AK37: the first pyridoacridine described capable of stabilizing the topoisomerase I cleavable complex

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Pyridoacridines are marine natural products that contain planar structures. Almost all are cytotoxic and capable of DNA intercalation. Several pyridoacridines have demonstrated anti-cancer activity, being able to generate reactive oxygen species or to inhibit topoisomerase (Topo) II. Synthetic pyridoacridines were characterized and compared to other pyridoacridines as well as the Topo-inhibiting drugs (etoposide, 9-aminocamptothecin and wakayin) in a series of *in vitro* enzyme systems. We found AK37 was able to stabilize a DNA–Topo I cleavable complex, but not a DNA–Topo II cleavable complex. To our knowledge, this is the first report of a DNA–Topo I cleavable complex stabilizing pyridoacridine. Structure comparison studies demonstrated that this activity was lost when an extra 'F' ring was added, but activity was not affected when the 'D' ring was removed. AK37 inhibited the catalytic activity of both human Topo I and II. *Anti-Cancer Drugs* 15:907–913 © 2004 Lippincott Williams & Wilkins.

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Introduction

Pyridoacridine alkaloids are marine natural products, isolated from many sponges and ascidians, as well as a mollusk and a coelenterate [1–3]. The first pyridoacridine, amphimedine, was reported as a cytotoxic component of the sponge *Amphimedon* in 1983 by Schmitz *et al.* [4]. Since 1983, over 50 pyridoacridines have been described; however, the pharmacological activities of only about half this number have been studied.

Almost all the pyridoacridines reported are cytotoxic to mammalian cells in culture. Some possess anti-viral (e.g. dercitin) [5] or fungicidal (e.g. meridine) [6] activities. More specific activities include stabilization of the topoisomerase (Topo) II–DNA cleavable complex, promotion of Topo II-mediated DNA catenation (e.g. neoamphimedine [7]), inhibition of Topo I and II catalytic activity or generation of reactive oxygen species (e.g. ascididemin [8,9]), while others stimulate calcium release from the sarcoplasmic reticulum (e.g. cystodytin A [10]), interact with the adenosine receptor or inhibit neuronal differentiation in cultured cells [11]. Almost all pyridoacridines are capable of DNA intercalation due to their planar structures [12,13]. However, their structures can vary extensively. Slightly different pharmacophores may possess significantly different activities, as seen with the amphimedines [7,14]. Until now, no pyridoacridine has been reported to stabilize the DNA–Topo I cleavable complex.

Previously, Lindsay *et al.* reported the structural requirements for the biological activity of the pyridoacridine, ascididemin [15]. Matsumoto *et al.* reported the mechanism of action of ascididemin [8,9], demonstrating that it and its analog BC109 cleaved DNA through the generation of reactive oxygen species (ROS), independent of the Topo enzymes. Two other analogs, BC31 and BC21, were also described by Matsumoto *et al.* and, while they were cytotoxic, they did not generate ROS nor stabilize the Topo II cleavable complex [8,9].

Here we have described the activity of AK37 (previously reported as BC31) (Fig. 1). AK37 differs from ascididemin only slightly in that one nitrogen has been replaced by a carbon bonded to hydrogen. Originally we hypothesized that AK37 would be cytotoxic as a result of non-specific inhibition of Topo I and II catalytic activity, but not the stabilization of either the Topo I– or II–DNA cleavable complexes. However, this hypothesis was only partially correct. We show here that while AK37 was able to inhibit the catalytic activity of both Topo I and II, it was also able to stabilize the DNA–Topo I cleavable complex. This is the first pyridoacridine described with this activity.

