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Acetyl-Boswellic Acids Are Novel Catalytic Inhibitors of Human Topoisomerases I and IIα

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ABSTRACT

Acetyl-boswellic acids (acetyl-BA) are pentacyclic triterpenes derived from the gum resin of frankincense. We have previously shown that these compounds are effective cytotoxic agents, acting through a mechanism that appears to involve the inhibition of topoisomerase activity. We have now investigated the mechanism of action of acetyl-BA and show that these compounds are more potent inhibitors of human topoisomerases I and IIα than camptothecin, and amsacrine or etoposide, respectively. Our data demonstrate that acetyl-BA and, to a lesser extent, some other pentacyclic triterpenes, such as betulinic acid, ursolic acid, and oleanolic acid, inhibit topoisomerases I and IIα through a mechanism that does not involve stabilization of the cleavable complex or the intercalation of DNA. Surface plasmon resonance analysis revealed that topoisomerases I and IIα bind directly to an immobilized derivative of acetyl-BA. This acetyl-BA derivative interacts with human topoisomerases through high-affinity binding sites yielding K_D values of 70.6 nM for topoisomerase I and 7.6 nM for topoisomerase IIα. Based on our data, we propose that acetyl-BA inhibit topoisomerases I and IIα through competition with DNA for binding to the enzyme. Thus, acetyl-BA are a unique class of dual catalytic inhibitors of human topoisomerases I and IIα.

Topoisomerases are essential enzymes that control and modify the topological state of DNA. These enzymes act by sequential breakage and reunion of either one DNA strand (topoisomerase I) or both DNA strands (topoisomerase II) (Burden and Osheroff, 1998; Pommier et al., 1998). Topoisomerase-mediated strand passing leads to the reduction of DNA twists, as well as the relief of supercoiling, thereby allowing replication, transcription, and recombinant repair to take place (Burden and Osheroff, 1998; Pommier et al., 1998). Numerous studies have shown that rapidly proliferating and transformed cells contain higher levels of topoisomerases (Muller et al., 1985; Burden and Osheroff, 1998), and pharmacological inhibition of these enzymes gained a special interest when it was realized that they are targets of various antitumor and antimicrobial drugs (Burden and Osheroff, 1998; Pommier et al., 1998).

Compounds that interfere with topoisomerases are widespread; some of these substances, such as the plant-derived camptothecin and podophyllotoxins have remarkable therapeutic efficacy as antitumor drugs. The mechanisms of interference with topoisomerase activity are quite different and can be divided into two classes: topoisomerase poisons and catalytic inhibitors (Capranico et al., 1997). Poisons stabilize the covalent enzyme-DNA complex and block rejoining of the DNA break. These compounds promote the accumulation of damaged DNA in the cells and, therefore, possess a mutagenic potential (Baguley and Ferguson, 1998). Catalytic inhibitors of topoisomerases are compounds that prevent binding of enzyme to DNA through interaction either with topoisomerase (Benchokroun et al., 1995; Boege et al., 1996; Frydman et al., 1997; Fortune and Osheroff, 1998) or with DNA (Gatto et al., 1996; Sim et al., 1997; Sorensen et al., 1997). Moreover, substances that interfere with binding or release of ATP during the catalytic cycle of topoisomerase II (Tanabe et al., 1991; Roca et al., 1994) also belong to this class of inhibitors.

The gum resin of *Boswellia serrata* contains boswellic acids (BA) and other pentacyclic triterpenes, which have a chemical structure that closely resembles that of steroids. Recently, we have found that 3-*O*-acetyl-11-keto-β-BA (AKβBA) as well as the structurally related 3-*O*-acetyl-β-BA (AβBA) are cytotoxic for the human glioma cell lines U87 MG and U373 MG (Heldt et al., 1997). Subsequent studies performed by us and others have confirmed these results and have shown that BA as well as other pentacyclic triterpenes are effective anticancer agents. Thus, acetyl-BA exhibit cytotoxic effects on human leukemia HL-60 cells (Shao et al., 1998; Hoernlein et al., 1999). Betulinic

ABBREVIATIONS: BA, boswellic acid(s); AKβBA, 3-*O*-acetyl-11-keto-β-boswellic acid; AβBA, 3-*O*-acetyl-β-boswellic acid; EMSA, electrophoretic mobility shift assay; TAE, Tris-acetate-EDTA; biotinyl-AC-αBA, 3-*O*-(*N*-(+)-biotinyl-6-aminocapropyl)-α-boswellic acid; SPR, surface plasmon resonance; AαBA, 3-*O*-acetyl-α-boswellic acid.

acid is cytotoxic to human melanoma (Pisha et al., 1995), neurodermal tumors (Fulda et al., 1997), and leukemia L1210 cells (Noda et al., 1997), and ursolic and oleanolic acids inhibit tumor growth in irradiated mice (Hsu et al., 1997). In relation to the mechanism of action of AβBA and AKβBA, we observed that the induced cytotoxicity did not directly correlate with the reported ability of these compounds to inhibit 5-lipoxygenase (Safayhi et al., 1992) but did correlate to morphological changes within the nucleus that are consistent with the inhibition of topoisomerases (Heldt et al., 1997; Hoernlein et al., 1999). Indeed, nuclear extracts from U87 MG and U373 MG glioma cells contain high levels of topoisomerase activity, which is inhibited by the presence of acetyl-BA, strongly suggesting that these compounds are topoisomerase inhibitors (Heldt et al., 1997).

In this report, we further investigate the mechanism of action of acetyl-BA and demonstrate that these compounds, as well as some other pentacyclic triterpenes, are highly potent inhibitors of both human topoisomerases. We found that the inhibitory efficacy of acetyl-BA on topoisomerases I and IIα is at least comparable with that of camptothecin and amsacrine or etoposide, respectively. Moreover, we also found that acetyl-BA neither stimulate the formation of DNA-strand breaks in the presence of topoisomerases nor intercalate into DNA. Rather, our results show that acetyl-BA impair activity of topoisomerases I and IIα through direct interaction with the enzymes and strongly suggest that these compounds compete with DNA for binding to topoisomerase. Thus, our data identify acetyl-BA as novel dual catalytic inhibitors of human topoisomerases.

Experimental Procedures

Materials. Purified human topoisomerase I (100 kDa; specific activity, 4 U/ng of protein), topoisomerase IIα (170-kDa isoform; specific activity, 44 U/μg of protein), marker DNA, catenated kinetoplast DNA and supercoiled pRYG DNA were purchased from TopoGEN Inc. (Columbus, OH). Topoisomerases were free of nuclease contamination and migrated on SDS-polyacrylamide gel electrophoresis as single bands of the given molecular mass. Supercoiled pBR322 DNA was from Amersham Pharmacia Biotech (Freiburg, Germany) and DNase I (specific activity, 2 U/μg of protein) from bovine pancreas was from Roche Molecular Biochemicals (Mannheim, Germany); amsacrine, ATP, BSA, and chloroquine were from Sigma (Munich, Germany); etoposide and camptothecin from Calbiochem (Bad Soden, Germany); proteinase K from Life Technologies (Karlsruhe, Germany). Various pentacyclic triterpenes (HPLC grade 99%) were obtained from Roth (Karlsruhe, Germany). Acetyl-BA were isolated from the gum resin of African frankincense (Winterstein and Stein, 1932), purified by reversed-phase HPLC and characterized by mass spectrometry and one- and two-dimensional NMR. The purity of the acetyl-BA was generally >99%. The compounds were dissolved in dimethyl sulfoxide (Fluka, Deisenhofen, Germany); control samples contained equivalent amounts of solvent. All other reagents were of analytical grade.

