Quinobenoxazines: A Class of Novel Antitumor Quinolones and Potent Mammalian DNA Topoisomerase II Catalytic Inhibitors†

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Received April 15, 1994; Revised Manuscript Received July 5, 1994®

ABSTRACT: The antineoplastic quinobenoxazines A-62176 and A-74932 were shown to be potent inhibitors of mammalian DNA topoisomerase II *in vivo*. This was demonstrated by their selective inhibition of the SV40 DNA replication stages that require topoisomerase II. Neither drug stabilized a covalent complex of the enzyme with SV40 DNA, which suggests that they are not poisons of DNA topoisomerase II. A-77601, an analog having little antitumor activity, barely inhibited DNA topoisomerase II *in vivo*, even at high concentrations. These findings were supported by *in vitro* studies which showed that A-62176 and A-74932, but not A-77601, strongly inhibited the catalytic activity of mammalian DNA topoisomerase II. A-62176 did not cause topoisomerase II-mediated DNA strand breaks *in vitro* under conditions in which adriamycin produced extensive DNA breakage. The antineoplastic and topoisomerase inhibitory activities of the quinobenoxazines correlate with their ability to unwind DNA. A-62176 antagonized the poisoning of topoisomerase II by VM-26 *in vivo* and *in vitro*, but had no effect on DNA breakage induced by camptothecin, a DNA topoisomerase I poison. A-62176 and A-74932 thus inhibit DNA topoisomerase II reactions at a step prior to the formation of the "cleavable complex" intermediate. These findings indicate that stabilization of the DNA topoisomerase II–DNA cleavable complex is not necessary for the antitumor activity of this class of quinolones and that the catalytic inhibition of DNA topoisomerase II may contribute significantly to the anticancer activity of other DNA topoisomerase II inhibitors.

A-62176 and its prodrug A-74932 are two members of a new family of quinolones, the quinobenoxazines (structures in Table 1). They show good activity against a large number of human cancer cell lines, several mouse intraperitoneal tumors (including the multidrug resistant P388/ADR line), and human tumor xenografts (Chu et al., 1992). A-74932 was substantially less toxic to mice than adriamycin, cisplatin, and methotrexate (Chu et al., 1992). The quinobenoxazines did not cause topoisomerase II-mediated DNA strand breaks, indicating that they are not poisons of this enzyme (L. Shen, unpublished data). Topoisomerase poisons stabilize a reaction intermediate, the cleavable complex, in which the topoisomerase is covalently attached to the DNA; after addition of a protein denaturant, the DNA is broken, and the enzyme is covalently bound to the revealed 5′ ends (Liu, 1989). Topoisomerase II poisons such as adriamycin, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA),[1] and VM-26 are useful anticancer drugs. Quinolones are bacterial DNA topoisomerase II (DNA gyrase) poisons (Kreuzer & Cozzarelli, 1979). More recently some quinolones were also shown to stabilize the mammalian topoisomerase II–DNA cleavage complex (Kohlbrenner et al., 1992; Barrett et al., 1989; Elsea et al., 1993; Gootz et al., 1994). Thus, the failure of quinobenoxazines to stabilize the mammalian topoisomerase II–DNA cleavage complex was surprising.

As shown in this study, low doses of A-62176 and A-74932 caused heavy accumulations of highly catenated SV40 daughter chromosomes. This is a signature of topoisomerase II inhibition (DiNardo et al., 1984; Varshavsky et al., 1983; Snapka, 1986). High concentrations of A-77601, an analog with very low anticancer activity, caused only marginal inhibition of topoisomerase II *in vivo*. Unlike several well-known DNA topoisomerase II poisons (Shin et al., 1990), these drugs did not induce covalent linkage of topoisomerase II to replicating SV40 chromosomes. Our *in vitro* studies using purified mammalian DNA topoisomerase II showed that A-62176 strongly inhibits DNA topoisomerase II reactions without stabilizing a cleavable complex. The antineoplastic activity of the quinobenoxazines was shown to correlate with their strength as topoisomerase II catalytic inhibitors and with their ability to unwind DNA.

Other drugs which inhibit topoisomerase II without significantly stabilizing cleavable complexes have been reported to have anticancer activity. ICRF-193 does not intercalate into DNA, but appears to interact with the topoisomerase itself (Ishida et al., 1991; Roca et al., 1994). This drug and its analogs have anticancer activity and are undergoing clinical trials. ICRF-193 inhibits DNA topoisomerase II reactions at a step prior to DNA cleavage (Tanabe et al., 1991). This compound slows the late stages of SV40 replication known to require DNA topoisomerase II (Ishimi et al., 1992a) and acts as an antagonist to DNA topoisomerase II poisons, preventing them from stabilizing the cleavable complex. ICRF-193 gave a 50% inhibition of calf thymus DNA topoisomerase II decatenation at 2 μM, a concentration which does not detectably increase topoisomerase II-mediated DNA strand breaks *in vitro*. However, at approximately a 10-fold higher concentration (30 μM), ICRF-193 did cause weak topoi-

† This work was supported by Ohio State University Comprehensive Cancer Center Grant P30CA 16058-19.

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® Abstract published in *Advance ACS Abstracts,* September 1, 1994.