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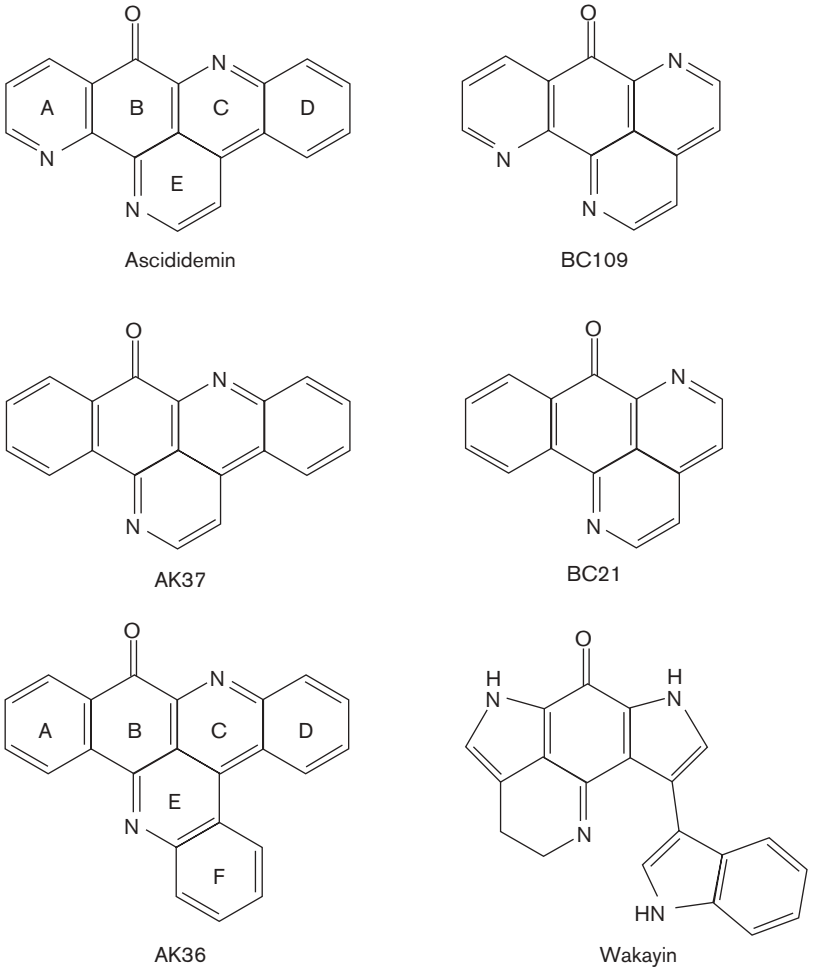
Fig. 1



Structures of acsididemin, BC109, AK37, BC21, AK36 and wakayin. Ring designations of ascididemin and AK36 are shown.

Topo I is an important enzyme target in cancer chemotherapy because it facilitates DNA relaxation. It is important in DNA repair, transcription and replication. However, there are few drugs that selectively target Topo I. Currently used Topo I drugs include the camptothecin derivatives, irinotecan and topotecan. They are approved for the treatment of colorectal and ovarian cancer, respectively, and stabilize the DNA–Topo I cleavable complex without first intercalating DNA [16–19].

Because of the clinical potential of new Topo I drugs, we made further comparisons of AK37 to other similar structures; AK36, which had an extra benzenoid ring, or BC21, which has a deleted benzenoid ring. We also compared AK37 to other known DNA–Topo I cleavable complex stabilizers. 9-aminocamptothecin (9AC) is a water soluble camptothecin derivative, similar to the camptothecins mentioned above [16–19]. Wakayin is a bispyrroloiminoquinone that stabilizes DNA–Topo I cleavable complexes, in addition to intercalating DNA and inhibiting both Topo I- and II-mediated DNA relaxation [20].

Materials and methods

Chemicals and reagents

AK37 and AK36 were synthesized by Dr Avi Koller in the chemical laboratory of Dr Yoel Kashman and initial cytotoxic analysis was reported [21]. Drug standards were purchased from Sigma (St Louis, MO). Radioactive thymidine was purchased from New England Nuclear (Boston, MA). Restriction enzymes and buffers were purchased from New England Biolabs (Beverly, MA). All additional chemicals were purchased from Sigma or Baker Chemical (Springfield, NJ). Radiolabeled (4.4×10^3 c.p.m./g) ^{3}H replicative form (rf) of M13 mp19 was isolated by the alkaline lysis method as described [8,9]. 9AC was the generous gift of Dr Monroe Wall of

Research Triangle Park. Wakayin, BC31 and BC21 were previously studied by our group [8,9,15,20,22].