DNA Relaxation and Decatenation. Topoisomerases were assayed by relaxation of supercoiled plasmid DNA (Trask et al., 1984). Relaxation of 250 ng of supercoiled pBR322 DNA by topoisomerase I (2 U) was performed in 20 μl of topoisomerase I relaxation buffer [10 mM Tris·HCl, pH 7.9, 1 mM EDTA, 150 mM NaCl, 0.1% (w/v) BSA, 0.1 mM spermidine, 5% (v/v) glycerol] in the presence and absence of varying amounts of the test compounds, dissolved in dimethyl sulfoxide (5% (v/v) final concentration). Reactions were started by addition of DNA. Control groups were either DNA alone or DNA treated with topoisomerase. Relaxation of pRYG DNA with topoisomerase IIα (Spitzner et al., 1990) was performed in topoisomerase IIα relaxation buffer [50 mM Tris·HCl, pH 8.0, 0.5 mM ATP, 10 mM $MgCl_2$, 120 mM NaCl, 0.5 mM dithiothreitol] essentially as with topoisomerase I. One unit of either topoisomerase relaxed 250 ng of corresponding substrate DNA in 30 min at 37°C under standard reaction conditions. In the samples with amsacrine, DNA was added before the addition of enzyme. After 30 min at 37°C, the reaction was terminated by addition of 1% (w/v) SDS and digested with 50 μg/ml proteinase K at 55°C for 30 min. DNA was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and separated on 1% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, pH 8.0, and 2 mM EDTA) at 2 V/cm for 3.5 h. Gels were stained with 5 μg/ml ethidium bromide, destained, and photographed using Polaroid 665 film or a gel-imaging system for numerical quantification by densitometry scanning (Herolab, Wiesloch, Germany). For the quantification of the inhibitory effects on the catalytic activity of topoisomerase IIα in relaxation assays, only the changes of the monomeric form of pRYG DNA were considered. For the analysis of decatenation, 125 ng of catenated kinetoplast DNA was incubated with topoisomerase IIα (2 U) in 20 μl of topoisomerase II relaxation buffer at 37°C for 60 min. Samples were separated on gels containing 1 μg/ml ethidium bromide. Numerical data for drug-induced effects were expressed as percent difference from control samples. Data are expressed as mean ± S.E.

Measurement of DNase I Activity. Bovine DNase I (0.4, 2.0, 4.0 U/ml) was incubated with 400 ng of pBR322 DNA in 20 μl of buffer (50 mM Tris·HCl, pH 7.5, 10 mM $MnCl_2$, and 50 μg/ml BSA) in the presence of various amounts of acetyl-BA (10–100 μM) for 15 min at 37°C. The reaction was stopped by addition of 25 mM EDTA (final concentration) followed by agarose gel electrophoresis as described above.

Measurement of Topoisomerase-Mediated DNA Cleavage. Reaction mixtures contained an excess of enzymes (i.e., 100 U of topoisomerase I and 10 U of topoisomerase IIα). Topoisomerase IIα reactions were performed in buffer especially optimized for the detection of cleavage (30 mM Tris·HCl, pH 8.0, 3 mM ATP, 15 mM mercaptoethanol, 8 mM $MgCl_2$, and 60 mM NaCl) (TopoGEN). Samples, which contained two inhibitors, were assembled in this order: AαBA, topoisomerase, second compound (camptothecin or etoposide). Reactions were started by addition of DNA and terminated with prewarmed SDS [1% (w/v) final concentration]. After digestion with proteinase K, open circular and linear DNA were separated from intact supercoiled and relaxed form by agarose gel electrophoresis in the presence of 1 μg/ml ethidium bromide under the same conditions as for the relaxation assay.

Analysis of Topoisomerase-DNA-Binding by Electrophoretic Mobility Shift Assay (EMSA). EMSAs were basically performed as described elsewhere (Boege et al., 1996; Osheroff, 1986). In brief, supercoiled pBR322 DNA was incubated in 20 μl of relaxation topoisomerase I buffer with or without excess of topoisomerase I (100 U) in the presence of the compounds indicated in Fig. 6 (10 μM) at 37°C for 6 min. The reaction was started by addition of DNA. The samples containing two inhibitors were assembled in the order AαBA, topoisomerase, second compound (camptothecin or etoposide). Samples were immediately loaded onto the 1% agarose gel in Tris-acetate-EDTA buffer with 1 μg/ml ethidium bromide and separated by electrophoresis for 6 h at 2 V/cm. Additional control samples containing DNA and enzyme but no test compounds were terminated with SDS and digested with proteinase K to confirm that the DNA shift was caused by enzyme-DNA interaction. EMSA in the presence of topoisomerase IIα (6 U) was performed in 20 μl of topoisomerase II relaxation buffer without ATP essentially as described for the topoisomerase I. Some experiments also were performed in the presence of ATP to define any possible impact of ATP on inhibitory effects of acetyl-BA. DNA electrophoresis was performed in 1% TAE-agarose, pH 6.4. At this pH, topoisomerase IIα is positively charged (pI = 6.5) (Boege et al., 1994), ensuring a stronger shift. Similar results were also obtained when electrophoresis was performed at pH 7 and 8.

Measurement of DNA Intercalation. Intercalation was determined by the unwinding assay (Pommier et al., 1985). Supercoiled pBR322 DNA was relaxed with 300 U of topoisomerase I at 37°C for 15 min in topoisomerase I relaxation buffer. To confirm full relaxation of DNA, one sample (lane 2) was terminated with SDS after 15 min. Inhibitors were added (20 μM each acetyl-BA or the intercalator amsacrine) and the incubations were continued for another 60 min. Parallel experiments ensured that topoisomerase I retained its activity in the presence of the compounds used. The reaction was terminated by addition of 1% (w/v) SDS and followed by digestion with proteinase K as described above. The compounds were removed by extraction with chloroform/isoamyl alcohol (24:1). For a better resolution of topoisomers, DNA was separated on 1% agarose Tris-phosphate-EDTA buffer (36 mM Tris·HCl, pH 7.8, 1 mM EDTA, and 30 mM NaH_2PO_4) gel with 0.2 μg/ml chloroquine for 15 h at 0.4 V/cm. After removal of chloroquine, the gel was stained with ethidium bromide and photographed as described above.

Surface Plasmon Resonance Analysis of Acetyl-BA-Topoisomerase Interaction. Measurements were performed on the IBIS optical sensor device (XanTec Analysensysteme, Muenster, Germany). The instrument uses surface plasmon resonance (SPR) to measure changes in the refractive index of p-polarized light (670 nm) close to the sensor surface. These changes in refractive index are related to the amount of macromolecules bound to the sensor surface. The signal is recorded in millidegrees. A response of 120 m° represents a change in surface protein of approximately 1 ng/mm^2. AαBA, used for immobilization onto a SPR sensor chip, was deacetylated and coupled to 6-aminocaproic acid anhydride. The product, 3-*O*-(6-aminocaproyl)-α-BA, reacted with (+)-biotin-*N*-hydroxysuccinimidyl ester to yield the conjugate 3-*O*-(*N*-(+)-biotinyl-6-aminocaproyl)-α-boswellic acid (biotinyl-AC-αBA). The conjugate was subsequently bound to neutravidin and the resulting complex immobilized on the sensor surface according to standard procedures. For the SPR analysis of topoisomerase binding, a planar carboxylated sensor chip covered with a maleic acid-ethylene copolymer (XanTec Analysensysteme, Muenster, Germany) was used. Immobilization was carried on to a density equivalent to a sensor response of 490 ± 23 m°. Both topoisomerase I (Lot no. MR159) and topoisomerase IIα (Lot no. AP159) were thawed on ice and transferred by gel filtration into corresponding relaxation buffers before each experiment. Measurements were performed with the indicated concentrations of topoisomerases at 20°C in 50 μl of relaxation buffer. Topoisomerase IIα measurements were performed in absence of ATP. The increase of the response after injection of enzyme reflects binding to the immobilized ligand. After recording of the association, the liquid phase was replaced by assay buffer and dissociation was monitored for another 200 to 300 s. Binding of plasmid DNA (pBR322 or pRYG, 30 μg/ml) to surface-bound biotinyl-AC-αBA was measured by application of 50 μl of each DNA solution over the sensor surface. After each measurement, the sensor chip was regenerated with 1 M NaCl in 0.1 M NaOH. There were no mass transport limitations during the measurements as confirmed by the analysis with the software supplied with the instrument.

