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Topoisomerase II-Mediated DNA Cleavage Activity and Irreversibility of Cleavable Complex Formation Induced by DNA Intercalator with Alkylating Capability

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SUMMARY

A group of chrysophanol and emodin derivatives with DNA-intercalating capability and with or without alkylating potential have been synthesized and shown to have antitumor activity *in vitro*. The topoisomerase II (Topo II)-mediated DNA cleavage activities induced by representative compounds 3-(2-chloroethylamino)methyl-1,8-dihydroxy-9,10-anthraquinone (SK-31690), 3-bis[(2-chloroethyl)amino]methyl-1,8-dihydroxy-9,10-anthraquinone (SK-31662), and 3-(2-hydroxyethylamino)methy-1,8-dihydroxy-9,10-anthraquinone (SK-31694), and their cytotoxicities, have been investigated. All three compounds inhibited the kinetoplast DNA decatenation catalyzed by DNA Topo II. These compounds inhibited leukemia cell growth and stimulated, in a dose-dependent manner from 0.5 to 60 μM, the formation of Topo II-DNA cleavable complexes, when 3′-^{32}P-labeled DNA was used. The mapping of Topo II-mediated DNA cleavage sites using *Hind*III-digested 3′-^{32}P-labeled DNA showed that, at 10 μM, these compounds induced protein-linked DNA breaks that correlated with cytotoxicity, with respect to their maximal efficacy or the reciprocal concentration for the half-maximal effect. The reversibility study showed that the amounts of protein-linked DNA cleavage induced by 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and VP-16 as well as SK-31694, which lacks alkylating potential, were markedly decreased during 30-sec exposure to 65° or 0.5 M NaCl. In contrast, protein-linked DNA cleavages induced by SK-31662, which has two alkylating functionalities, and by SK-31690, which has one alkylating functionality in its structure, cannot be reversed during the 15-min exposure to 65° or 0.5 M NaCl. These data suggest that Topo II is a major cellular target for cytotoxicity of these compounds. Furthermore, DNA intercalators with alkylating potential interact with Topo II-DNA cleavable complexes in an irreversible manner, with enhanced toxicity.

Mammalian DNA Topo II is the primary target of a number of potent antitumor drugs with diverse and unrelated chemical structures, including DNA intercalators such as *m*-AMSA, ellipticine, and anthracycline antibiotics (e.g., doxorubicin) (1–3) and nonintercalators such as the epipodophyllotoxins VP-16 and VM-26 (4). In mammalian cells, DNA Topo II inhibitors interfere with the breakage-reunion reaction mediated through Topo II by trapping a tight enzyme-DNA complex, termed the "cleavable complex," which is presumed to be the key covalent intermediary in the topoisomerase strand-passing reaction (5). Several lines of evidence indicated that the formation of this putative enzyme-DNA-drug ternary complex is reversible (3–6) and the stabilized cleavable complex apparently aborts the strand-passing activity of Topo II, which results in DNA breakage and the covalent linking of the tyrosyl group of topoisomerase homodimer to each 5′-phosphoryl end of the broken DNA (3–6). The level of Topo II-mediated DNA breakage *in vitro* correlated strongly with the level of protein-linked DNA breakage in cultured cells and the level of drug-induced cytotoxicity (7–9).

The structure-activity relationship studies on certain antitumor intercalating agents suggest that, although intercalation may be a necessary condition, it may not be sufficient for potentiation of the anticancer activity. Studies on the indole antibiotic CC-1065 (10, 11) indicated that the covalent binding of the drug to DNA seems to be important for its potent cytotoxic activity. Based on the "biooxidative alkylation" hypothesis, a group of *C*-methyl-modified derivatives of the anthraquinones chrysophanol and emodin and their various

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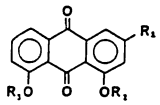
ABBREVIATIONS: Topo II, DNA topoisomerase type II; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; kDNA, kinetoplast DNA; Topo I, DNA topoisomerase type I; SK-31662, 3-bis[(2-chloroethyl)amino]methyl-1,8-dihydroxy-9,10-anthraquinone; SK-31690, 3-(2-chloroethylamino)methyl-1,8-dihydroxy-9,10-anthraquinone; SK-31694, 3-(2-hydroxyethylamino)methyl-1,8-dihydroxy-9,10-anthraquinone; XTT, 2′,3′-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide; SV40, simian virus 40; VP-16, 4′-demethylepipodophyllotoxin-4(4,6-*O*-ethylidene-β-D-glucopyranoside; SDS, sodium dodecyl sulfate; dThd, thymidine; BSA, bovine serum albumin; DTT, dithiothreitol; bp, base pairs.

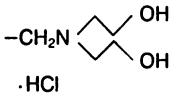
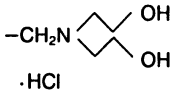
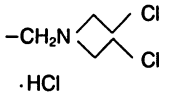
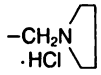
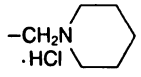
methyl ethers have been synthesized in this institution (12) (Table 1). Some of these compounds are intercalating agents with covalent bond-forming capability that may interact with DNA, leading to alkylation of DNA; they have potent antitumor activity *in vitro*, and some of them have shown antitumor activity *in vivo* (12). In our previous report, the comparison of the ratios of potencies (IC_{50}) for cell growth inhibition and inhibition of [^{3}H]dThd incorporation into DNA in HL-60 cells suggested that the representative compounds exert their initial effects mainly on processes other than precursor deprivation in DNA synthesis (12). In this study, we extended our studies into the mechanism of action of the selected compounds by examining the effects of the compounds on DNA Topo I and II, the topoisomerase-mediated DNA cleavage sites, and the reversibility of proposed DNA-Topo II-drug cleavable complexes, compared with those of the standard intercalator and nonintercalator Topo II inhibitors. We found that some of these compounds are potent inhibitors of Topo II of HL-60 cells and the DNA-Topo II cleavable complex formation stabilized by the compounds with alkylating group(s) on their side chain was irreversible.