[1] Abbreviations: SV40, simian virus 40; [^{3}H]dT, [*methyl*-^{3}H]thymidine; DMSO, dimethyl sulfoxide; k-DNA, kinetoplast DNA; EDTA, ethylenediaminetetraacetate; VM-26, teniposide; m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; DTT, dithiothreitol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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somerase II-mediated DNA strand breaks (Tanabe et al., 1991). Merbarone, another antineoplastic drug, appears to have a similar mechanism (Drake et al., 1989). This catalytic inhibitor also acts as an antagonist of DNA topoisomerase II poisons. At low concentrations, it strongly inhibits DNA topoisomerase II without stabilizing cleavable complexes. At high concentrations, it is a weak topoisomerase II poison.

In contrast, the quinobenoxazines A-62176 and A-74932 only decreased topoisomerase II cleavage complexes—even at high concentrations. The finding that these new antineoplastic quinolones are strong catalytic inhibitors of DNA topoisomerase II but not topoisomerase poisons supports the idea that catalytic inhibition may play an important role in the efficacy of other antineoplastic DNA topoisomerase II inhibitors.

MATERIALS AND METHODS

Virus Infection, Pulse-Labeling, Drug Treatments, and Preparation of SV40 DNA. African green monkey kidney cells (CV-1) were infected with plaque-purified SV40 (strain 777) as described (Snapka, 1986). Experiments were carried out 36 h after infection, the peak of viral DNA replication. Replicating viral chromosomes were labeled with [*methyl*-^{3}H]thymidine ([^{3}H]dT, 250 μCi/mL) for 30 min in serum-free medium. Drugs were added 15 min after the start of labeling. Intracellular reactions were stopped by addition of Hirt lysing fluid to the cell layers, and samples were processed as described (Snapka, 1986).

Cytotoxicity Studies. Cytotoxicity studies were carried out as described by Chu et al. (1992).

Chemicals and Reagents. VM-26 (teniposide; NSC 122819) was a gift from Bristol Myers. Camptothecin and adriamycin were purchased from Sigma Chemical Co. A-62176, A-74932, and A-77601 were synthesized at Abbott Laboratories. Purified calf thymus DNA topoisomerase II was kindly provided by D. Weigl and W. Kohlbrenner, previously at Abbott, and purified DNA topoisomerase I was purchased from BRL. ColE1 DNA was provided by J. Baranowski at Abbott, and P4 knotted DNA was provided by A. Saldivar and N. Wideburg at Abbott. Kinetoplast catenated DNA was from Topogen Inc.

GF/C Filter Assay. The GF/C filter assay measures protein cross-links to pulse-labeled SV40 DNA (Shin et al., 1990). It is based on the observation that proteins bind tightly to GF/C filters (Whatman) in 0.4 M guanidinium chloride. DNA is not retained under these conditions unless it is linked to a protein. At a 10-fold higher concentration of guanidinium chloride (4.0 M), DNA binds to GF/C filters quantitatively. Thus, filter binding in 4.0 M guanidinium chloride can be used as a measure of total labeled DNA in an aliquot. The assay is not sensitive to detergents and can be performed on Hirt extract supernatants. By filtering identical aliquots of labeled SV40 DNA in 0.4 M and 4.0 M guanidinium chloride, one can determine the fraction of SV40 DNA cross-linked to protein. Assays were performed with 10 μL aliquots of the Hirt supernatant. Results are reported as "percent of maximum binding" which is the percentage of total labeled DNA cross-linked to protein. This assay has been used to quantitate the covalent trapping of topoisomerases on SV40 DNA by topoisomerase poisons (Shin et al., 1990).

Drugs which inhibit topoisomerase II reactions at steps prior to DNA cleavage can prevent topoisomerase II poisons from stabilizing cleavable complexes (Tanabe et al., 1991). A-62176 was thus studied for its ability to prevent the topoisomerase II poison VM-26 from stabilizing cleavable complexes on SV40 DNA *in vivo*. Infected cells were labeled for 35 min with [^{3}H]dT (250 μCi/mL). A-62176 or solvent (DMSO) was added after 15 min of labeling, and VM-26 (100 μM) was added 5 min later. After 35 min, the labeling medium was removed, and SV40 DNA was selectively extracted by the method of Hirt (1967). Aliquots of the Hirt supernatant were assayed for protein–DNA cross-links. In the controls, protein was removed by treatment with proteinase K (0.1 mg/mL for 4 h at 45 °C). To test for A-62176 interference with topoisomerase I cleavable complexes stabilized by camptothecin, 40 μM camptothecin replaced the VM-26.

Gel Electrophoretic Analysis of SV40 DNA Replication Intermediates. For electrophoretic analysis, the Hirt supernatant was treated with proteinase K (0.1 mg/mL, 45 °C, 4 h). The DNA was extracted with chloroform–2-propanol (24:1) and precipitated with 2.5 volumes of ice-cold 95% ethanol. After brief drying under vacuum, the DNA pellet was dissolved in gel loading buffer. One- and two-dimensional gel electrophoresis and fluorography of SV40 DNA replication intermediates were performed as described (Snapka et al., 1991).

Assay of Inhibition of Catalysis by DNA Topoisomerase II. The P4 DNA unknotting assay for topoisomerase II was previously described (Shen et al., 1992). One unit is defined as the amount of enzyme required to unknot 0.1 μg of the knotted DNA substrate. Kinetoplast DNA decatenation by calf thymus DNA topoisomerase II (Miller et al., 1981) was also used for assessing inhibition by test compounds. The k-DNA decatenation reaction mixture (20 μL final volume) contains 50 mM Tris, pH 8.3, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 2 mM ATP, 30 μg/mL BSA, 0.2 μg of k-DNA, 1 unit of calf thymus DNA topoisomerase II, and specified amounts of the test compound. The reaction was initiated by addition of the enzyme, and incubation was at 37 °C for 30 min. The reactions were terminated by the addition of 5 μL of SDS–ficoll–bromophenol blue (2%–14%–0.08%). The sample was electrophoresed in a 1.4% agarose gel for 16 h and stained with ethidium bromide.