Analysis of the data was performed with the IBIS kinetic evaluation program. Using SPR biosensors, the kinetic parameters of a single-phase association can be determined by nonlinear regression of the data points as the most robust data analysis (O'Shannessy et al. 1993) by the equation:

$$Rt = \frac{k_a C R_{max}[1 - e^{-(k_a C + k_d)t}]}{k_a C + k_d} + R_0$$

where R is the SPR response, R_0 is response at the $t = 0$, C is the concentration of the analyte in M, k_a is the association rate constant in $M^{-1}\,s^{-1}$, and k_d is the dissociation rate constant in s^{-1} (O'Shannessy et al., 1993). The model allows determination of rate constants without reaching equilibrium during the experimental cycle. The relevant kinetic information was obtained from the parameter $k_s = (k_a C + k_d)$. A plot of k_s values versus concentration is used for linear regression to obtain the association rate constant from the slope and the dissociation rate constant from the y-intercept. Data from the entire association phase were used to determine the kinetic constants. Dissociation rate constants calculated from the dissociation phase yielded comparable results.

Results

Acetyl-BA Inhibit the Catalytic Activity of Topoisomerases I and IIα. The gum resin of *Boswellia serrata* contains both acetylated and nonacetylated forms of BA. Figure 1 shows the chemical structures of three of the acetylated forms of these compounds: acetyl-α-boswellic acid (AαBA), AβBA, and AKβBA. All three acetyl-BA inhibited human topoisomerases I and IIα in a concentration-dependent manner in DNA relaxation assays (Fig. 2A and B). In addition, equivalent inhibitory effects on topoisomerase IIα were demonstrated in decatenation assays, where the catalytic activity of topoisomerase IIα results in decatenated kinetoplast DNA yielding open circular DNA (Fig. 2C, upper band) and closed circular DNA (Fig. 2C, lower band) able to penetrate into the gel (Fig. 2C). A comparison of the relative efficacies of the three acetyl-BA showed that AαBA > AβBA > AKβBA (Fig. 2, A–C). The IC_{50} value for the inhibition of the catalytic activity of topoisomerases I and IIα by AαBA was ~3 μM ($n = 5$) and ~1 μM (relaxation, $n = 9$; decatenation, $n = 4$), respectively. Moreover, under these experimental conditions AαBA seemed to be more potent than camptothecin, amsacrine, or etoposide in inhibiting the activity of topoisomerases I or IIα.

To exclude nonspecific interactions with DNA-processing enzymes, we determined the effects of acetyl-BA, such as AαBA, AβBA, and AKβBA on the catalytic activity of bovine DNase I. In contrast to topoisomerases, acetyl-BA (10–100 μM) did not impair the activity of DNase I (0.4–4.0 U/ml; data not shown).

We next compared AαBA with the structurally related pentacyclic triterpenes shown in Fig. 3 for topoisomerase inhibition. Whereas AαBA effectively inhibited DNA relax-

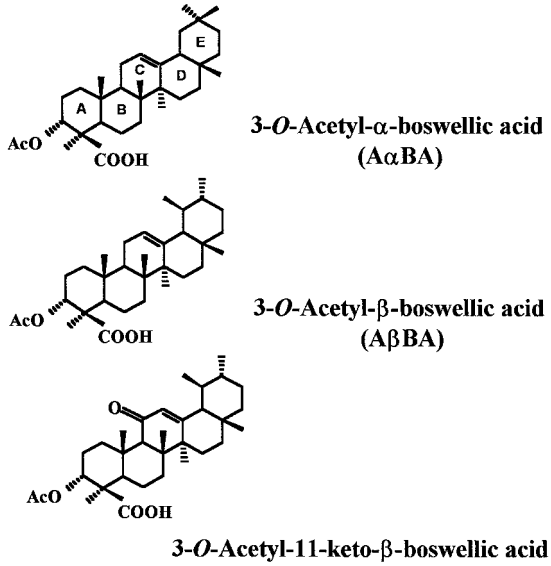

Fig. 1. Chemical structures of acetyl-BA.

ation by both topoisomerases I and IIα, neither amyrin isoform nor 18-β-glycyrrhetinic acid had significant effects in the concentrations used (Fig. 4). Similar to acetyl-BA, the other pentacyclic triterpenes tested inhibited both topoisomerases. The most effective of these compounds was betulinic acid having an IC_{50} value of ~43 μM and ~5 μM for topoisomerases I and IIα, respectively (Fig. 4). Considering the structural features of the various pentacyclic triterpenes used in this study, the above results suggest that the shared pentacyclic ring conformation is important but not sufficient for the inhibition of topoisomerases. Moreover, our results also suggest that the combination of the carboxyl group at the fourth carbon (ring A) and the α position of the two methyl groups at ring E is important for enhancing the inhibitory activity of the molecule toward both topoisomerases: AαBA possesses the highest inhibitory efficacy.

Acetyl-BA Do Not Induce Topoisomerase-Mediated DNA-Strand Breaks. The catalytic cycle of human topoisomerases consists of several distinct steps. Compounds such as camptothecin and etoposide interfere with the religation step and stabilize the enzyme-DNA cleavable complex. These compounds are known as topoisomerase poisons because their action results in an alteration of topoisomerase function leading to DNA breakage (Capranico et al., 1997). To investigate whether acetyl-BA are such poisons, we measured formation of topoisomerase-induced DNA-strand breaks. Figure 5A shows, as expected, that camptothecin stabilized the topoisomerase I cleavable complex, resulting in the generation of open-circle plasmid DNA. In contrast, open-circle DNA was not observed with either AαBA or AKβBA (1 and 100 μM), even when a wider concentration range of these compounds was used (0.1–1000 μM, data not shown). Surprisingly, both acetyl-BA antagonized formation of open-circle DNA in the presence of equimolar concentrations of camptothecin, suggesting that acetyl-BA were acting at a step upstream of camptothecin.

Similar results were obtained in experiments with topoisomerase IIα (Fig. 5B). Etoposide blocks topoisomerase IIα-mediated DNA religation, which could be monitored by the appearance of linear DNA. Neither AαBA nor AKβBA (1 and 100 μM) increased the level of DNA scission, but both of them prevented formation of cleavable complex in the presence of etoposide. Experiments performed with AβBA and betulinic and oleanolic acids (100 μM) demonstrated no stabilization of enzyme-DNA cleavable complexes, indicating the same mechanism of action for different pentacyclic triterpenes