Materials and Methods

Enzymes, DNA substrates, and chemicals. Nuclear extracts from 0.35 M NaCl precipitation were obtained from 1×10^9 HL-60 cells

TABLE 1
Structures and some biological effects of chrysophanol derivatives



Compound	R_1	R_2	R_3	IC_{50} for cell growth	IC_{50} for [^{3}H]dThd incorporation
				μM	μM
SK-31671	$-CH_2N(CH_3)_2 \cdot HCl$	H	H	2.8	9.3
SK-31661	$-CH_2N(CH_2CH_3)_2 \cdot HCl$	H	H	1.8	14.5
SK-31660		H	H	3.3	13.7
SK-31653		H	CH_3	20.7	8.9
SK-31662		H	H	0.14	1.7
SK-31669		H	H	1.14	10.2
SK-31665		H	H	2.8	63.3
SK-31694	$-CH_2NHCH_2CH_2OH \cdot HCl$	H	H	0.86	14.9
SK-31690	$-CH_2NHCH_2CH_2Cl \cdot HCl$	H	H	7.5	7.0
SK-31666	$-CH_2Br$	H	H	7.1	11.6
SK-31824	$-CH_2N(CH_2CH_2Cl)_2 \cdot HCl$	H or CH_3[a]	H or CH_3[a]	0.52	5.4
SK-31833	$-CH_2N(CH_2CH_3)_2 \cdot HCl$	H or CH_3[a]	H or CH_3[a]	6.9	180

[a] These compounds (SK-31824 and SK-31833) are monomethoxyl derivatives, i.e., either 1-OCH_3 or 8-OCH_3. It is difficult to determine whether the methyl group is in the R_2 or R_3 position.

in suspension during early logarithmic phase of cell growth (0.5×10^6/ml), by using a modified technique of Glisson *et al.* (13), for use as a Topo II source. Calf thymus Topo I, *Eco*RI, *Hind*III, larger fragment *Escherichia coli* DNA polymerase I, T4 polynucleotide kinase, and pBR322 DNA were purchased from GIBCO-Bethesda Research Laboratories (Bethesda, MD). kDNA was isolated from *Crithidia fasciculata* as previously described (14). [α-^{32}P]dATP was purchased from NEN (Boston, MA). *m*-AMSA and VP-16 were the products of Sigma (St. Louis, MO), and VM-26 was a gift from Dr. A. M. Casazza of Bristol-Myers Co. (Wallingford, CT). The modified derivatives of chrysophanol and emodin or their various methyl ethers, denoted as SK compounds, were synthesized by the Organic Chemistry Laboratory of this institution (12). Most of these compounds were dissolved in dimethylsulfoxide; the final concentration of dimethylsulfoxide in the reaction mixture was 0.5–1%, which had a negligible effect on Topo II activity and produced negligible cytotoxicity.

End-labeling of DNA. *Eco*RI-restricted pBR322 DNA was labeled at the 3′ end using [α-^{32}P]dATP (800 Ci/mmol) and the large fragment of *E. coli* DNA polymerase I, as described by Liu *et al.* (5). End-labeling at the 5′ end was achieved by a procedure in which another set of *Eco*RI-linearized pBR322 DNA was dephosphorylated with alkaline phosphatase and then phosphorylated with T4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol), as described previously (5). The DNA was purified by passage through a Sephadex G-50 column (Pharmacia). For the Topo II cleavage site-mapping studies, the resulting 3′-end-labeled DNA was subsequently cut with *Hind*III, to generate a large DNA fragment (4333 bp) labeled at only one end and a small DNA fragment (33 bp) that will run off the gels.

Evaluation of antitumor activity in cultured cells. The effects of the compounds on cell growth inhibition were determined in HL-60 and L1210 cells in a 72-hr incubation, by XTT-tetrazalium microculture assay, as described by Scudiero *et al.* (15). After incubation with phenazine methosulfate-XTT solution at 37° for 6 hr, absorbance at 450 nm and 630 nm was detected with a microplate reader (EL 340; Bio-Tek Instruments Inc., Winooski, VT). The IC_{50} and dose-effect relationships of the compounds for antitumor activity were calculated by a median-effect plot (16, 17), using a computer program developed in this laboratory (18). The effects of the compounds on incorporation of [^{3}H]dThd into DNA in cultured cells were measured by a previously described method (19).

Topoisomerase activity assay. Topo I and II relaxation activities were determined by following the procedures described by Liu and Miller (20) and Hirose *et al.* (21). The reaction mixture (50 μl), containing 10 mM Tris, pH 7.9, 50 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 1 mM ATP, 30 μg/ml BSA, 15 μg/ml SV40 DNA or pBR322 DNA, and the known amounts of the Topo I or Topo II, was incubated at 30° for 15 min. The reaction was stopped by the addition of SDS to 1%, EDTA to 10 mM, and proteinase K to 25 μg/ml; the mixture was further incubated at 37° for 30 min, mixed with dye solution containing 5% sucrose and 0.01% xylene cyanol, and analyzed on a 1% agarose gel using TBE (89 mM Tris-borate, 2 mM EDTA) containing 0.1% SDS.