Assays for DNA Nicking and Cleavage by Topoisomerases. Procedures for assay of DNA cleavage by topoisomerase II have been described (Shen et al., 1992). The assay of DNA nicking by topoisomerase I was performed as previously described (Fostel et al., 1992), with minor modifications. Supercoiled ColE1 DNA (0.25 μg) was incubated with the drug and DNA topoisomerase I (10 units) in (final volume 20 μL) 50 mM Tris-HCl, pH 7.5, 120 mM KCl, 1 mM EDTA, 1 mM DTT, 30 μg/mL BSA, and 10 mM $MgCl_2$. After 10 min at 37 °C, the reaction was stopped by the addition of 2 μL of warm solution containing 2 mg/mL proteinase K and 2.5% SDS. After 30 min at 37 °C, 5 μL of ficoll–bromophenol blue solution was added. The reaction mixture was electrophoresed on a 1% agarose gel with 0.5 μg/mL ethidium bromide in the running buffer.

DNA Unwinding Assay. The unwinding assay utilized plasmid DNA and a eukaryotic type I topoisomerase (Pommier et al., 1987). The relaxed form of ColE1 DNA was prepared using calf thymus DNA topoisomerase I (Otter & Cozzarelli, 1983). Unwinding by quinolones was tested in two sets running on the same agarose gel. One set used supercoiled DNA, and the other set used relaxed DNA. Unwinding, indicated by a topoisomer shift, should be identical for both sets for a given concentration of drug. ColE1 DNA (0.25 μg) was incubated with the drug for 10 min at room temperature in (20 μL) 50 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM Na-EDTA, 1 mM DTT, 30 μg/mol BSA, and 10 mM $MgCl_2$. An excess of topoisomerase I (about 20 relaxation units) was then added and incubation continued for an additional 60 min at 37 °C

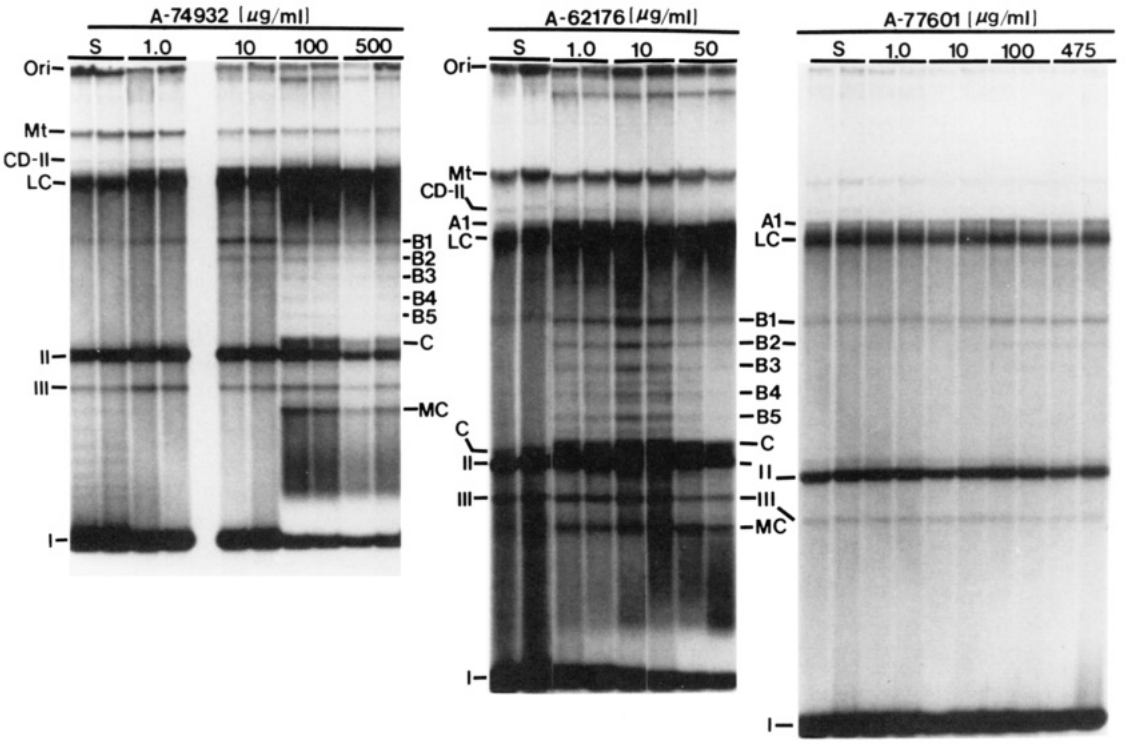


FIGURE 1: Accumulation of catenated SV40 daughter chromosomes in the presence of quinobenoxazines. S, solvent controls; Ori, origin of electrophoresis; Mt, mitochondrial DNA; I, form I (superhelical) SV40 DNA; II, form II (nicked circular) SV40 DNA; III, form III (linear) SV40 DNA; LC, late Cairns replication intermediates (late replication intermediates with the terminus region unreplicated); A1, A-family catenated SV40 dimer (both daughter chromosomes nicked) with a catenation linking number of 1; B1–B5, B-family catenated dimers (one daughter chromosome nicked and one superhelical) with the catenation linking number indicated by the number; C, unresolved C-family catenated dimers (both daughter chromosomes superhelical) and form I circular dimer (head-to-tail dimer); CD-II, form II circular (head-to-tail) dimer; MC, a pseudoband at the point where B-family dimer bands are no longer resolved.