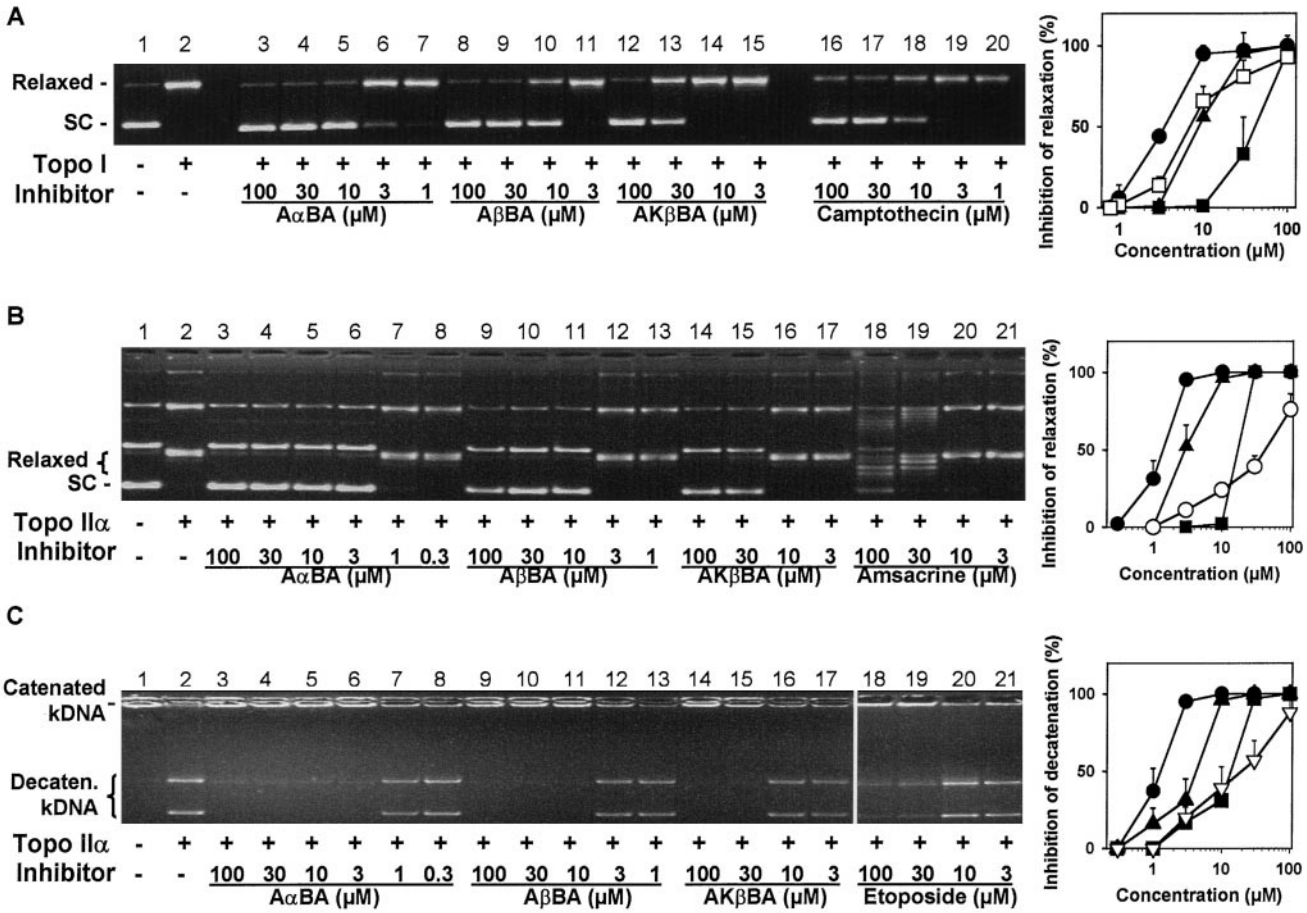

Fig. 2. Inhibitory effects of acetyl-BA on the catalytic activity of topoisomerases. A, the inhibitory effects of acetyl-BA on topoisomerase I was determined in relaxation assays. The effects on topoisomerase IIα activity were measured by both DNA relaxation assays (B) and decatenation of kinetoplast DNA (C). Control samples contained substrate DNA (lane 1) and DNA with enzyme (lane 2). Substrate DNA was incubated with 2 U of either topoisomerase I or IIα in the presence of various concentrations of the acetyl-BA. The standard inhibitors camptothecin (A), amsacrine (B), and etoposide (C) served as positive control samples. Numerical data for the compound-induced effects as percent difference from control are shown on the right panels (AαBA, ●; AβBA, ▲; AKβBA, ■; camptothecin, □; amsacrine, ○; etoposide, ▽). pRYG DNA used in B exists as monomers, dimers, and trimers; the changes of monomeric DNA were considered for quantification only. Data presented are mean ± S.E. of five (A), nine (B), and four (C) experiments.

(data not shown). Taken together, the above experiments demonstrate that acetyl-BA are not topoisomerase poisons.

Acetyl-BA Prevent Binding of Topoisomerases I and IIα to the Substrate DNA. We next investigated whether acetyl-BA directly interfere with binding of topoisomerase I (Fig. 6A) or IIα (Fig. 6B) to DNA using an EMSA. Excess topoisomerase was used in either case to ensure a stronger shift. Acetyl-BA alone did not interfere with the electrophoretic mobility of plasmid DNA. Both topoisomerases formed complexes with plasmid DNA, and treatment of these complexes with SDS and proteinase K released the DNA. Figure 6 also demonstrates that acetyl-BA inhibited the formation of these enzyme-DNA complexes. AKβBA was less effective than AαBA in respect to topoisomerase IIα inhibition in accordance with the data from the DNA relaxation assays above. AαBA and AKβBA inhibited the binding of DNA by topoisomerase IIα in both the presence (data not shown) and absence of ATP (Fig. 6B). The topoisomerase IIα-DNA complex was relatively immobile and was retained close to the application slot (Fig. 6B, lane 2). Similar to AαBA and AKβBA, AβBA, betulinic acid, and oleanolic acid also inhibited topoisomerase-DNA complex formation (data not shown). In contrast, DNA binding of topoisomerase I was not affected by camptothecin (Fig. 6A, lane 7) nor was that of topoisomerase IIα affected by etoposide (Fig. 6B, lane 5). These observations are consistent with the mechanism of action of these compounds, both camptothecin and etoposide do not interfere with the binding and scission steps of either topoisomerase. When added before camptothecin and etoposide, AαBA prevents binding of either topoisomerase to DNA, suggesting that it inhibits the formation of tertiary complexes between enzyme, topoisomerase poison (camptothecin or etoposide), and DNA, further supporting the notion that acetyl-BA inhibit the DNA-binding step of both topoisomerases.

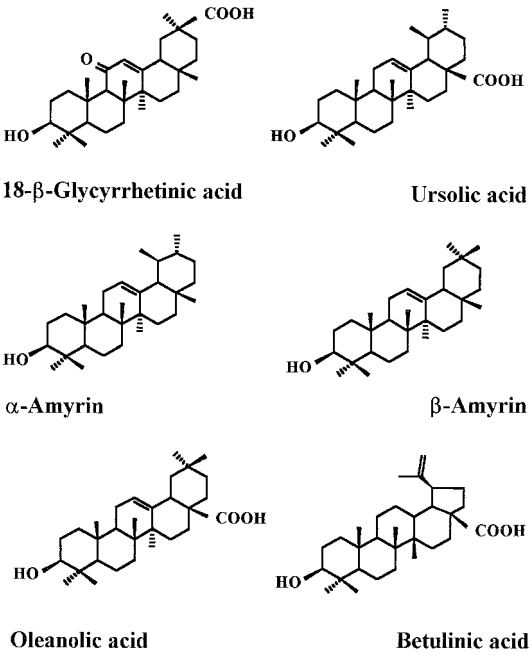

Fig. 3. Chemical structures of related pentacyclic triterpenes.

Acetyl-BA Do Not Intercalate into DNA. To elucidate further the mechanism of topoisomerase inhibition, we investigated the DNA binding characteristics of acetyl-BA. We employed a DNA unwinding assay to assess any possible impact of acetyl-BA on the superhelical state of closed circular DNA. This assay is based on the ability of intercalating compounds to unwind the DNA duplex and thereby change the DNA twist (Waring, 1981). These drug-induced changes in DNA twist also induce structural tension in the DNA