SDS-KCl co-precipitation of topoisomerase-DNA complex and complex reversibility. SDS-KCl co-precipitation of double-strand DNA-topoisomerase complexes, using a nuclear extract preparation as Topo II source, and of single-strand DNA-topoisomerase complexes, using purified Topo I, was performed as described by Liu *et al.* (5). The reaction mixture (50 μl), containing 40 mM Tris, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM DTT, 0.5 mM EDTA, 30 μg/ml BSA, 15–20 μg of protein of nuclear extract, 3′- or 5′-end-labeled [^{32}P] pBR322 DNA (0.15–0.2 μg), and various concentrations of drugs to be tested, was incubated at 37° for 30 min. Reactions were terminated by addition of a stop solution containing 2% SDS, 2 mM EDTA, and 0.5 mg/ml salmon sperm DNA, and the precipitations of topoisomerase-DNA complexes were achieved by addition of 0.25 M KCl. The resulting pellets were resuspended, and radioactivity was determined by scintillation counting. For the precipitation of single-strand DNA-topoisomerase complexes, 0.2 N NaOH was included in the stop solution to denature the DNA, and 0.4 M Tris·HCl, pH 7.9, was included in the KCl solution to neutralize NaOH. For heating and high salt reversal studies, the reaction mixture was heated to 65° or exposed to 0.5 M NaCl for the indicated time intervals, respectively, before termination of the reaction. The rest of the procedure was the same as the standard SDS-KCl co-precipitation assay described above.

The SDS-KCl co-precipitation assay was also used to measure the reversibility of protein-DNA complexes in intact cultured cells, by the modified procedure described by Rowe *et al.* (8), for reversal experiments in this study.

Inhibition of kDNA decatenation. kDNA was isolated from *C. fasciculata* by published methods (14, 22). The standard reaction mixture for kDNA decatenation assay, containing 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 30 μg/ml BSA, 5 mM ATP, 1.5 μg of kDNA, and 5 μg of protein of nuclear extracts, in 50 μl, was incubated at 37° for 30 min (23). Reaction was terminated by the addition of 5 μl of 10% SDS and 1 mg/ml proteinase K, and the reaction mixture was further incubated at 37° for 30 min, followed by electrophoreses on 1% agarose with TBE buffer. After staining with ethidium bromide (5 μg/ml), gels were photographed under UV illumination, using Polaroid type 55 film.

Mapping of Topo II cleavage sites. In order to map the sites of cleavage mediated by Topo II, the 3′-end-labeled pBR322 DNA was restricted with *Hind*III, to remove a 31-bp fragment. The reaction mixture for Topo II cleavage site mapping was identical to that for SDS-KCl co-precipitation of topoisomerase-DNA complexes. The incubated reaction mixture was terminated by addition of 10% SDS (5 μl) and proteinase K (1.7 mg/ml; 3 μl) and was further incubated at 50° for 30 min. DNA samples were analyzed on a 1% agarose gel in TBE buffer. All DNA samples were extracted with phenol before loading onto gels. The gel drying and autoradiography were done as previously described (4).

Results

Inhibitory effect of SK compounds on cell growth. The antitumor activity of representative chrysophanol and emodin derivatives on HL-60 cells is summarized in Table 1. The compound SK-31662, which has two alkylating groups in its methyl side chain, is among the most potent compound, with a IC_{50} of 0.14 μM. SK-31694, which has a hydroxyethylamino group in its methyl side chain, also shows potent antitumor activity, with a IC_{50} of 0.86 μM. A comparison of the potencies of these compounds for cell growth inhibition and inhibition of [^{3}H]dThd incorporation into DNA in HL-60 cells showed, to different extents, an inverse relationship between cell growth inhibition and inhibition of initial dThd incorporation into DNA, except for those compounds with chloroethyl amino side chain(s). No obvious inverse relationship between cell growth inhibition and inhibition of [^{3}H]dThd incorporation into DNA was observed for SK-31671 and SK-31660.

Effects of SK compounds on DNA relaxation. The effects of SK compounds on DNA relaxation *in vitro* were studied in the presence and absence of Topo I. In the presence of increasing concentrations of different SK compounds listed in Table 1, the results indicated that Topo I relaxation activity was inhibited, as evidenced by the disappearance of relaxed SV40 DNA, compared with the untreated controls. Fig. 1 is the result with SK compound SK-31694 and Topo I. Similar results were obtained for these SK compounds when the nuclear extract, which contains Topo I and II activities, was used as an enzyme source (data not shown). The relative inhibitory potencies for DNA relaxation were then used to design subsequent