Cell culture

The human colon tumor cell line (HCT-116), human epidermoid-nasopharyngeal tumor cell line (KB), human melanoma cell line (SK-mel-5), human breast cancer cell line (MCF7), human ovarian multi-drug resistant cell lines (A2780 and A2780AD), and Chinese hamster ovary cell lines (CHO): AA8 (wild-type) and EM9 (single strand break repair deficient) were used. HCT-116, KB, SK-mel-5, MCF7, EM9 and AA8 cell lines were purchased from ATCC. A2780 and A2780AD cell lines were provided by Dr Jindrich Kopecek (University of Utah). All cell lines, except HCT-116, were maintained in α-MEM. CHO cell lines were supplemented with 10% fetal bovine serum, and the others were supplemented with 2.5% fetal bovine serum and 7.5% calf sera (Atlanta Biologicals, Atlanta, GA). All cultures received 100 U/ml penicillin and 100 µg/ml streptomycin. HCT-116 cells were maintained in McCoy's medium similarly enriched with 2.5% fetal bovine serum, 7.5% newborn serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown at 37°C as monolayers in 75-cm^2 culture flasks and detached with trypsin before seeding into microtiter culture plates.

MTT cell cytotoxicity assay

Cytotoxicity was established in a MTT assay as performed by Mosmann [23] and modified by others [20,24]. Drugs were dissolved in 100% DMSO at initial concentrations of 10 mM and serially diluted. The final concentration of DMSO in the cell culture wells was 1% or less. Cells were seeded in 200 µl of growth media in Corning 96-well microtiter plates at 30 000 (CHO) or 20 000 cells/well (human cell lines). Four hours after seeding, cells were treated, each dose in quadruplicate, with 2 µl of drug (CHO cell lines) or 1 µl of drug (human cell lines). CHO cell lines were re-fed at 18 h. After 72 h, all cultures were re-fed with 100 µl McCoy's medium and 11 µl MTT (5 mg/ml in PBS, pH 7.4) was added to each well. The plates were incubated for 4 h at 37°C. Viable cells reduced MTT to a purple formazan product that was solubilized by the addition of 100 µl DMSO to aspirated culture wells. The absorbance at 540 nm was measured for each well using a Bio-Rad MP450 plate reader. Following subtraction of absorbance from blank culture cells, the average absorbance for each set of drug-treated wells was compared to the average absorbance of the control wells to determine the fractional survival at any particular drug concentration. The inhibitory concentration 50 (IC_{50}) was defined as the drug concentration that yielded a fractional survival of 50%.

EtBr displacement assay

Using the protocol of McDonald *et al.* [9,20,25], the intercalation activity of molecules tested was determined as the ability to displace EtBr from DNA. Reactions were performed in 96-well plates containing 25 µg/ml salmon sperm DNA, 0.5 µg/ml EtBr and a log range of sample concentrations. A decrease in fluorescence at 600 nm correlated with the displacement of EtBr from the DNA. The K_s value was determined as the concentration of sample required to decrease EtBr fluorescence by 50%, determined using a logit-log plot [26].

Isolation of human Topo I from human placenta

Human Topo I was isolated from human placentas as described [27].

Isolation of human Topo II from yeast

Human Topo IIα was induced and isolated from yeast strain BCY 123, containing plasmid YEpWOb6 as described [7,22]. Enzymatic activity was determined by assaying for decatenation of kinetoplast DNA [28]. The purified 170-kDa species of Topo II was identified by denaturing PAGE analysis [29].

Quantification of DNA cleavage

DNA cleavage assays were performed as described [7,22]. In brief, 20 µl volumes containing 50 mM Tris–HCl (pH 7.5), 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 30 µg/ml bovine serum albumin, 2 mM DTT, 500 ng of radiolabeled and supercoiled rf M13 mp 19 DNA, and 100–150 ng purified Topo I or 80–120 ng purified Topo II (unless otherwise noted) was treated with drug (in DMSO) and incubated at 30°C for 30 min. The reactions were stopped by the addition of 2 µl of 1.5 mg/ml proteinase K in 0.5% SDS and incubated at 37°C for 60 min. The DNA was resolved by electrophoresis in 0.8% agarose (containing 50 ng EtBr/ml TAE) to separate the nicked, linear, relaxed and supercoiled isomers. EtBr-stained DNA was visualized by its fluorescence under UV light.

Time and concentration dependence of DNA cleavage

To determine Topo I time or concentration dependency of DNA cleavage *in vitro*, DNA cleavage assays were performed as described above using different time points of incubation (0, 0.5, 1.0, 2.0, 4.0, 8.0 and 30 min) or increasing concentrations of AK37 (0.1–500 µM). It is common to use micromolar concentrations in these analyses in order to provide enough DNA damage to visualize in the cleavage gel. However, lesser cleavage accomplished by much lower drug concentrations is sufficient to induce cytotoxicity in cultured cell lines [30,31].