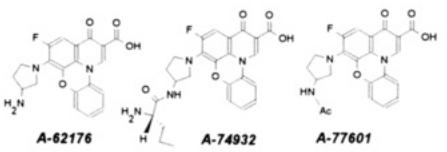
before addition of the SDS–ficoll–dye mixture (5 μL) and electrophoresis. The unwinding concentration is defined as the drug concentration that causes a shift of the topoisomers from the original position to the middle position between the relaxed and the supercoiled DNA bands.

RESULTS

Inhibition of DNA Topoisomerase II in Vivo. When either A-62176 or A-74932 was added to pulse-labeled SV40-infected cells during the pulse-labeling, intense bands of catenated dimers were seen (Figure 1). Catenated dimers are topologically linked SV40 daughter chromosomes. The daughter chromosomes are linked once for each turn of the Watson–Crick helix that was not removed before completion of DNA replication. Catenated dimers are grouped in three families depending on the superhelicity of the daughter chromosomes (Varshavsky et al., 1983). In the A-family, each daughter chromosome contains a "nick" or single-strand DNA break. B-family dimers are composed of one superhelical and one nicked ring. In the C-family, both daughter chromosomes are superhelical. One-dimensional electrophoresis resolves A- and B-family catenated dimers into ladders of bands (Varshavsky et al., 1983). This is because each increase in catenation linking number causes a discrete increase in compactness. For the A- and B-families of catenated dimers, it is usually possible to assign linking numbers to bands by counting from the known positions of the A-1 and B-1 dimers. The C-family dimers are normally not resolved from one another and are only partially resolved from the form I circular (head-to-tail) dimer. Circular dimers are double-sized SV40 genomes which are normally present in trace amounts in SV40 lytic infections. After the addition of A-74932, the first intensification of catenated dimer bands (B-family), indicating slight DNA topoisomerase II inhibition, was seen at 10 μg/mL. At 100 μg/mL, this drug caused accumulations of highly catenated dimers and late Cairns structures (replication intermediates in which only the terminus region remains to be replicated). A-62176 is a much stronger inhibitor of DNA topoisomerase II *in vivo*. Highly catenated SV40 dimers accumulated at a drug concentration of only 1 μg/mL. The levels of catenation at 10 μg/mL A-62176 were comparable to those obtained with 100 μg/mL A-74932, showing that A-62176 is a stronger inhibitor of DNA topoisomerase II by about an order of magnitude. A-77601 is similar in structure to A-62176 and A-74932 but has only marginal anticancer activity (Table 1). It caused barely detectable increases in catenated SV40 dimer bands at 475 μg/mL, indicating that it is almost inactive as a DNA topoisomerase II inhibitor (Figure 1). At this same concentration, A-77601 gave barely detectable (3%) protein cross-linking to labeled SV40 DNA. Neither A-62176 nor A-74932 gave protein cross-links to SV40 DNA at any concentration.

The identity of the catenated dimer bands was confirmed by neutral-chloroquine two-dimensional gel analysis of SV40 DNA replication intermediates from cells treated with 50 μg/mL A-62176 (Figure 2). The first-dimension electrophoresis was carried out as in Figure 1, to separate viral replication intermediates on the basis of size and compactness. The gel was then soaked in a buffer containing chloroquine, and electrophoresis was carried out at a right angle to the first-dimension separation (Snapka et al., 1991). Chloroquine unwinds DNA, causing superhelical forms to become less compact. This results in pronounced changes in electrophoretic mobility for covalently closed circular forms. Since chloroquine does not change the topology of forms with single- or double-strand breaks, their electrophoretic mobility is not greatly affected. The A-family catenated dimers formed a straight diagonal, with the more highly catenated forms

Table 1: Cytotoxicity, Decatenation Inhibition, and DNA Unwinding for the Quinobenzoxazines[a]

	Quinobenoxazine		
	A-62176	A-74932	A-77601
Minimum DNA unwinding conc. (10 mM $MgCl_2$)	0.5	3	128
Decatenation Inhibition**	0.45	2.0	>100
HT29 (human colon cancer cell line) IC_{50}	0.1*	0.2*	1.4
A546 (human breast cancer cell line) IC_{50}	0.26 *	0.6*	2
P-388 (leukemia cell line) IC_{50}	0.02*	0.04*	0.5
Structure			

[a] Unit = micrograms per milliliter. One asterisk, from Chu et al. (1992). Two asterisks, drug concentration that inhibits 50% of the conversion of catenated to decatenated k-DNA catalyzed by calf thymus topoisomerase II.

predominating. The B-family catenated dimer ladder was also well resolved. However, the A- and B-family dimer ladders overlap at the highest catenation levels (Snapka et al., 1991). The "MC" band apparent in the one-dimensional gel is the point at which B-family dimers are no longer resolved (Snapka et al., 1991). In the neutral-chloroquine gel, this is the point at which the B-dimer ladder shifts sharply to the right. This behavior indicates that B-dimers lose superhelicity with increased catenation (Snapka & Permana, 1993). The C-family dimers also intensified (relative to untreated cells, Figure 2A), but remained unresolved from the circular dimer band. A ladder of bands extended out of the region of C-family dimers and downward, past the form III band. This ladder may represent C-family catenated dimers with unusually high levels of catenation, knotting, or a combination of both. The shifting of the form I band to the left in Figure 2B relative to the one in Figure 2A is typical of intercalators (Snapka et al., 1991; Chu & Hsu, 1992). This shift reflects increased negative superhelicity due to unwinding of the DNA helix by the intercalated drugs *in vivo*. The linking number is changed by intercalators *in vivo* because of the presence of topoisomerase I. A very similar two-dimensional neutral-chloroquine gel electrophoresis pattern was obtained with A-74932 (data not shown). A secondary effect of A-62176 is also evident in Figure 2; no early or intermediate replication structures are present. This means that label incorporated into intermediate Cairns structures by the time of drug addition chased into the late Cairns structure and catenated dimers. Label was not incorporated into new replicons after addition of the drug.