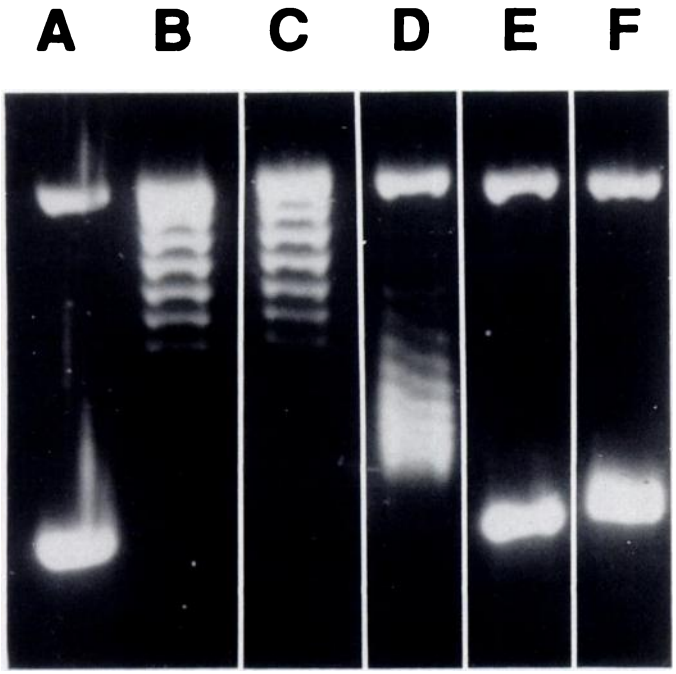


Fig. 1. Effect of SK-31694 on DNA relaxation in the presence of purified Topo I. *Lane A,* SV40 DNA only; *lane B,* DNA and Topo I; *lanes C–F,* SK-31694 at 10, 20, 40, and 80 μM, respectively. Similar results were obtained in two separate experiments.

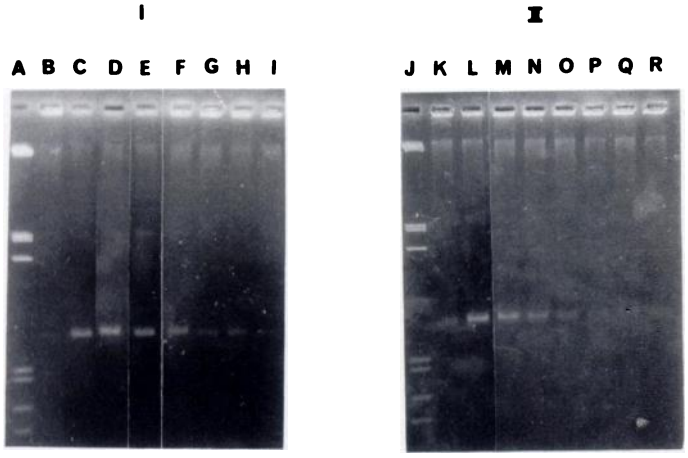


Fig. 2. Inhibitory effects of SK-31662 and SK-31694 on kDNA decatenation using Topo II nuclear extracts of HL-60. I, *Lane A, Hind*III-digested DNA markers; *lane B,* nuclear extract and kDNA; *lane C,* nuclear extract, kDNA, and ATP; *lanes D–I,* 1, 5, 10, 20, 50, and 100 μM SK-31662, respectively. II, *Lane J, Hind*III-digested DNA markers; *lane K,* nuclear extract and kDNA; *lane L,* nuclear extract, kDNA, and ATP; *lanes M–R,* 1, 5, 10, 20, 50, and 100 μM SK-31694, respectively. Similar results were obtained from two separate experiments.

studies with the KCl-SDS precipitation and kDNA decatenation methods.

Inhibitory effect of SK compounds on kDNA decatenation catalyzed by Topo II. The effect of SK compounds on Topo II activity was studied by using a Topo II-mediated kDNA decatenation reaction, converting thousands of connected minicircles into a free minicircle. The inhibition of the decatenation reaction by the compounds results in a progressive disappearance of free minicircles of kDNA in the gel. As shown in Fig. 2, in the presence of HL-60 cell nuclear extracts, compounds SK-31694 and SK-31662 inhibited kDNA decatenation in a dose-dependent manner, with a full-inhibition concentration of about 35 and 50 μM, respectively. The rest of the SK compounds listed in Table 1 have relatively weak inhibitory effects on the kDNA decatenation reaction, with full-inhibition concentrations from 120 to 400 μM (data not shown).

Effect of SK compounds on stimulation of topoisomerase-DNA complex formation. Previous studies showed that Topo I is linked to the 3′ end of the broken DNA and Topo II is linked to the 5′ end of broken DNA when Topo I and II inhibitors were used to induce topoisomerase-mediated DNA cleavage, in the SDS-KCl co-precipitation assay, to quantitate the amounts of precipitated covalently bound protein-topoisomerase complexes. To test whether the cleavage products in the presence of these SK compounds have protein covalently bound to the 5′ end or the 3′ end, the 5′-end-radiolabeled DNA and 3′-end-labeled DNA were used to identify the effects of the SK compounds on Topo I and Topo II, respectively. As shown in Fig. 3, when 3′-end-labeled DNA was used, dose-dependent increases of covalently bound protein-topoisomerase complexes were observed after treatment with the SK compounds listed in Table 1. The fold increases were