Reversal of DNA–Topo I cleavage

The ability of heat or high salt to reverse Topo I drug-induced DNA cleavage was determined using the cleavage reactions described above. After the 30-min incubation and prior to the addition of SDS and proteinase K, the reactions were either heated to 63°C

or brought to a concentration of 0.5 M NaCl for 0.5–10 min. At this point, SDS and proteinase K were added and incubated as described above prior to running the reactions on an EtBr agarose gel. Under heat or high salt, the enzyme detaches from the DNA leaving an intact double strand helix.

Topoisomerase inhibition assays

Reactions were prepared as described above with the exception that the concentrations of Topo I or the test compound were altered as described. Reactions were incubated at 30°C for 30 min prior to the addition of an SDS-loading dye, which was followed directly by gel electrophoresis in a 0.8% agarose gel without EtBr. To visualize the DNA, EtBr was added after DNA electrophoresis by soaking the gel for 20 min in water containing EtBr (1 mg/ml).

Table 1 IC_{50} values determined for AK37 in mammalian cell lines (IC_{50} values in μM were determined using the MTT microtiter plate assay)

Cell line	IC_{50}
HCT-116	30
SK-mel-5	0.13
KB	11
MCF7	7.7
A2780wt	12
A2780AD	30
AA8	18
EM9	4.0

Values are the average of two to three independent experiments, $n=4$ for each experiment.

Results

AK37 cytotoxicity

AK37 and AK36 were tested for cytotoxicity in the HCT-116 cell line. AK36 was not toxic in the HCT-116 cell line up to 100 μM. The cytotoxicity of AK37 was further determined in several other human tumor cell lines, in addition to a set of mutated CHO cell lines: AA8 and EM9. Table 1 lists the IC_{50} values extrapolated from the cytotoxic curves generated by using a log range of drug concentration to treat the cells. The differential at the IC_{50} concentrations was approximately 4-fold in the CHO cell lines, indicating the production of single-strand breaks in treated cells, indicative of a drug that stabilizes DNA–Topo I cleavable complexes.

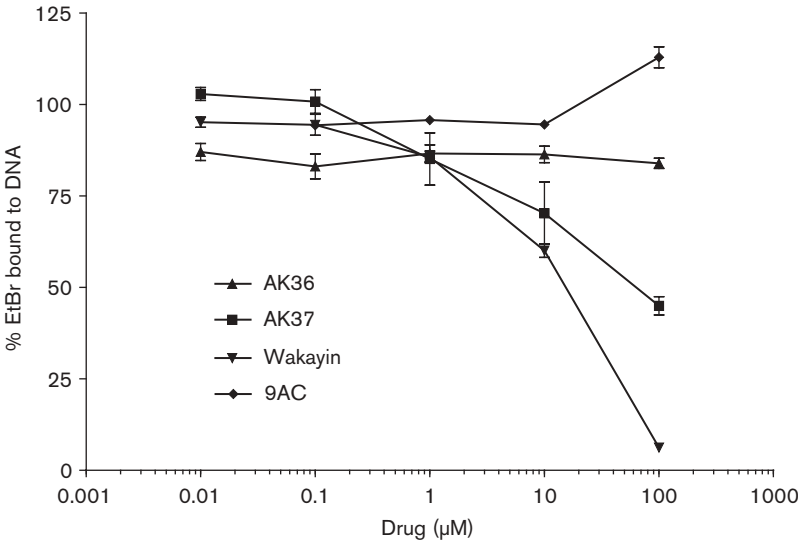
Assessment of DNA intercalation

AK37, AK36, wakayin and 9AC were evaluated for their ability to displace intercalated EtBr from DNA, as a measure of DNA intercalation (Fig. 2). As drugs intercalate DNA, EtBr is displaced, resulting in a decrease in absorbance at 600 nm [9,20,25]. AK36 and 9AC did not intercalate DNA at tested concentrations and the K_s values were not determined. Both AK37 and wakayin were able to displace EtBr from DNA, the latter completely. The K_s values for AK37 and wakayin were 70.8 and 11.2 μM, respectively.