A-62176 Does Not Cause Topoisomerase II-Dependent DNA Strand Breaks. Since the SV40 results suggested that A-62176 and A74932 were strong inhibitors of topoisomerase II but not topoisomerase II poisons, we continued the studies using *in vitro* assays. The effects of the drugs on DNA strand cleavage by purified calf thymus topoisomerase II were investigated. Two strong DNA topoisomerase II poisons, A-75272 (Shen et al., 1992) and m-AMSA, and the weak DNA topoisomerase II poison adriamycin all gave dose-dependent increases in topoisomerase II-mediated DNA double-strand breaks. This was indicated by an increase in intensity of the form III (double-strand linear) DNA band (Figure 3). In contrast, increasing concentrations of A-62176 caused a progressive decrease in DNA strand breaks, below the background level due to the topoisomerase alone. This is expected for a drug that inhibits topoisomerase II at a stage prior to the formation of the cleavable complex.

A-62176 Is an Inhibitor of DNA Topoisomerase II Unknotting and Decatenation Reactions. A-62176 detectably inhibited unknotting by DNA topoisomerase II at 0.39 μg/mL and strongly inhibited unknotting at 1.56 μg/mL and higher concentrations (Figure 4A). A-77601, an analog with a very similar structure but lower anticancer activity, negligibly inhibited unknotting at concentrations up to 100 μg/mL. The *in vitro* catalytic inhibition of DNA topoisomerase II by A-62176 was also evident in the decatenation assay (Figure 4B). Here inhibition by A-62176 was detected at 0.5 μg/mL. Higher concentrations of this drug completely blocked

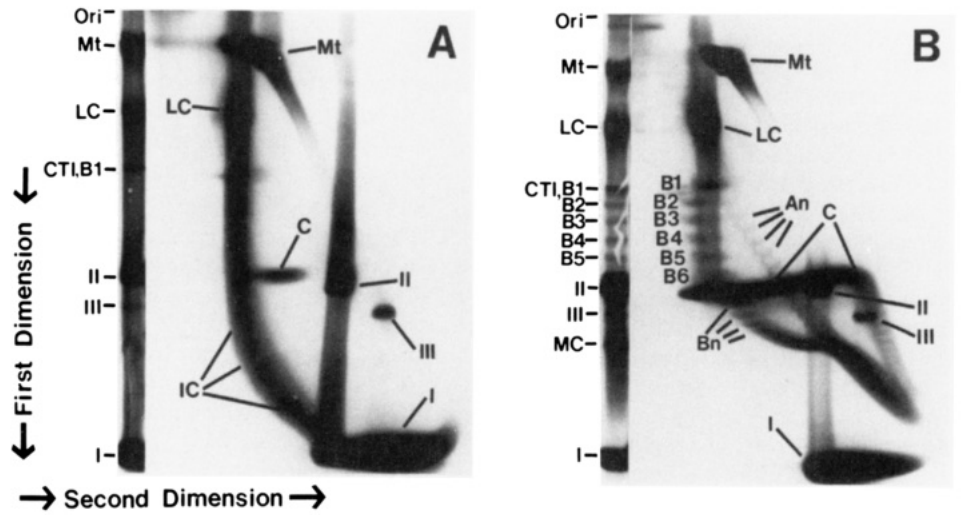


FIGURE 2: Two-dimensional neutral-chloroquine gel electrophoresis of normal SV40 DNA replication intermediates (A) and intermediates resulting from exposure to 50 μg/mL A-62176 (B). The first-dimension separation is shown on the left side of each two-dimensional separation for comparison. As shown, the first-dimension gel was run from top to bottom, and the second dimension (in chloroquine) was run from left to right. CT-1, form I circular (head-to-tail) trimer; C, unresolved form I circular dimer and C-family catenated dimers; IC, intermediate Cairns structures ("theta" replication intermediates in which the unreplicated region is superhelical); NC, nicked Cairns structures (same as IC, but with single-strand DNA breaks in the parental strands); A, highly catenated A-family dimers; B, highly catenated B-family dimers. Other abbreviations same as in Figure 1.