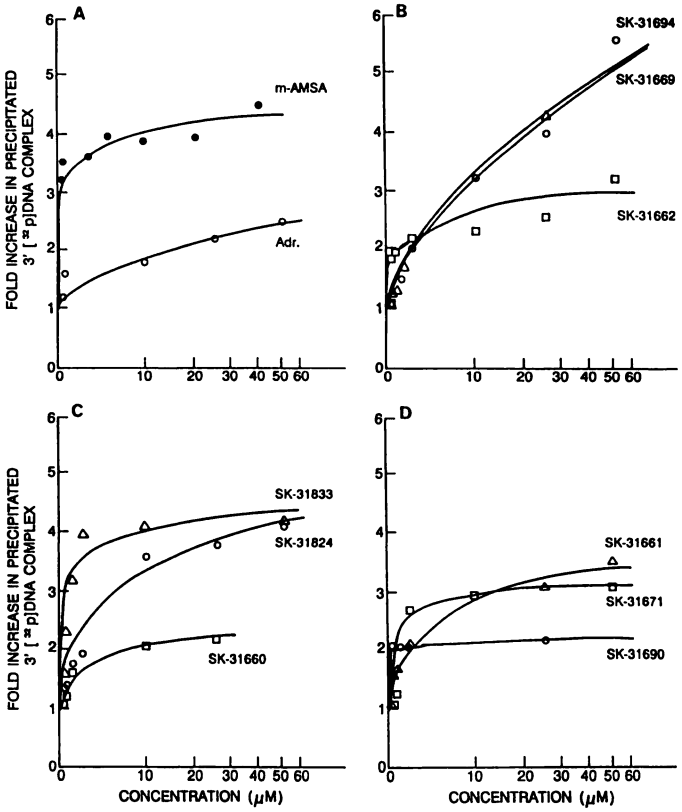


Fig. 3. Quantitative KCl-SDS precipitation of covalent Topo II-DNA complexes induced by *m*-AMSA, Adriamycin, and nine representative SK compounds. Fold increases in precipitation of [3′-^{32}P]-DNA-Topo II complex in the presence of various concentrations of each compound, compared with the control (without the compounds), were determined. Experimental conditions are detailed in Materials and Methods. Similar results were obtained in a separate experiment. The mean and variations from two or three experiments using four to six concentrations ranged from 0.045 to 0.099 for SK-31669, 0.34 to 0.48 for SK-31660, 0.049 to 0.11 for SK-31690, 0.028 to 0.13 for SK-31662, 0.18 to 0.44 for SK-31661, 0.13 to 0.45 for SK-31671, 0.025 to 0.12 for SK-31824, 0.02 to 0.099 for SK-31833, 0.26 to 0.76 for SK-31694, 0.085 to 0.2 for *m*-AMSA, and 0.16 to 0.27 for Adriamycin (*Adr.*), respectively. These variations are about 15% or less of the mean.

comparable to those of *m*-AMSA. SK-31669 and SK-31694 produced the most stimulation of Topo II cleavage, but with high half-maximal stimulatory concentrations, whereas the stimulation by SK-31690, SK-31662, SK-31671, and SK-31833 reached maximum stimulation at very low concentrations (i.e., low K_m values), which were very similar to that observed with *m*-AMSA. In general, very low K_m values suggest very high affinity of binding. Further increase of the concentrations of these SK compounds produced no greater stimulation of the protein-DNA complex formation but, instead, frequently resulted in inhibition of protein-DNA complex formation (data not shown). When 5′-end-labeled DNA and purified Topo I were used to test these compounds, no radioactivity above the background was recovered in the precipitates, whereas radioactivity (protein-DNA-complexes) of 3.5-fold above background was recovered when 50 μM camptothecin, a known topo I inhibitor, was used under the same experimental conditions (data not shown).

Mapping of Topo II cleavage sites in the presence of compounds SK-31662 and SK-31694. In order to map the locations of Topo II-mediated DNA cleavages induced by SK-31662 and SK-31694, pBR322 DNA was first labeled at the 3′ end at the *Eco*RI site and then cut with *Hind*III to generate a DNA fragment (4333 bp), as detailed in Materials and Methods. As shown in Fig. 4, in the absence of any drug, HL-60 Topo II cuts DNA at same site (Fig. 4, *lane B*). In the presence of increasing concentrations of *m*-AMSA, Topo II cleaves DNA at multiple sites (Fig. 4, *lanes C–F*). In the presence of 10 and 25 μM SK-31662, the Topo II-mediated DNA cleavage site patterns (Fig. 4, *lanes G–L*) were somewhat different from those induced by *m*-AMSA, in which *m*-AMSA induced two more DNA cleavage sites, as evidenced by two more light bands in the positions between 1375 bp and 947 bp and at about 800 bp. With further increases in the concentration of SK-31662 (50 and 100 μM) (Fig. 4, *lanes K* and *L*), certain cleavage sites that could be observed at lower concentrations had disappeared. The cleavage site patterns (Fig. 4, *lanes M–S*) induced by SK-31694 were somewhat different from those observed with *m*-AMSA and SK-31662, in which many fewer cleavage sites were identified. Similar to SK-31662, certain cleavage sites disappeared when the higher concentrations of SK-31694 were used. These observations are basically in agreement with the results from SDS-KCl co-precipitation, in which the DNA cleavages were inhibited to different extents at concentrations of SK-31662 and SK-31694 higher than 60 μM. The reasons for the observed inhibition of DNA cleavage in the mapping assay at higher concentrations of SK compounds are unknown. The dose range differences between the quantitative SDS-KCl co-precipitation assay and the mapping of Topo II-mediated DNA cleavage sites, in terms of observed inhibition of DNA cleavage activity of Topo II, may be due in part to the different sensitivities of these two methods. In addition, the size of broken DNA resulting from Topo II-mediated DNA cleavage in the presence of Topo II inhibitor may be too small to detect in the 1% agarose gel electrophoresis used in this study. If this is the case, the small-size broken DNA induced by SK-31662 and SK-31694 at high concentrations may run off the gels; therefore, the cleavage sites cannot be mapped completely.