Quantification of *in vitro* DNA cleavage

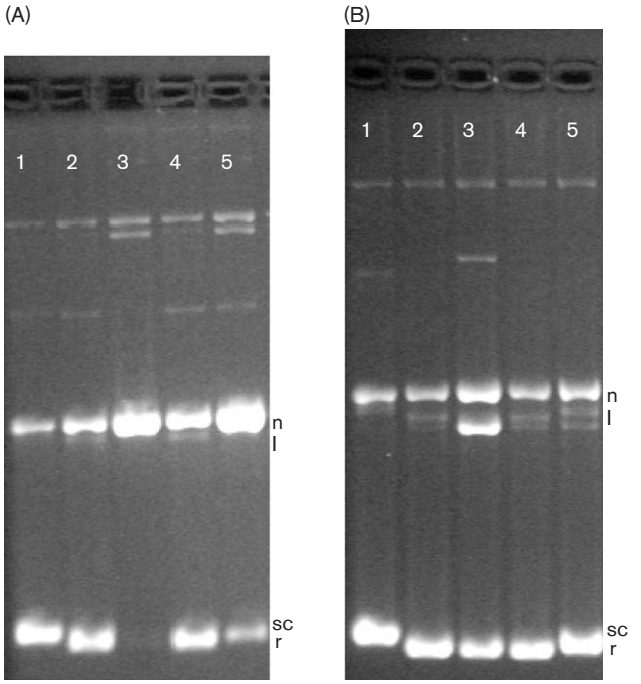
AK36 and AK37 were assayed in DNA cleavage gel assays to determine the specificity of Topo I or II inhibition, or alternatively, the ability to produce DNA-damaging ROS.

Fig. 2



EtBr displacement by the synthetic pyridoacridines as a measure of DNA intercalation. Intercalation data comparing AK37 to an inactive analog (i.e. AK36), an intercalating DNA–Topo I cleavable complex stabilizer (i.e. wakayin) and a non-intercalating DNA–Topo I cleavable complex stabilizer (i.e. 9AC).

Fig. 3



Topo I and II DNA cleavage gels of the synthetic pyridoacridines. (A) Cleavage assay gel of tested compounds using Topo I. Lanes are as follows from left to right: (1) DNA only, (2) DNA + Topo I, (3) DNA + Topo I + 9 μM 9AC, (4) DNA + Topo I + AK36 and (5) DNA + Topo I + AK37. (B) Cleavage assay gel of tested compounds and Topo II. Lanes are as follows from left to right: (1) DNA only, (2) DNA + Topo II, (3) DNA + Topo II + 50 μM etoposide, (4) DNA + Topo II + AK36 and (5) DNA + Topo II + AK37. All compounds were tested at 100 μM, unless noted. Bands representing DNA isoforms separated by electrophoresis are indicated as n = nicked, l = linear, sc = supercoiled and r = relaxed.

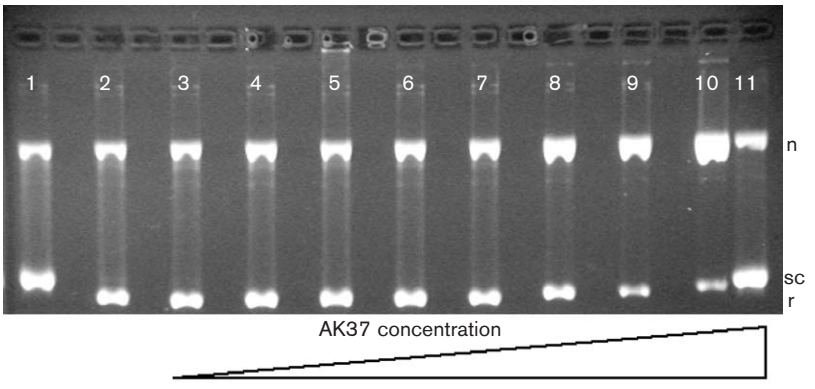
Figure 3 shows both Topo I and II cleavage gels, where cleavage is indicated by increased DNA in nicked or linear bands. Enzyme inhibition is indicated by increased DNA in the supercoiled bands. While there may have been small increases in DNA cleavage observed in both Topo I and II gels for the majority of the compounds, the differences were not significant. AK36 did not stabilize Topo I or II–DNA cleavable complexes.