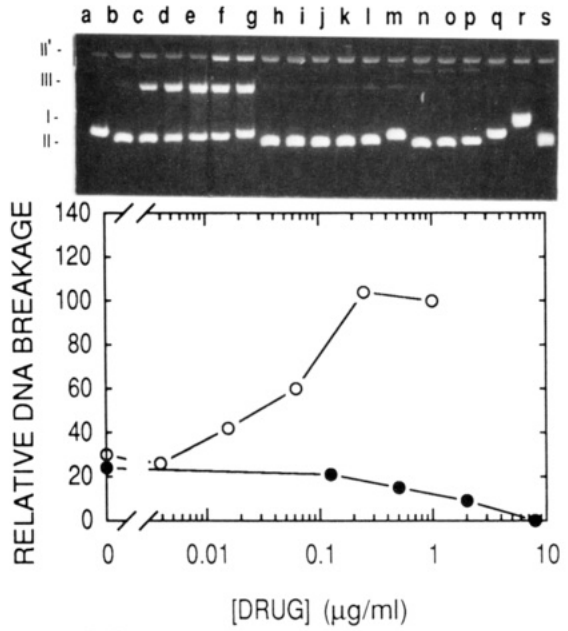


FIGURE 3: Calf thymus topoisomerase II-mediated DNA cleavage, a, ColE1 DNA only; b, h, and n, no drug controls containing DNA and topoisomerase II only; c–e, A-75272 at 2, 10, and 50 μg/mL; f–g, m-AMSA at 25 and 100 μg/mL; i–m, adriamycin at 0.004, 0.016, 0.06, 0.25, and 1.0 μg/mL; o–s, A-62176 at 0.125, 0.5, 2, 8, and 32 μg/mL. The graph shown in the bottom panel is the densitometer trace of the linearized DNA (form III) in the presence of adriamycin and A-62176 expressed as the relative amount of the form III intensities. Forms I, II′, and II denote supercoiled, nicked circle, and relaxed circle, respectively. The gel was run in the presence of 0.5 μg/mL ethidium bromide.

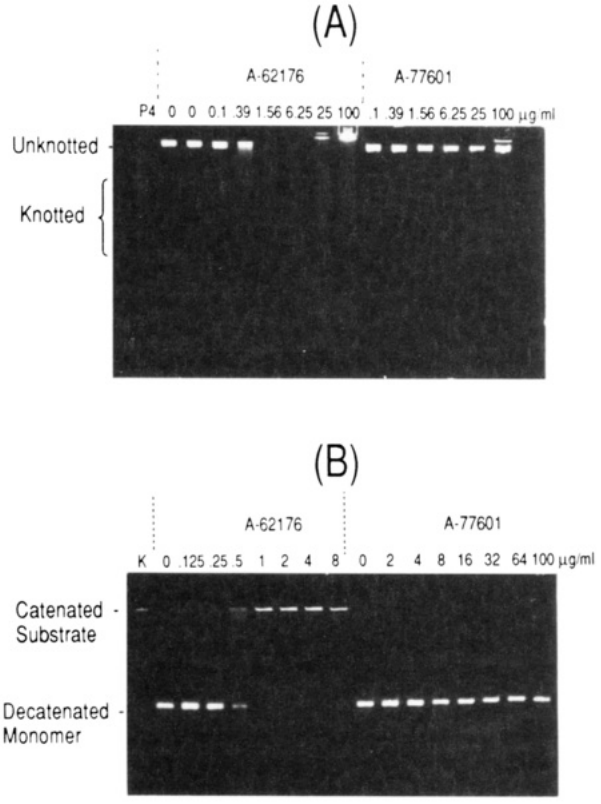


FIGURE 4: Inhibition of the catalytic functions of calf thymus DNA topoisomerase II by A-62176 and A-77601. The inhibitory potency was assayed by P4 DNA unknotting assay (A) and by k-DNA decatenation assay (B). All lanes contained topoisomerase II except the ones labeled with P4 or K. Numbers above each lane denote the concentrations of the drugs.

decatenation. A-77601 showed no inhibition of decatenation up to 100 μg/mL. Thus, the *in vitro* assays with purified topoisomerase II support the SV40 results indicating that A-62176 and A-74932 are strong inhibitors of mammalian topoisomerase II, but not topoisomerase II poisons. A-77601, the analog with little anticancer activity, was only a very weak inhibitor of topoisomerase II reactions *in vitro*. Again, this agrees well with the SV40 data.

DNA Unwinding by the Quinobenoxazines. Quinolone antibacterials are known to be DNA-targeted agents as demonstrated by a radioligand binding method using pure DNA (Shen & Pernet, 1985). Quinolone–DNA interaction in the presence of magnesium can result in a DNA unwinding effect at high drug concentrations (Tornaletti & Pedrini, 1988). The actual mode of such an interaction, whether due to DNA intercalation or due to binding to major or minor grooves, is still unclear. Some cellular components, such as polyamines or histones, can prevent the DNA unwinding effect of antibacterial quinolones (Shen et al., 1989a,b). Thus, the role of DNA unwinding in the inhibition of DNA gyrase, especially *in vivo*, remains to be established. In this DNA unwinding study, sufficient topoisomerase I was added to ensure excess DNA relaxing activity even in the absence of magnesium ions. The linking number of DNA is changed when it is unwound in the presence of topoisomerase I. DNA unwinding due to drug binding is thus reflected by a change in linking number. After drug binding, the topoisomerase is inactivated by addition of SDS. Subsequent electrophoresis removes the bound drug molecules and reveals a distribution of topoisomers whose average superhelicity is a function of DNA unwinding by the test drug. The usefulness of this test has been demonstrated with many DNA-binding agents, including the strong intercalator ethidium bromide (Shen et al., 1989b). The results (Table 1) show that the DNA unwinding strength of the quinobenoxazines correlates with their antineoplastic activity and their ability to inhibit DNA topoisomerase II. The unwinding effect may be maximized in the presence of magnesium ions, but in contrast to the antibacterial quinolones, polyamines do not prevent DNA unwinding by the quinobenoxazines.