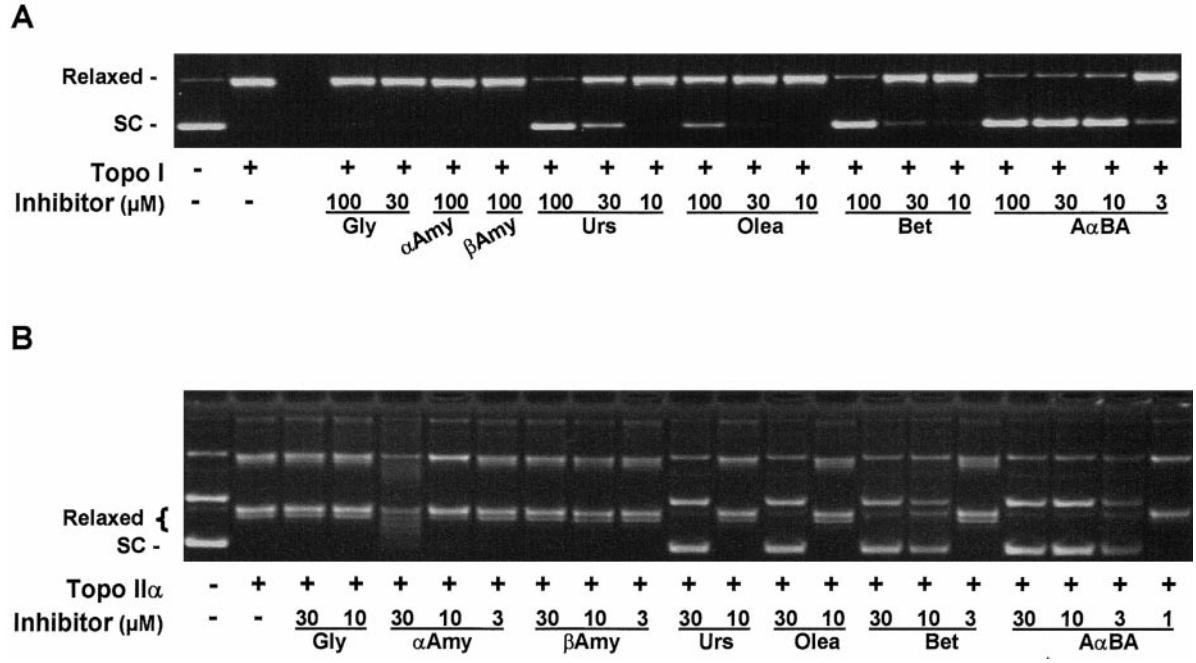

Fig. 4. Inhibitory effects of pentacyclic triterpenes on the catalytic activity of topoisomerases. DNA relaxation assay showing the inhibitory effects of pentacyclic triterpenes on activity of topoisomerases I (A) and IIα (B). Control samples contained supercoiled plasmid DNA (lane 1) and DNA incubated with 2 U of enzyme (lane 2). As in Fig. 2, supercoiled DNA was incubated with 2 U of either topoisomerase I or topoisomerase IIα for 30 min at 37°C in the presence of the following pentacyclic triterpenes: 18-β-glycyrrhetinic acid (Gly), ursolic acid (Urs), oleanolic acid (Olea), and betulinic acid (Bet) acid, or α- and β-amyrin (Amy). Samples containing AαBA were included for comparison. One of five comparable experiments is shown.

backbone; this tension can be relieved by topoisomerases. On removal of both topoisomerase and intercalating agent, the unwinding effect of the intercalating compound is no longer present and the DNA returns to a supercoiled state. Figure 7 shows that the classical intercalator amsacrine affected the gaussian distribution of the DNA topoisomers by shifting them down (i.e., into the supercoiled state); however, neither AαBA nor AKβBA had any effect, suggesting that the mechanism through which acetyl-BA inhibit topoisomerases was independent of DNA intercalation. Similar results were obtained using AβBA, betulinic acid, and oleanolic acid (data not shown). Furthermore, acetyl-BA did not impair staining of DNA by ethidium bromide (not shown), which is known to bind through the minor grove. Thus, acetyl-BA interfere with human topoisomerases through a mechanism different from that of agents that either intercalate DNA or bind to the minor grove of DNA. To determine whether this mechanism involved direct binding of acetyl-BA to topoisomerases, we performed binding experiments using SPR.

Binding of an Acetyl-BA Derivative to Topoisomerases I and IIα as Measured by SPR. We immobilized a derivative of AαBA to the surface of the plasmon resonance sensor chip by creating a biotinyl-AC-αBA (Fig. 8A). This compound added to the fluid phase of the relaxation assays was fully active in inhibiting topoisomerase activity, showing an IC_{50} value of ~12 μM and ~2 μM for the inhibition of topoisomerases I and IIα, respectively (Fig. 8, B and C).

Fig. 9 shows the binding curves of topoisomerases I and IIα to biotinyl-AC-αBA linked to the sensor chip surface. There was no unspecific binding of topoisomerases to the sensor surface after the activated carboxymethyl groups had been blocked with ethanolamine (data not shown). Furthermore, once bound, there was no detectable dissociation of the immobilized BA from the sensor surface even after many cycles of binding and regeneration. The binding of topoisomerase I to biotinyl-AC-αBA was concentration-dependent (Fig. 9A) and followed a one-phase reaction. The apparent rate constants for the single class high affinity binding sites were determined as: $k_a = 9.1 \times 10^4\ M^{-1}\,s^{-1}$ and $k_d = 6.5 \times 10^{-3}\ s^{-1}$. The apparent equilibrium dissociation constant (K_D) was calculated as 70.6 nM. The kinetics of topoisomerase I binding to biotinyl-AC-αBA were slower than that for topoisomerase IIα (Fig. 9B), and removal of the nonbound topoisomerase I resulted in a similarly slow dissociation of the complex. In some experiments, topoisomerase I was mixed with either biotinyl-AC-αBA (40 μM) or pBR322 DNA (30 μg/ml) and then applied to the sensor surface. In those experiments, no binding to the immobilized ligand could be detected (data not shown), demonstrating the specificity of the reaction. In accordance with the unwinding assay, binding of pBR322 and pRYG plasmid DNA to biotinyl-AC-αBA was undetectable (data not shown).

Fig. 9B shows the binding of topoisomerase IIα to the immobilized biotinyl-AC-αBA. Kinetic analysis of the binding revealed a single-phase interaction between enzyme and ligand. The apparent rate constants for the high affinity binding site was calculated to be: $k_a = 4.2 \times 10^6\ M^{-1}\,s^{-1}$ and $k_d = 3.2 \times 10^{-2}\ s^{-1}$. The apparent equilibrium dissociation constant was determined to be $K_D = 7.6$ nM. No binding of topoisomerase IIα to immobilized boswellic acid was detected on preincubation with either biotinyl-AC-αBA (40 μM) or pBR322 DNA (30 μg/ml) (data not shown).

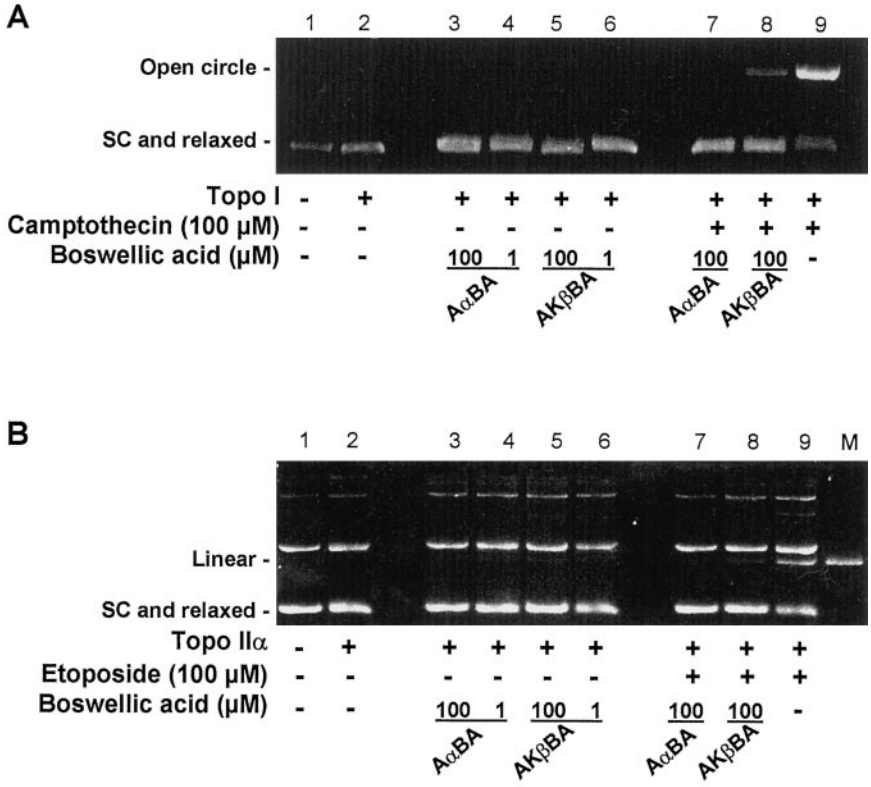

Fig. 5. Acetyl-BA do not induce topoisomerase-mediated DNA-strand breaks. A, topoisomerase I; supercoiled pBR322 DNA was incubated with an excess of topoisomerase I (100 U) in 20 μl of assay buffer in the presence or absence of the indicated compounds. Control samples were DNA alone (lane 1) and DNA with topoisomerase I (lane 2). Lanes 3 and 4 show the effects of AαBA. Lanes 5 and 6 show the effects of AKβBA. Lane 9 shows the formation of open-circle DNA in the presence of 100 μM camptothecin as a positive control. Lanes 7 and 8 show that equimolar concentrations of AαBA or AKβBA added to the reaction mixture before camptothecin antagonize the formation of the cleavable complex. B, topoisomerase IIα; the assay was performed as described for topoisomerase I but using pRYG DNA, topoisomerase IIα (10 U), and a special buffer containing 3 mM ATP. The topoisomerase II poison etoposide was used as a positive control (lane 9). Formation of the linear DNA by topoisomerase IIα in the presence of etoposide was antagonized by the addition of equimolar amounts of AαBA and AKβBA before etoposide (lanes 7 and 8, respectively). Cleavable complex formation was monitored by appearance of linearized DNA (lane M contains a marker). One of three representative experiments is shown.

