATA caused physiological changes which somehow might affect the formation of drug-induced DNA–protein complexes, nuclei from untreated DC-3F cells were exposed to amsacrine after only 10 min pretreatment with ATA (this is now possible because we have solved the problem whereby the drug had to penetrate the cytoplasmic membrane). The results (Fig. 8) are basically the same as for whole cells. Furthermore, the effects of ATA on living cells and isolated nuclei are analogous to what we observed *in vitro* with purified enzyme (Fig. 5).

ATA protects DC-3F cells from the cytotoxic action of amsacrine

The decrease in amsacrine-induced DNA–protein complexes in ATA-treated cells (Fig. 8) is accompanied by a decrease in the cytotoxic effect of amsacrine (Fig. 9). The ED_{50} of amsacrine is 28 nM for control DC-3F cells and 82 nM for ATA-treated DC-3F cells.

Colony formation

The effect of ATA on the survival of the Chinese hamster cell line DC-3F and the 9-OHE resistant subline DC-3F/9-OHE is shown in Fig. 10. DC-3F/9-OHE cells are cross-resistant to DNA topoisomerase II inhibitors such as amsacrine and etoposide and have been shown to have altered DNA topoisomerase II activity [14, 17, 30, 31]. DC-

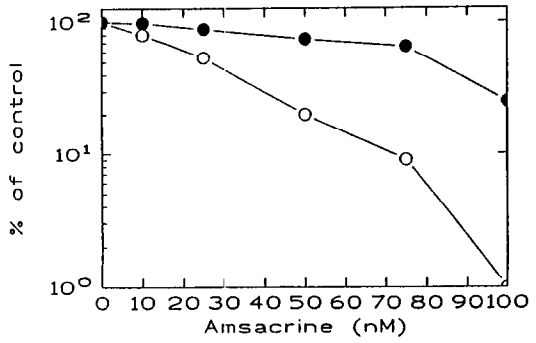


Fig. 9. Effect of ATA on amsacrine cytotoxicity in DC-3F cells. Control (○) or ATA-treated (●) DC-3F cells were grown in the absence or presence of ATA (5 μM) for 18 hr and then exposed to amsacrine for 3 hr. Cell survival was determined by colony formation. The standard error of the data shown was <5%.

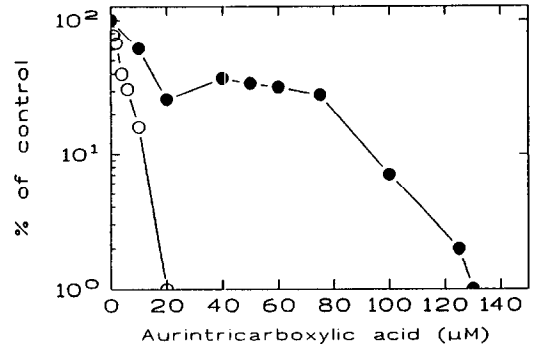


Fig. 10. Cytotoxic effect of ATA on DC-3F (○) and DC-3F/9-OHE (●) cells. The cytotoxic effects of ATA were determined by colony formation in the continuous presence of the drug. The standard error of the data shown was <5%.

3F/9-OHE are approx. 5-fold more resistant to ATA than the parental DC-3F cells (Fig. 10); the ID_{50} for DC-3F is 3 μM whereas the ID_{50} for DC-3F/9-OHE is 16 μM. The difference in cytotoxicity can not be due to a reduced drug uptake mediated by the P-glycoprotein since the DC-3F/9-OHE subline employed in these studies does not express the MDR phenotype [15].

DISCUSSION

The present study was undertaken in an attempt to elucidate the mechanism(s) of action of ATA. We show here that ATA is a potent inhibitor of the nuclear enzyme DNA topoisomerase II *in vitro*. Our results further indicate that ATA interacts with topoisomerase II in living DC-3F Chinese hamster fibrosarcoma cells, and that this interaction plays a role in the cytotoxic activity of ATA.

ATA is a potent inhibitor of purified topoisomerase II with an ID_{50} of approx. 75 nM as measured by relaxation assays. In comparison, under similar experimental conditions, approximately 500-fold more amsacrine and 100-fold more suramin are required to obtain a similar inhibition of catalytic activity.

The unusual potency of ATA may be explained by its ability to interfere with more than one step in the catalytic cycle of topoisomerase II. ATA prevents the binding of topoisomerase II to DNA. This might be due to electrostatic interactions between the enzyme, which contains basic regions, and the polyanionic ATA. In contrast, no binding was detected between ATA and DNA, although low-affinity binding cannot be excluded.