A-62176 Prevents Stabilization of the Topoisomerase II–DNA Cleavable Complex by VM-26. VM-26 stabilizes the topoisomerase II–DNA cleavable complex so that denaturation with SDS results in a covalent protein–DNA cross-link and a double-strand DNA break (Liu, 1989). In SV40-infected CV-1 cells, these complexes can be quantitated as protein cross-links to pulse-labeled SV40 DNA using the GF/C filter assay. Addition of 100 μM VM-26 caused approximately 45% of the pulse-labeled DNA to be retained on the filter (Figure 5A). Predigestion of the Hirt supernatant with proteinase K eliminated the retention of labeled DNA on the filter. This confirms that filter retention was due to cross-linked protein (the topoisomerase II subunits). A-62176 caused a dose-dependent reduction in the level of VM-26-induced protein–DNA cross-links (Figure 5A). Thus, A-62176 is an antagonist of VM-26. The IC_{50} (50% inhibitory concentration) for A-62176 blocking of VM-26-induced protein–DNA cross-links was 3.8 μg/mL, and the maximum reduction in cross-links was 86%. A-62176 had little or no effect on camptothecin-induced topoisomerase I–DNA cross-links (Figure 5B). A-74932 showed a similar inhibition of VM-26-induced protein–DNA cross-links and had no effect on camptothecin-induced protein–DNA cross-links (data not shown). For A-74932, the IC_{50} was 7.2 μg/mL, and maximum reduction of VM-26-induced protein–DNA cross-links was 95%. *In vitro* studies with purified topoisomerase II verify the antagonist activity against VM-26. A-62176 caused a dose-dependent decrease in VM-26-induced topoisomerase

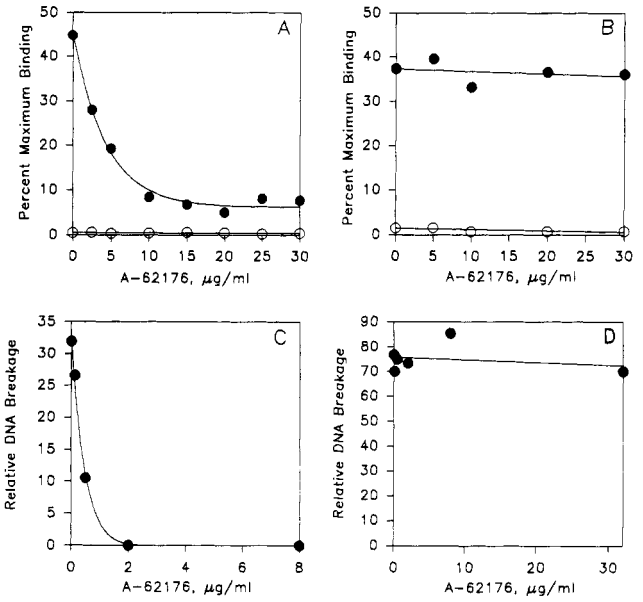


FIGURE 5: A-62176 acts as an antagonist of VM-26 but not of the DNA topoisomerase I poison camptothecin. (A) *In vivo* protein–SV40 DNA cross-links caused by exposure to VM-26 (100 μM) are reduced by treatment with A-62176. (B) *In vivo* protein–DNA cross-links caused by exposure to camptothecin (40 μM) are not affected by pretreatment with A-62176. Samples were taken directly from Hirt supernatants of drug-treated, SV40-infected cells and assayed for protein–DNA cross-links (●), or were assayed for protein–DNA cross-links after proteinase K digestion (○). (C) A-62176 prevents VM-26-induced DNA topoisomerase II cleavable complexes *in vitro*. (D) A-62176 does not affect camptothecin-stabilized topoisomerase I–DNA complexes *in vitro*.

II-mediated double-strand DNA breaks *in vitro* (Figure 5C) but had no effect on camptothecin-induced DNA topoisomerase I-mediated single-strand breaks (Figure 5D). Thus, A-62176 prevents the strong topoisomerase II poison VM-26 from stabilizing cleavable complexes both *in vivo* and *in vitro*. This indicates that A-62176 inhibits the topoisomerase II reaction at steps which precede DNA strand breakage.

DISCUSSION

In SV40 DNA replication, catalytic inhibition of DNA topoisomerase II causes slowed replication of the terminus region and accumulation of catenated daughter chromosomes (Snapka et al., 1988). But DNA topoisomerase II inhibitors do not seem to slow replication of the first 95% of the viral genome and do not cause the extensive breakage of replication forks typical of camptothecin. This has led to a model in which DNA topoisomerase I acts at or just ahead of replication forks to remove superhelical stress during replication of most of the genome, with DNA topoisomerase II taking over this function and acting from behind the forks as replication is completed (Snapka et al., 1988). DNA topoisomerase II then separates the daughter chromosomes. This model has recently been supported by studies of SV40 *in vitro* replication in which the activities of the topoisomerases were controlled directly (Ishimi et al., 1992b).

In addition to inhibiting decatenation of SV40 daughter chromosomes, DNA topoisomerase II poisons cause dose-dependent protein–DNA cross-links to pulse-labeled SV40 chromosomes (Shin et al., 1990). The protein–DNA cross-links are the drug-stabilized covalent topoisomerase II–DNA cleavable complexes in which the topoisomerase is covalently attached to the DNA at the site of a DNA strand break. The antineoplastic DNA topoisomerase inhibitors differ significantly in the relative strengths of catalytic inhibition and levels of protein–DNA cross-linking (topoisomerase poisoning). Studies with SV40 DNA replication seem to show a rough inverse relationship between strength of catalytic inhibition (as measured by accumulations of catenated daughter chromosomes) (Snapka, 1986; Snapka et al., 1988) and topoisomerase poisoning (as measured by drug-induced protein cross-links to labeled viral genomes) (Shin et al., 1990). The strongest catalytic inhibitors of topoisomerase II-dependent steps in SV40 DNA replication tend to be the weakest topoisomerase II poisons. The drug-stabilized cleavable complex must contribute to DNA topoisomerase II inhibition, but pre-DNA cleavage steps in the topoisomerase II reaction may be rate-limiting *in vivo*.