AK37 inhibited the Topo II-mediated relaxation of supercoiled DNA without causing an increase in Topo II–DNA cleavage [9]. However, the analysis showed that AK37 was able to stabilize the DNA–Topo I cleavable complex, resulting in DNA nicking and inhibition of Topo I catalytic activity (i.e. relaxation). This was unexpected since no pyridoacridines were known to stabilize DNA–Topo I cleavable complexes. Therefore, we explored this mechanism using quantitative cleavage assays.

Time and concentration dependence of AK37-Topo I DNA cleavage

AK37 was assayed to determine the time required to see Topo I-mediated DNA cleavage *in vitro*. Cleavage was complete within 30 s of initiation of the 30°C incubation. This was comparable to 9AC, which also cleaves DNA via Topo I within 30 s at 30°C and is characteristic of Topo I drugs [32]. AK37 cleavage was dependent on drug concentration as well (Fig. 4). AK37 was incubated in the reactions at concentrations of 0.1–500 μM in the presence of Topo I and at 500 μM without Topo I. Increasing amounts of DNA cleavage were observed with increasing amounts of AK37. AK37 at 500 μM in the absence of Topo I did not produce an increase in the nicked band of DNA resolved in the gel, illustrating the requirement for Topo I.

Fig. 4



Concentration dependence of AK37-induced DNA–Topo I cleavage. (A) Cleavage assay gel, lanes are as listed from left to right: (1) DNA only, (2) DNA + Topo I, (3) DNA + Topo I + 0.1 μM AK37, (4) DNA + Topo I + 0.5 μM AK37, (5) DNA + Topo I + 1 μM AK37, (6) DNA + Topo I + 5 μM AK37, (7) DNA + Topo I + 10 μM AK37, (8) DNA + Topo I + 50 μM AK37, (9) DNA + Topo I + 100 μM AK37, (10) DNA + Topo I + 500 μM AK37 and (11) DNA + 500 μM AK37. Bands representing DNA isoforms separated by electrophoresis are indicated as n = nicked, l = linear, sc = supercoiled and r = relaxed.

Reversal of AK37 cleavable complexes

It is characteristic of stabilized DNA–Topo I cleavable complexes to be reversed by heat or high salt concentrations [32]. The AK37 DNA–Topo I-stabilized cleavable complexes were reversed in heat or high salt. The amount of nicked DNA detected in the gel was diminished within 30 s of incubation at 63°C or following the addition of salt to the reaction (0.5 M NaCl, Fig. 5). It is interesting to note that when the 9AC complex was reversed it produced relaxed plasmid, whereas the reversal of AK37 Topo I complexes released supercoiled plasmid, suggesting these two compounds may act to stabilize the DNA–Topo I cleavable complex by different mechanisms.

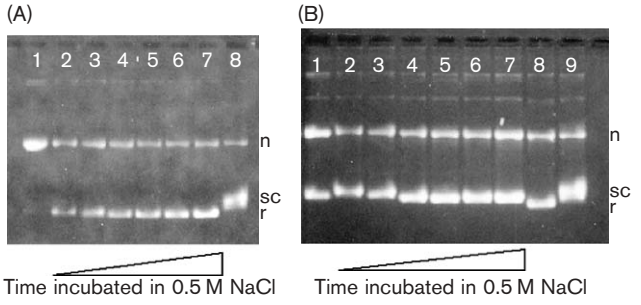
Ability of BC21 to stabilize DNA–Topo I cleavable complexes *in vitro*

It was hypothesized that BC21 (i.e. AK37 minus the 'D' ring) would retain the Topo I-directed activity of AK37 because the analogous asididemin pharmacophore minimized by removing the 'D' ring (i.e. BC109) retained its activity [9]. Small amounts of BC21 remaining from the studies of Matsumoto *et al.* provided a means to test this hypothesis. It was determined that, indeed, both AK37 (i.e. BC31) and BC21 stabilize the DNA–Topo I cleavable complex at 100 μM (data not shown).

Catalytic inhibition of the Topos

AK37 inhibits the Topo I-mediated relaxation of supercoiled DNA *in vitro* at a concentration between 2.5 and 5 μM. AK36 did not inhibit Topo I relaxation of DNA up to 100 μM. Both AK36 and AK37 inhibited Topo II catalytic activity; however, it was found that it required a great deal more AK36 than AK37 to see this effect on Topo II inhibition, 500 versus 10 μM (data not shown).