Discussion

In this article, we show that acetyl-BA inhibit human topoisomerases I and IIα. We further describe the molecular mechanism of this inhibition, demonstrating that acetyl-BA inhibit topoisomerase action by directly binding to the enzyme, not by binding to DNA or by complex formation with enzyme and DNA. Our data suggest that acetyl-BA inhibit both topoisomerases I and IIα using the same mechanism; that is, by competing with DNA for topoisomerase binding. This inhibition seems to be specific, because acetyl-BA did not affect the activity of bovine DNase I. Thus, we propose acetyl-BA as a new class of topoisomerase inhibitors.

Pentacyclic triterpenes are widespread in nature and are a part of our daily diet as constituents of fruits and vegetables. Some pentacyclic triterpenes are known to possess antitumor activity, but the mechanism through which these compounds achieve this effect has not been elucidated (Pisha et al., 1995; Fulda et al., 1997; Heldt et al., 1997). Boswellic acids belong to the class of pentacyclic triterpenes, and we have recently described the acetyl-BA, AKβBA, to induce cell cytotoxicity through a mechanism involving the inhibition of topoisomerase I (Hoernlein et al., 1999). We isolated and characterized several different BA and found that not only did these compounds inhibit topoisomerase I, but also topoisomerase IIα activity. Analysis of the structure-activity relationship suggested that the general pentacyclic ring structure of the BA was important for topoisomerase inhibitory activity but was in itself not sufficient because β-amyrin was inactive and α-amyrin had only a negligible effect on topoisomerases IIα. AαBA and AβBA differ from α and β-amyrin in that they are acetylated and carboxylated on positions 3 and 4 of ring A, respectively. This indicates that the nature and arrangement of the side groups is important. Our study suggests that carboxylation of the pentacyclic ring structure, and particularly on rings A and D, is necessary for topoisomerase inhibition. We found that those compounds that contain a carboxyl group (betulinic acid, ursolic acid, oleanolic acid, and acetyl-BA) all inhibit topoisomerases, although β-amyrin was not active. That 18-β-glycyrrhetinic acid was not an effective topoisomerase inhibitor could be attributed to either

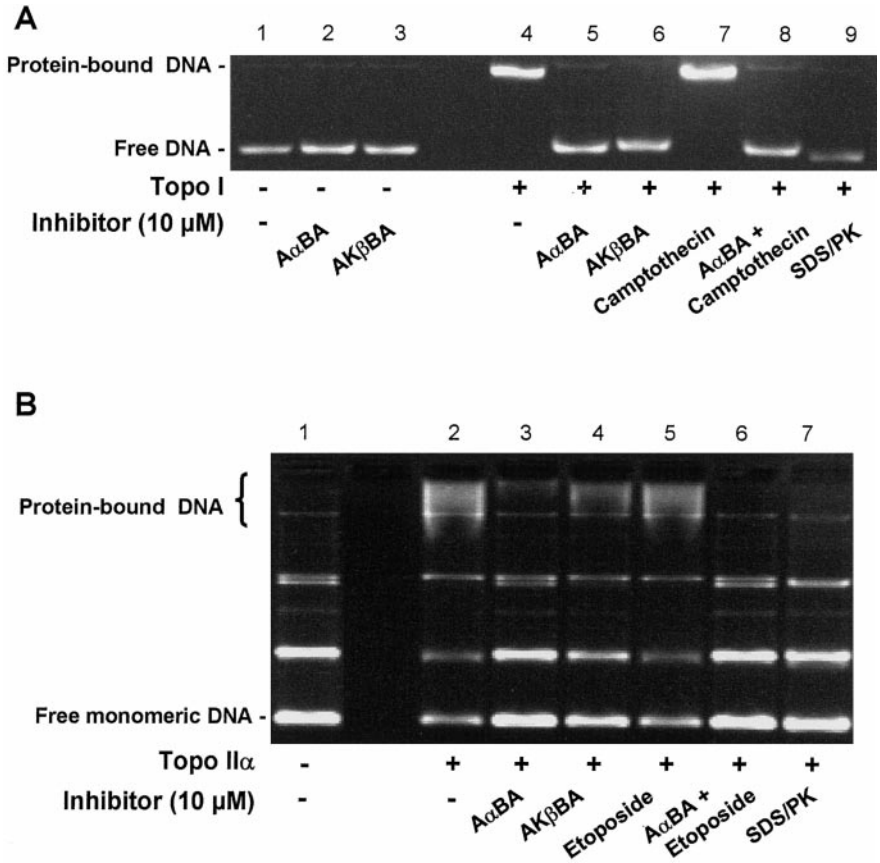

Fig. 6. Acetyl-BA prevent binding of topoisomerases to substrate DNA. EMSAs of topoisomerase I (A) and topoisomerase IIα (B) incubated with appropriate DNA are shown. A, topoisomerase I; samples contained pBR322 DNA, 10 μM each inhibitor, and excess of topoisomerase I (100 U) to allow the strongest possible DNA-shift. Control samples were of DNA alone (lane 1) and DNA with topoisomerase I (lane 4). To the samples of lane 2 and 3 DNA was added together with AαBA or AKβBA (10 μM) to show that acetyl-BA had no influence on the pBR322 mobility (similar results were obtained with pRYG DNA; data not shown). AαBA was added to the sample in lane 8 before camptothecin. The reactions were started with the addition of DNA and incubated for 6 min at 37°C. Samples were separated on 1% TAE-agarose gel electrophoresis in the presence of ethidium bromide for 6 h. Under these conditions supercoiled and relaxed free DNA had similar mobility, and protein-bound DNA migrated more slowly. The control sample containing DNA and topoisomerase I (lane 9) was terminated with SDS, and topoisomerase was digested with proteinase K. The resulting nonbound, relaxed DNA migrates in ethidium bromide gel slightly faster than supercoiled DNA. B, topoisomerase IIα; assays were performed with pRYG DNA and 10 U of enzyme in 20 μl of assay buffer in the presence of Mg^{2+} ions, but without ATP. The reaction was carried out as described above for topoisomerase I. Binding of topoisomerase IIα to DNA in the presence of etoposide (lane 5) was used as a positive control. AαBA was added to sample 6 before etoposide. Denaturation of topoisomerase IIα with SDS and subsequent digestion with proteinase K released the protein-bound DNA (lane 7). One of three representative experiments is shown.

the carboxylation on ring E or to the keto group at position 11 on ring C. Because AKβBA also contains this keto group and is the least effective of the acetyl-BA, it is possible that this position of the pentacyclic triterpenes is important for enzyme inhibition. Considering the structural differences between the compounds tested in connection with their relative efficacy, we would propose that pentacyclic triterpenes could serve as backbones for the rational design of specific topoisomerase inhibitors.

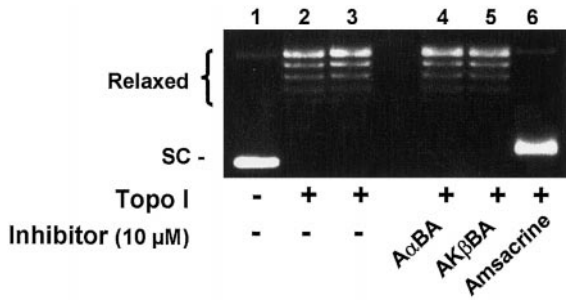

Fig. 7. Acetyl-BA do not intercalate into DNA. Interaction of acetyl-boswellic acids with DNA was measured in an unwinding assay. Negatively supercoiled pBR322 DNA was relaxed in the presence of excess of topoisomerase I (300 U) for 15 min. Test compounds were added after 15 min of full relaxation and incubated for another 60 min. The negative control contained DNA alone (lane 1). Full DNA relaxation induced by topoisomerase I after 15 min and 75 min is shown in lanes 2 and 3, respectively. Lanes 4 to 6 show the effects of the indicated compounds at the concentration of 20 μM on the helical state of relaxed DNA. The intercalator amsacrine was used as a positive control (lane 6). The compounds were removed with organic solvent. For better separation of the DNA topoisomers, the electrophoresis was carried out in the presence of chloroquine for 12 h. One of three experiments is shown.