A-62176 caused heavy accumulations of very highly catenated dimers at only 1 μg/mL. A-74932 caused similar accumulations of catenated dimers at higher concentrations (>10 μg/mL). In contrast, 40 μM ellipticine causes lower levels of catenation (Snapka, 1986). In view of the proposed inverse relationship between topoisomerase poisoning and catalytic inhibition, one would expect that these two quinolones would be much weaker topoisomerase poisons than ellipticine. Neither drug caused detectable protein–DNA cross-links to replicating SV40 genomes at any concentration, suggesting that they are not topoisomerase II poisons. The *in vitro* studies demonstrated clearly that A-62176 is a strong catalytic inhibitor of topoisomerase II but is not a topoisomerase poison. A-62176 did not increase topoisomerase II-mediated double-strand DNA breaks, and at high concentrations reduced them below the background level seen in the presence of the topoisomerase alone. This shows that A-62176 prevents DNA topoisomerase II from forming cleavable complexes. Thus, A-62176 and A-74932 conform well to the proposed inverse relationship between catalytic inhibition of topoisomerase II and topoisomerase II poisoning *in vivo*.

A-77601, which has low antineoplastic activity, showed only slight evidence of DNA topoisomerase II catalytic inhibition and topoisomerase poisoning *in vivo* at very high concentrations. In the *in vitro* studies, A-77601 had no effect on decatenation and only a negligible effect on the unknotting reaction. Thus, the strength of the quinobenoxazines as catalytic inhibitors of topoisomerase II (both *in vivo* and *in vitro*) parallels their effectiveness as anticancer drugs. The strength of the quinobenoxazines as DNA topoisomerase II inhibitors and as anticancer drugs also correlates with their ability to unwind DNA (Table 1).

A-62176 and A-74932 both acted as efficient antagonists of VM-26-induced protein cross-links to SV40 DNA *in vitro* and *in vivo*. Neither drug had a significant effect on camptothecin-induced protein–DNA cross-links. These results show that A-62176 and A-74932 prevent the formation of topoisomerase II–DNA cleavable complexes even in the presence of strong DNA topoisomerase II poisons which stabilize them. A-62176 and A-74932 thus interfere with steps preceding the formation of the cleavable complex.

A large body of evidence indicates that drug-stabilized topoisomerase II–DNA complexes are cytotoxic [for reviews, see Drlica and Franco (1988) and Liu (1989)]. Correlations between cleavable complex levels and cytotoxicity have been made within given series of analogs such as the acridines (Bakic et al., 1986; Covey et al., 1988). However, when different structural classes of DNA topoisomerase II inhibitors are considered, the relationship between topoisomerase poisoning and cytotoxicity or effectiveness as anticancer agents is less clear (Ross et al., 1979). DNA topoisomerase II is an essential enzyme for eukaryotic cells; nothing else can separate newly replicated chromosomes. Inhibition of DNA topoisomerase II blocks the decatenation of newly replicated SV40 and yeast

plasmid chromosomes (Varshavsky et al., 1983; DiNardo et al., 1984; Snapka, 1986). Inhibition of type II DNA topoisomerase also prevents the separation of cellular chromosomes of *Escherichia coli* (Steck & Drlica, 1984; Adams et al., 1992), yeasts (Holm et al., 1985; Uemura & Yanagida, 1986), and mammalian cells (Charron & Hancock, 1991; Downes et al., 1991; Clarke et al., 1993).

The drugs described in this study, A-62176 and A-74932, were found to be precleavage catalytic inhibitors of DNA topoisomerase II. They are similar to ICRF-193 in their ability to inhibit DNA topoisomerase II-dependent steps of SV40 DNA replication, their ability to inhibit DNA topoisomerase reactions *in vivo* without formation of cleavable complexes, and their ability to act as antagonists of known DNA topoisomerase II poisons. ICRF-193, in the presence of ATP, stabilizes topoisomerase II in a conformation that is unable to bind circular DNA (Roca et al., 1994). However, the quinobenoxazines are structurally unrelated to ICRF-193 and merbarone, they are clearly strong DNA-interacting agents, and they are not DNA topoisomerase II poisons at any concentration. The antineoplastic quinobenoxazines, ICRF-193, and merbarone are all precleavage catalytic inhibitors of DNA topoisomerase II (Tanabe et al., 1991; Drake et al., 1989). Precleavage catalytic inhibition may also play a role in the cytotoxicity and anticancer activity of DNA topoisomerase poisons such as ellipticine and adriamycin. These topoisomerase II poisons slow the steps of SV40 replication which require topoisomerase II just as the precleavage catalytic inhibitors do (Snapka, 1986). In addition, weak topoisomerase poisons such as ellipticine can act as antagonists of strong topoisomerase II poisons (Shin et al., 1990). This suggests that topoisomerase poisons can also inhibit the pre-DNA cleavage steps of topoisomerase II reactions. In this sense, they may interfere with their own topoisomerase poisoning activity. The precleavage catalytic inhibitors underscore the need to reevaluate the exclusive role of the cleavable complex in the anticancer activity of classical DNA topoisomerase II inhibitors such as adriamycin.

ACKNOWLEDGMENT

We thank Dr. Nicholas Cozzarelli for helpful comments on the manuscript.

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