ATA also inhibits topoisomerase II-catalysed ATP hydrolysis. Interestingly, suramin, which like ATA is a polyaromatic, polyanionic compound [32], has only a marginal effect on topoisomerase II-catalysed ATP hydrolysis, whereas its effect on the binding between DNA and topoisomerase II is comparable to that of ATA (Bojanowski K and Larsen AK, unpublished results). These mechanistic differences may explain the almost 100-fold difference in potency between the two compounds.

ATA does not stabilize the covalent DNA–enzyme reaction intermediate (the cleavable complex) as has been shown to be the case for classical inhibitors of topoisomerase II such as amsacrine, etoposide and the ellipticines. In contrast, ATA strongly inhibits amsacrine or etoposide-induced cleavable complex formation in a concentration-dependent manner. This appears to be a direct effect on the enzyme rather than due to drug–drug interactions between ATA and amsacrine or etoposide, since the endogenous formation of cleavable complexes is inhibited as well.

We then wanted to know if ATA penetrates into the nucleus and interacts with nuclear topoisomerase II in living DC-3F Chinese hamster cells. No covalent DNA–protein complexes are observed after 18 hr at doses up to 5 μM. However, the same dose of ATA is able to reduce the number of amsacrine-induced DNA–protein complexes in DC-3F cells. To exclude the possibility that the long preincubation period with ATA caused physiological changes which might somehow affect the formation of drug-induced DNA–protein complexes, nuclei from untreated DC-3F cells were prepared and exposed to amsacrine after only 10 min pretreatment with ATA. The results are basically the same as for whole cells. Therefore, it is excluded that our observations with whole cells could be due to artifacts such as differences in drug uptake, down-regulation of topoisomerase II, cell cycle perturbations etc, but appear instead to be due to a direct effect of ATA on topoisomerase II. This effect is analogous to what we observe *in vitro* with purified topoisomerase II. Subsequent studies have shown that ATA protects DC-3F cells from the cytotoxic effects of amsacrine. In this case the protective effects of ATA are likely to be a direct result of the interaction between ATA and topoisomerase II which leads to a reduction in the initial toxic insult, the amsacrine-induced cleavable complex. The ability of ATA to protect murine leukemia cells from doxorubicin-induced cell death [8] may be explained in a similar manner.

Finally, we wanted to determine whether the interaction between ATA and topoisomerase II plays a role in the cytotoxic effects which are observed at higher doses and/or longer exposure times. Cells resistant to 9-OHE, which have been shown to have altered topoisomerase II activity [14, 17, 30, 31] are approx. 5-fold more resistant to the effects of ATA than sensitive DC-3F parental cells as shown by colony formation assays in the continuous presence of the drug. Interestingly, the dose–response curve for 9-OHE resistant cells shows a biphasic response, which may indicate the presence of at least two targets in these cells.

ATA has been shown to protect a variety of different cell types from apoptotic cell death [5–8]. The protective effect is usually attributed to ATA's ability to inhibit endogenous endonucleases [5, 6, 8, 9]. This interpretation is based on: (a) the ability of ATA to inhibit numerous different nucleases *in vitro* and; (b) the lack of endonuclease activity in ATA-treated cells as determined by the absence of small DNA fragments (a DNA ladder) which often, but not always [33, 34], accompanies apoptotic cell death [9]. However, similar results would be obtained if ATA was acting on enzymes upstream from the endonuclease(s), rather than directly on the endonuclease(s) as such. This possibility is supported by the observation that ATA also protects cell types where apoptosis is not accompanied by a substantial degree of DNA fragmentation [7].

DNA topoisomerase II is an enzyme that regulates chromatin structure by transient breakage, strand passage and resealing of double stranded DNA [35]. In addition to its catalytic activity, topoisomerase II appears to be a structural component of the nuclear matrix [36] where the protein is associated with the DNA at the matrix attachment regions which maintain the DNA loops [37–39]. It has recently been shown that internucleosomal fragmentation is preceded by cleavage of DNA to 50 Kb fragments, which probably represents chromatin loops [33, 34]. Topoisomerase II therefore seems an attractive candidate for involvement in the early steps of apoptosis.

In this study, we demonstrate that ATA is a potent inhibitor of DNA topoisomerase II *in vitro* and that the drug interacts with topoisomerase II in living DC-3F cells. These findings raise the possibility that the protective effects of ATA towards apoptotic cell death might, at least in part, involve DNA topoisomerase II.

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