Inhibition of human topoisomerases by acetyl-BA seems to be specific, because they did not impair the activity of DNase I. DNase I-related enzymes, which are members of the family of Ca^{2+}- and Mg^{2+}-dependent endonucleases, have recently been implicated in DNA fragmentation during apoptosis (Mannherz et al. 1995). Thus, the lack of inhibition of DNase I by acetyl-BA is consistent with our earlier observation that acetyl-BA induce DNA fragmentation and apoptosis in HL-60 and CCRF-CEM cells (Hoernlein et al., 1999).

Our observation that acetyl-BA inhibit topoisomerases I and IIα suggests that these compounds may have a mechanism of action that is similar to those of other dual topoisomerase inhibitors. In general, such inhibitors interact directly with DNA and include agents that intercalate DNA, or bind into the minor groove (Pilch et al., 1997; Pommier et al., 1998; Xu et al., 1998). For example, topoisomerase IIα inhibition is strongly correlated with the ability of a compound to intercalate DNA, whereas drug binding to the minor groove is essential for the inhibition of topoisomerase I (Pilch et al., 1997; Xu et al., 1998). In either case, such substances stabilize the enzyme-DNA cleavable complex and interfere with the scission-religation step; hence, these compounds are referred to as topoisomerase poisons. In this respect, topoisomerase poisons may induce DNA breakage in addition to their topoisomerase inhibitory function, leading to the significant toxicities associated with these compounds (Baguley and Ferguson, 1998). Our data show that acetyl-BA neither directly bind to DNA nor promote DNA breakage. Thus, this observation places acetyl-BA apart from other dual topoisomerase inhibitors and explains the low toxicity and the low incidence

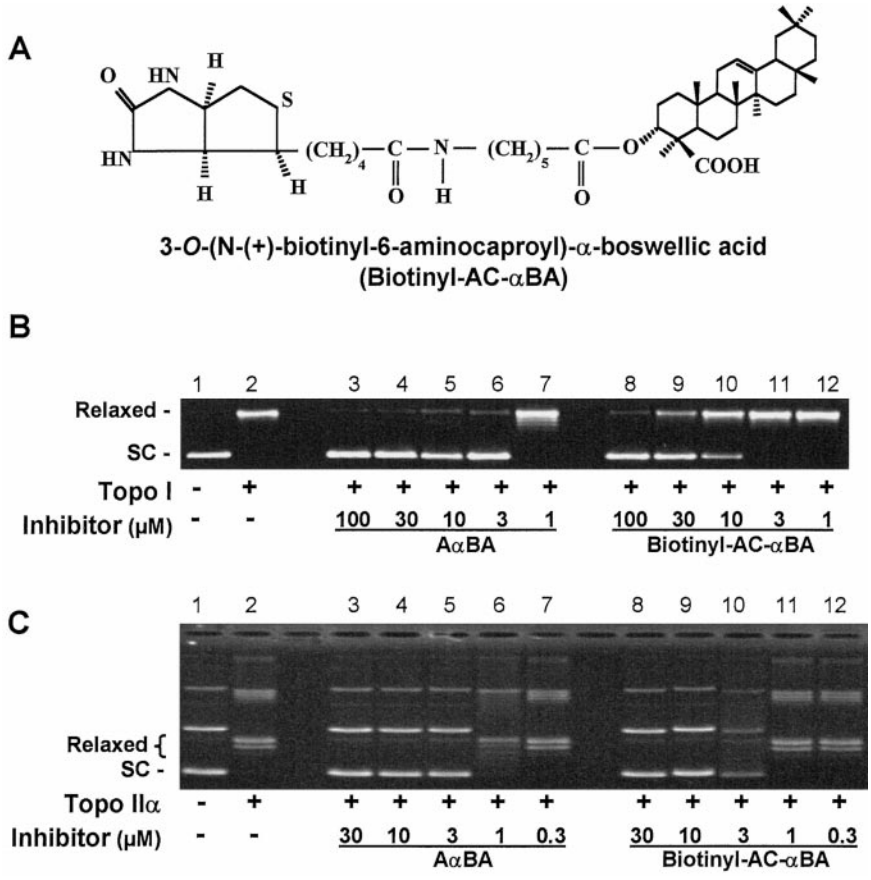

Fig. 8. Inhibitory effects of biotinyl-AC-αBA on the catalytic activity of topoisomerases. A, structure of biotinyl-AC-αBA. B and C, DNA relaxation assays showing the inhibitory effects of biotinyl-AC-αBA on topoisomerase I and IIα activity, respectively. Control samples were supercoiled DNA (lane 1) and DNA with corresponding topoisomerase (lane 2). Samples contained the indicated concentrations of either AαBA (lanes 3–7) or biotinyl-AC-αBA (lanes 8–12). Biotinyl-AC-αBA exhibited inhibitory activity on the topoisomerase I with IC_{50} ~12 μM. The original compound, AαBA, inhibited enzyme with IC_{50} ~3 μM. One of four experiments is shown. Biotinyl-AC-αBA inhibited the catalytic activity of topoisomerase IIα with approximately the same efficacy as AαBA (IC_{50} ~2 μM, n = 8).

of side effects associated with the use of phytopharmacological drugs containing these compounds (Gupta et al., 1998).

We propose that the mechanism through which acetyl-BA impair topoisomerase function is by direct binding through a single class of high-affinity binding sites to each enzyme. Indeed, acetyl-BA inhibit the enzyme-DNA complex formation as shown by EMSA, and directly bind to topoisomerases I and IIα as demonstrated by SPR, a reaction that was inhibited if the enzymes were preincubated with DNA. Thus, our data suggest that acetyl-BA might compete with DNA for the same binding sites on topoisomerases, thereby acting as catalytic inhibitors.

Compared with topoisomerase IIα, the interaction of immobilized biotinyl-AC-αBA with topoisomerase I followed slower association and dissociation kinetics, giving a 9-fold higher value for K_D. A similar difference was observed in the topoisomerase relaxation assays with biotinyl-AC-αBA. The other three acetyl-BA tested also inhibited topoisomerase IIα more effectively than topoisomerase I, which indicates that the inhibitory effect of acetyl-BA on human topoisomerases correlates with the binding characteristics to either enzyme.

Our observation that acetyl-BA inhibit both human topoisomerases is surprising because it seems to suggest similar structural or functional domains. However, although topoisomerases I and IIα have similar functions, these enzymes are completely different. On the other hand, some poisons such as actinomycin D, intoplicine, nitidine, and others act against both topoisomerases (Withoff et al., 1996; Pommier et al., 1998) suggesting some structural characteristics that might be shared by both enzymes. As far as the effects of acetyl-BA on human topoisomerase I and IIα are concerned, the common mechanistic features are obviously related to the first steps of the catalytic cycle: DNA binding and/or conformational changes, either of which might be affected by acetyl-BA. Interestingly, recent studies of the crystal structure of human topoisomerase I revealed the existence of three