Fig. 5



AK37 and 9AC DNA–Topo I cleavable complex reversal by high salt concentration in EtBr-containing agarose gels. (A) Reversal of 9AC DNA–Topo I cleavage. Lanes are as follows from left to right: (1) DNA + Topo I + 4.5 μM 9AC at 0 min salt, (2) DNA + Topo I + 4.5 μM 9AC at 1.0 min salt, (3) DNA + Topo I + 4.5 μM 9AC at 2.0 min salt, (4) DNA + Topo I + 4.5 μM 9AC at 5.0 min salt, (5) DNA + Topo I + 4.5 μM 9AC at 10.0 min salt, (6) DNA + Topo I + 4.5 μM 9AC at 20.0 min salt, (7) DNA + Topo I and (8) DNA only. (B) Reversal of AK37 DNA–Topo I cleavage. Lanes are as follows from left to right: (1) DNA + Topo I + 100 μM AK37 at 0 min salt, (2) DNA + Topo I + 100 μM AK37 at 1.0 min salt, (3) DNA + Topo I + 100 μM AK37 at 2.0 min salt, (4) DNA + Topo I + 100 μM AK37 at 5.0 min salt, (5) DNA + Topo I + 100 μM AK37 at 10.0 min salt, (6) DNA + Topo I + 100 μM AK37 at 20.0 min salt, (7) DNA + Topo I + 100 μM AK37 at 40.0 min salt, (8) DNA + Topo I and (9) DNA only. Bands representing DNA isoforms separated by electrophoresis are indicated as n = nicked, l = linear, sc = supercoiled and r = relaxed.

Discussion

AK37 demonstrated a specific DNA–Topo I-directed activity *in vitro*, it acted much like a clinical Topo I drug. It selectively stabilized the DNA–Topo I cleavable complex, but not the Topo II–DNA cleavable complex, in a time- and concentration-dependent manner. Similar to 9AC, AK37 could produce this stabilized cleavable complex within 30 s and have this complex fully reversed by heat or salt within 30 s, characteristic of Topo I poisons [32]. However, unlike 9AC, the product of the AK37 DNA–Topo I cleavable complex reversal was supercoiled DNA, not relaxed DNA, suggesting that the state of the AK37 cleavable complex is significantly different from that of 9AC. As one might expect, we found that AK37 could also inhibit Topo I catalytic activity. AK37 can also inhibit the catalytic activity of Topo II [9]. BC21, the 4-ring version of the 5-ring AK37, was also able to stabilize DNA–Topo I cleavable complexes, indicating that the 4-ring structure contains the pharmacophore of AK37.

AK37 was cytotoxic to several cultured tumor cell lines. Cytotoxicity that results from DNA strand breakage occurs at much lower doses of drug than used *in vitro* [30,31]. High concentrations of drug and Topo are used *in vitro* to allow visual detection of the nicked DNA following electrophoresis. We observed that AK37 was exceptionally toxic in the SK-mel-5 cell line and would like to study this further to determine if other melanoma cell lines will exhibit similar sensitivity. In addition, AK37 had enhanced cytotoxicity in the mutated cell line (EM9) that is sensitive to agents that cause single strand DNA breaks (i.e. Topo I poisons). Because the previous data was indicative of DNA–Topo I cleavable complex stabilization and not ROS generation, the cell data strengthened the conclusion that AK37 is a Topo I poison in mammalian cells. While these experiments were not identical to those reported earlier [15], these data contradict the earlier conclusion that AK37 is not a Topo I-directed drug. The results presented here show clearly that AK37 is capable of DNA–Topo I cleavable complex stabilization.

Overall, we conclude that AK37 stabilizes DNA–Topo I cleavable complexes, but that it is not a pure Topo I inhibitor. We conclude the extra 'F' ring on AK36 prevents it from being able to intercalate DNA and so it is less cytotoxic. This ring also abrogates the ability of AK36 to stabilize DNA–Topo I cleavable complexes. In comparison to asididemin, the alteration of the nitrogen in the 'A' ring changes AK37 from being a ROS-generating

drug (ascididemin) to a DNA–Topo I cleavable complex stabilizing drug. In addition, the loss of the 'D' ring from AK37, to form BC21, does not prevent DNA–Topo I cleavable complex stabilization and therefore is not necessary for its Topo I activity.

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