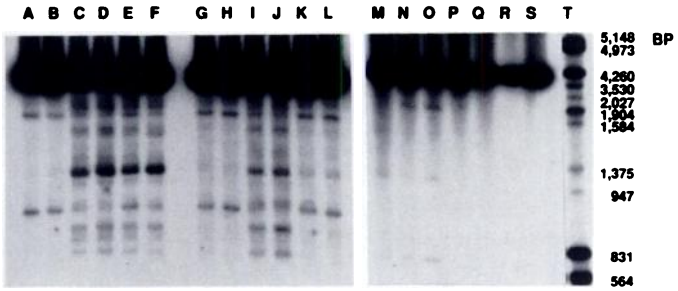


Fig. 4. Comparison of the mapping of Topo II-mediated DNA cleavage sites induced by *m*-AMSA, SK-31662, and SK-31694, using nuclear extract of HL-60 cells as enzyme source. *Lane A*, DNA control, no drug, no enzyme; *lane B*, no drug; *lanes C–F*, 2, 5, 10, and 50 μM *m*-AMSA, respectively; *lanes G–L*, 0.5, 2, 10, 25, 50, and 100 μM SK-31662, respectively; *lanes M–S*, 0.5, 2, 10, 25, 50, 100, and 200 μM SK-31694, respectively; *lane T*, DNA marker. Similar results were obtained in a separate experiment.

Irreversibility of SK-31662-induced protein-linked DNA breakage *in vitro*. It has been demonstrated that topoisomerase-mediated DNA strand breaks induced by many antitumor drugs, including intercalators that bind DNA and nonintercalators that do not bind DNA, were reversible (3–6). The unique alkylating functional groups in the structure of SK-31662 side chains prompted us to examine whether the topoisomerase-mediated DNA strand breaks induced by the compound are reversible. The SDS-KCl co-precipitation method in partially purified enzyme systems and cultured cell systems was used to follow the rate of reversal of SK compound-induced DNA breaks. As presented in Figs. 5 and 6, both heating and high salt (0.5 M NaCl) led to a rapid time-dependent reversal of the DNA cleavage induced by *m*-AMSA and VP-16. These results are consistent with the previous reports and the cleavable complex hypothesis (3–6). However, the Topo II-mediated DNA breaks induced by SK-31662, which has two alkylating groups, and SK-31690, which has one alkylating group in the methyl side chains, cannot be reversed either by heating (Fig. 5) or by high salt (Fig. 6), as evidenced by no change in the amount of cleaved DNA products during the 10-min exposure to 65° or 0.5 M NaCl; the DNA breaks induced by SK-31694 (Fig. 5A), which does not have an alkylating group in its methyl side chains, similar to *m*-AMSA and VP-16, were reduced after brief exposure of the reaction mixtures to 65° or 0.5 M NaCl (the data for the rest of the SK compounds in Table 1 are not shown). Similar results were obtained in the intact cultured L1210 cells, using the modified methods described in Materials and Methods (the results for one representative SK compound are shown in Fig. 7).

Discussion

It has been well established that 9-hydroxyelliptcine, one of the metabolites of ellipticine, is a potent anticancer agent, whereas 2-*N*-methyl-9-hydroxyellipticinium is among the most potent ellipticine analogues (24). This compound is easily oxidized by peroxidases to 9-oxo-2-methylellipticium (25), which is highly electrophilic and is able to alkylate DNA. It was found in this report, by comparing the IC_{50} of these compounds, that the presence of both the potent intercalating and alkylating functionalities in the chrysophanol and emodin structures, like SK-31662, increased their cytotoxicity against tumor cells *in vitro* to a great extent. Previous studies in this institute have shown that those SK compounds that lack potent intercalating capabilities, due to the presence of bulky methoxy groups, or lack alkylating potential had greatly decreased cytotoxicity

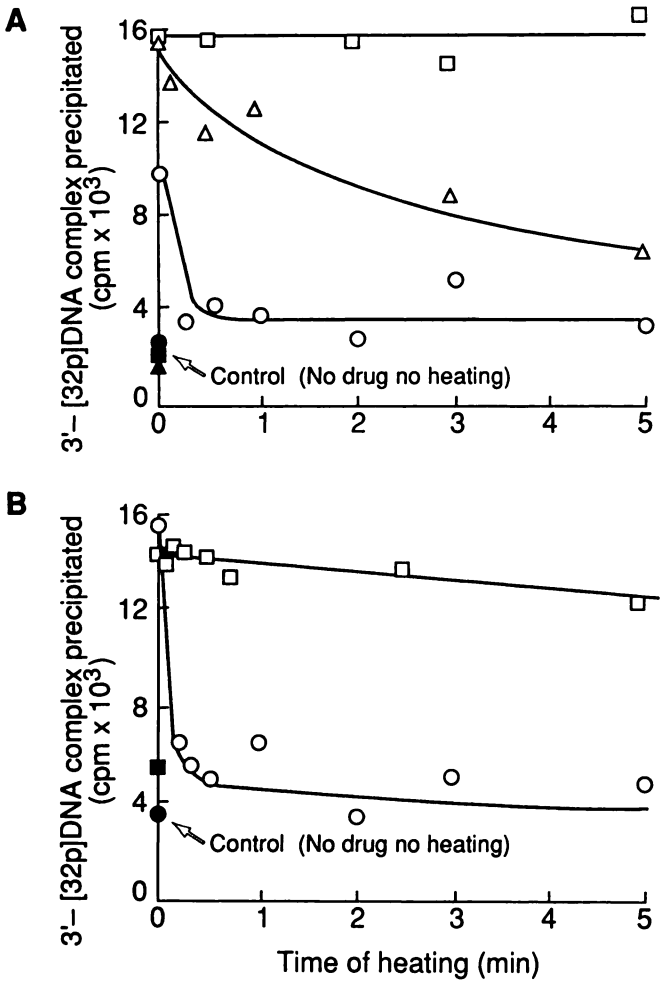


Fig. 5. Heat reversal of drug-induced Topo II-mediated DNA cleavage in the presence of nuclear extracts from HL-60 cells, as measured by KCl-SDS co-precipitation of cleavable complexes, using [3′-^{32}p]pBR322 DNA as substrate. A, ○, 10 μM *m*-AMSA; △, 10 μM SK-31694; □, 10 μM SK-31662. B, ○, 100 μM VP-16; □, 10 μM SK-31690. The precipitated radioactivity in the controls (no drug, no heating) is indicated by the corresponding *filled symbols* on the *ordinate*. Similar results were obtained in a separate experiment.