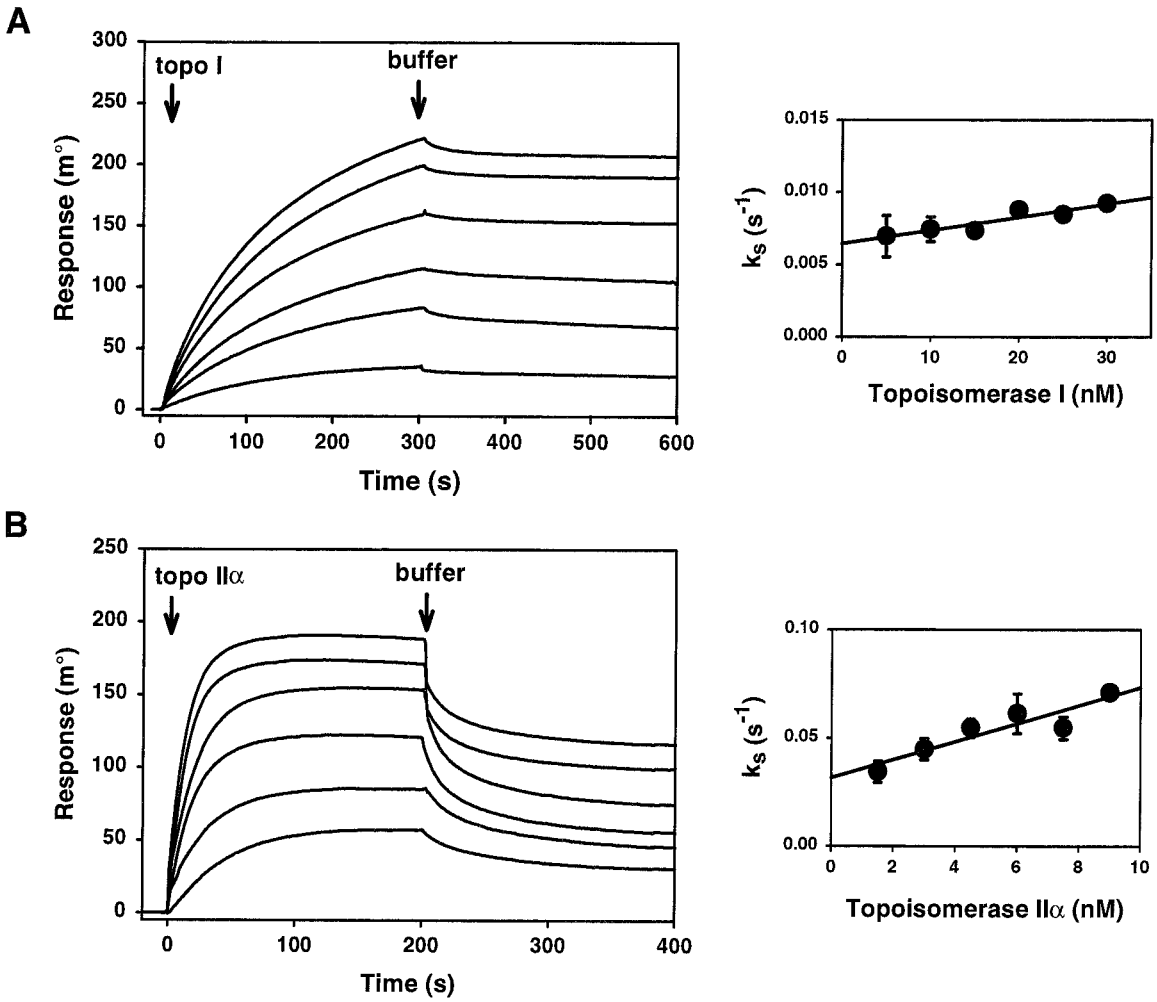

Fig. 9. Binding of topoisomerases to an immobilized acetyl-BA derivative. A, topoisomerase I; immobilization of the biotinyl-AC-αBA/neutravidin complex to the sensor surface was carried on to 490 ± 23 m°. The overlay plot shows association (0–300 s) and dissociation (300–600 s) phases of the interaction between topoisomerase I and immobilized biotinyl-AC-αBA. Six concentrations of topoisomerase I were measured (from the bottom to the top, 5, 10, 15, 20, 25, and 30 nM) and tracings of a typical experiment are shown. The rates were calculated from the entire association phase using an integrated rate method resulting in determination of the values k_s. Association rate constant (k_a) was calculated from the slope of the curve k_s versus concentration (right) and the dissociation constant (k_d) from the y-intercept: $k_a = 9.1 \times 10^4\ M^{-1}\ s^{-1}$ and $k_d = 6.5 \times 10^{-3}\ s^{-1}$. The equilibrium dissociation constant K_D = 70.6 nM (n = 3 experiments). B, topoisomerase IIα. Tracings of a typical experiment show the binding of increasing amounts of topoisomerase IIα (from the bottom to the top, 1.5, 3.0, 4.5, 6.0, 7.5, and 9.0 nM) to immobilized biotinyl-AC-αBA. Measurements were performed in topoisomerase II assay buffer in the absence of ATP. Association was measured for 200 s then enzyme was replaced by buffer and dissociation was recorded for another 200 s. Calculated rate constants for the reaction are: $k_a = 4.2 \times 10^6\ M^{-1}\ s^{-1}$ and $k_d = 3.2 \times 10^{-2}\ s^{-1}$. The equilibrium dissociation constant K_D = 7.6 nM (n = 4 experiments).

β strands that are analogous to a three-stranded antiparallel β-sheet structure from yeast topoisomerase II. These structures located in close proximity to the DNA cleavage sites harbor putative DNA-binding domains and are believed to represent a common DNA-binding motif among DNA topoisomerases (Berger et al., 1998; Redinbo et al., 1999). They might therefore accommodate targets for acetyl-BA binding. In this context it is intriguing that the inhibitory activity of pentacyclic triterpenes is critically dependent on the carboxylic group carrying an electronegative potential. By the same token, it is known that a relatively large number of electropositively charged amino groups of topoisomerase I form protein-phosphate interactions with the base pairs adjacent to the DNA cleavage site (Redinbo et al., 1999). Further studies will have to clarify whether acetyl-BA can intercept any of those protein-phosphate interactions. Moreover, at present it cannot be excluded that on the basis of their electronegative potential acetyl-BA might interact with some site of the likewise positively charged A′ domain groove, the putative primary DNA binding region of topoisomerase II (Berger et al., 1998). Future studies with appropriate topoisomerase mutants and/or photocrosslinking should provide further insights into the site-directed molecular mechanism of acetyl-BA. In addition, such experiments are expected to help us better understand the specific features of topoisomerase-DNA interactions.

Previous work by us has demonstrated that acetyl-BA exert a cytotoxic effect on human malignant glioma (Heldt et al., 1997) and leukemia cell lines (Hoernlein et al., 1999). Furthermore, other pentacyclic triterpenes, including betulinic acid, exhibit antitumor effects (Pisha et al., 1995; Fulda et al., 1997; Hsu et al., 1997; Noda et al., 1997). Our data suggest that the previously observed cytotoxic effects of acetyl-BA and other pentacyclic triterpenes might be a result of their ability to inhibit the activity of human topoisomerases, particularly topoisomerase IIα, which is known to be essential for the survival of eukaryotic cells (Andoh and Ishida, 1998; Burden and Osheroff, 1998).

Poisons of topoisomerases I and IIα, such as camptothecin or etoposide, trap enzyme-DNA cleavable complexes, leading to DNA strand breaks and, by mechanisms not yet completely defined, finally to cell death. Even less is known about the mechanisms and events that link the inhibition of the catalytic activity of topoisomerases to cell death (Andoh and Ishida, 1998; Burden and Osheroff, 1998; Pommier et al., 1998). It has been shown that catalytic inhibitors of topoisomerase II, such as the bisdioxopiperazines ICRF-187 and ICRF-193, result in a failure of dividing cells to accomplish normal mitosis. This is caused by incomplete chromosome condensation and segregation leading to polyploidization and, finally, to cell death (Roca et al. 1994; Andoh and Ishida, 1998). The cytotoxicity of ICRF-187 seems to correlate with the inhibition of the catalytic activity of topoisomerase II. In addition, recent evidence indicates that accumulation of closed clamp formations trapped on DNA might interfere with transcription, or other metabolic processes, resulting in cell death (Andoh and Ishida, 1998; Jensen et al., 2000). The signaling and execution events by which acetyl-BA trigger apoptosis and cytotoxicity are currently the subject of intense investigations.

The ability of acetyl-BA to inhibit both topoisomerases simultaneously might result in an enhanced antitumor efficacy, specifically because topoisomerase I, unlike topoisomerase II, is a cell-cycle–independent enzyme (Burden and Osheroff, 1998; Hande, 1998; Pommier et al., 1998). Acting on different cellular targets, these compounds may possibly have advantages similar to clinical combination therapy. Indeed, preliminary data suggest that acetyl-BA might be more potent cytotoxic agents for glioma cell lines than the poisons camptothecin and etoposide (our unpublished data). It is intriguing that acetyl-BA are lipophilic; they might therefore penetrate the blood-brain barrier, making these compounds promising therapeutic agents for the treatment of malignant brain tumors. Clinical studies are now underway to assess the value of acetyl-BAs for the treatment of human astrocytomas and glioblastomas.

Acknowledgments

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