(12). These results support the proposed "biooxidative alkylation" mode of action by this class of compounds, i.e., that, as effective anticancer drugs, such modified molecules intercalate into DNA and alkylate DNA, probably in a covalent bond. These results also further strengthen our hypothesis that, although intercalation may be a necessary condition for the interaction between the drug and the DNA or DNA-protein complexes, it may not be a sufficient condition for anticancer potency, because nonintercalating agents like epipodohyllotoxins (VP-16 and VM-26) also have potent anticancer activity *in vitro*. We also found that modified molecules with two alkylating groups in their methyl side chains, such as SK-31662, have more potent activity against cancer cells *in vitro* than do those with only one alkylyting group, such as SK-31690. This finding suggests that alkylation at the target, presumably at specific deoxypolynucleotide bases and/or specific amino acid moieties, may play an important role in the cytotoxic potency of this class of compounds. The analysis of radioactively labeled SK-31662 for more detailed active site or binding site studies is planned.

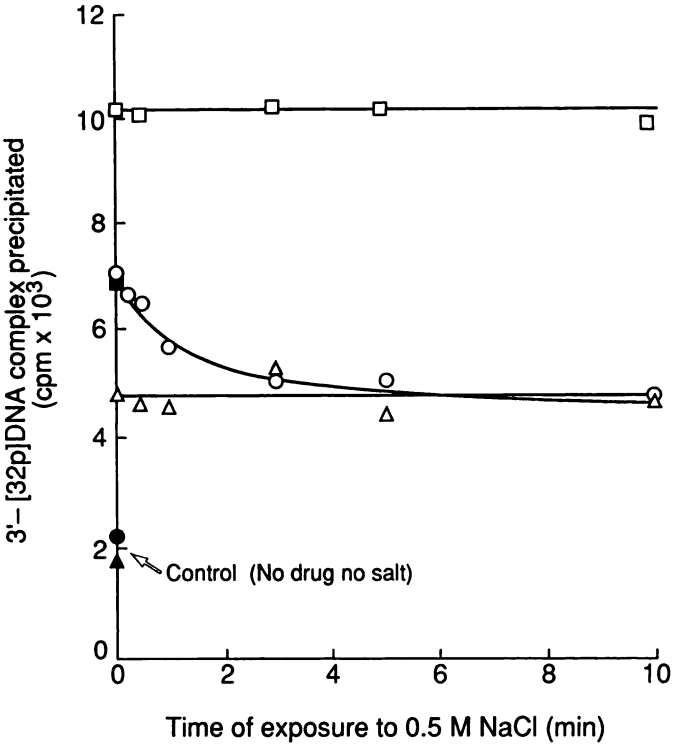


Fig. 6. High salt reversal of drug-induced Topo II-mediated DNA cleavage in the presence of nuclear extracts from HL-60 cells, as measured by KCl-SDS co-precipitation of cleaveable complexes, using [3′-^{32}p]pBR322 DNA as a substrate. ○, 10 μM *m*-AMSA; □, 10 μM SK-31690; △, 10 μM 31662. The precipitated radioactivity in the controls (no drug, no salt) is shown by the corresponding *filled symbols* on the *ordinate*. Similar results were observed in a separate experiment.

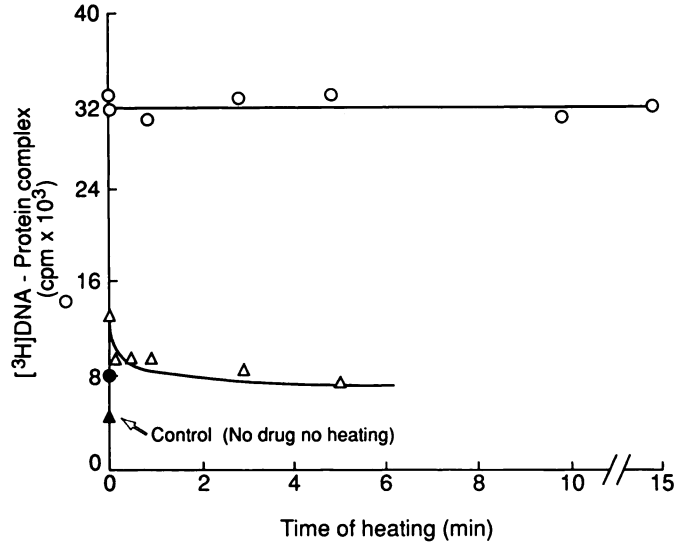


Fig. 7. Heat reversal of drug-induced protein-linked DNA breakage in intact L1210 cells measured by a modified KCl-SDS co-precipitation procedure, using [^{3}H]dThd-labeled cells, as described in Material and Methods. △, 100 μM VP-16; ○, 10 μM SK-31662. The precipitated radioactivity in the controls (no drug, no heating) is shown by the corresponding *filled symbols* on the *ordinate*. Similar results were observed in a separate experiment.

It has been documented by many laboratories (1–9) that Topo II may be the major cellular target for many anticancer drugs being used in clinical practice and under investigation and that camptothecin is a specific inhibitor of Topo I (26). Studies on the effects of the compounds SK-31662 and SK-31694 on DNA Topo II activity *in vitro* demonstrated that these two compounds inhibited Topo II activity, as evidenced by their inhibition of kDNA decatenation in the presence of Topo II from HL-60 cells. The effects of these compounds on Topo II may be achieved by the stimulation of cleavable complex formation of DNA and Topo II, as measured by a SDS-KCl co-precipitation assay. The data on mapping of Topo II-mediated DNA cleavage sites produced more evidence that these compounds induced DNA cleavage in the presence of Topo II, with the patterns of cleavage sites being different from those observed with *m*-AMSA.

The SK compounds appear to have no detectable effect on DNA Topo I, because these compounds fail to stimulate the cleavable complex formation of Topo I and DNA, although these compounds inhibited single-strand DNA relaxation in the presence of purified Topo I. It is possible that the observed inhibition of Topo I-mediated DNA relaxation by these compounds may result from the unwinding of supercoiled DNA due to an intercalation effect of the compounds. Interestingly, these compounds inhibited the stimulation of cleavable complex formation and the DNA cleavage mediated by Topo II in mapping experiments when the compound concentration reached certain higher levels. This type of dose-effect curve is totally different from that of *m*-AMSA, which does not exhibit inhibition of DNA cleavage even at high *m*-AMSA concentrations, but similar to those of ellipticine and 2-methyl-9-hydroxyellipticine (3). The basis of this observed inhibition of DNA cleavage by high concentrations of this class of compounds is not clear. It may be due to the fact that the active biooxidative alkylation reaction of these compounds can be easily achieved and, therefore, there may be strong drug-DNA interaction. This strong drug-DNA interaction, along with the intercalative capability of the compounds, may modify the DNA subtrate, which may halt its interaction or stop the turnover with Topo II enzyme. Another possibility is that the compounds directly inhibit the Topo II enzyme from interacting with DNA, thus hindering the access of the enzyme. In addition, DNA breaks are maximal at low or intermediate drug concentrations and decrease at higher concentrations (Fig. 4), whereas DNA-protein complexes do not decrease at high drug concentrations, as shown by the DNA-protein complex formation (Fig. 3). This difference raises a question about the nature of the DNA-protein complexes with respect to Topo II inhibition. One would expect a 1:1 stoichiometry between DNA breaks and DNA-protein complexes for Topo II cleavable complexes. In the presence of alkylating group(s), the cleavable complex formation may not be directly correlated with the cytotoxicity observed for these compounds.

Several lines of evidence have demonstrated that DNA Topo II is able to form a cleavable complex with DNA (3–6). Topo II-mediated DNA breakage induced by the drugs can be expected to result when protein denaturants such as SDS are used. The apparent DNA cleavage would be expected to reverse when the reaction condition is changed, e.g., exposure of the reaction mixture to an elevated temperature (65°) or to an increased salt concentration before the protein denaturant treatment. Consistent with the cleavable complex hypothesis, Hsiang and Liu (6) have shown that the cellular DNA lesion induced by Topo II poisons (DNA-Topo II cleavable complex induced by drugs) is indeed reversible. Our findings in this report, showing that the Topo II-mediated DNA cleavage induced by the intercalator *m*-AMSA and the nonintercalator VP-16 was reversible when the reaction mixtures were exposed to 65° or 0.5 M NaCl before the addition of SDS, are in agreement with the published observations (3–6). Interestingly, the reversibility studies on SK compounds using the same experimental conditions as used for *m*-AMSA and VP-16 showed that the Topo II-mediated DNA cleavages induced by those SK compounds that have two alkylating groups, like SK-31662, or only one alkylating group, like SK-31690, in their chloroethyl amino side chain(s) were not reversible by heating or high salt concentration, although the Topo II-mediated DNA cleavages induced by those compounds that lack alkylating ability, like SK-31694, were reversible, just as observed with *m*-AMSA and VP-16. The observed difference in terms of the reversibility of DNA cleavages induced by SK compounds with alkylating potential and the typical Topo II inhibitors strongly indicated that, besides the traditional site of action for the stimulation of cleavable complex formation (if the inhibition of Topo II by drug-stabilized complex formation is its major mechanism of cytotoxicity, as postulated), the SK compounds with alkylating potential may have additional site(s) of action on either DNA or enzyme (topoisomerases), which must be juxtaposed to the topo II-DNA cleavable complexes that are formed. It is possible that the intercalators with alkylating potential, like SK-31662, first intercalate into DNA, which enables the compounds to interact specifically with topo II and then alkylate either DNA or Topo II. The drug-enzyme interaction may result in an increase of residence time of the drug within the cleavable complex and, therefore, an increase of the half-life of the cleavable complex and, consequently, an increase of cytotoxicity.

The correlation between the reversibility of SK compound-induced Topo II-mediated DNA cleavages and structures of the alkylating group, as reported in this study, provides a new opportunity for more discriminating studies on the cleavable complex hypothesis, and the unique structures of these compounds may be used as a tool for exploring the binding site, the topological interrelationship among the DNA, DNA-Topo II, and its inhibitors, and their mechanism of interactions in terms of temporal and spatial events. Better understanding of the possible mode of drug-enzyme interaction may lead to the rational development of antitumor drugs specifically targeted at mammalian DNA Topo II.

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