Induction of Mammalian DNA Topoisomerase I Mediated DNA Cleavage by Antitumor Indolocarbazole Derivatives

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ABSTRACT: DNA topoisomerases have been shown to be important therapeutic targets in cancer chemotherapy. We found that KT6006 and KT6528, synthetic antitumor derivatives of indolocarbazole antibiotic K252a, were potent inducers of a cleavable complex with topoisomerase I. In DNA cleavage assay using purified calf thymus DNA topoisomerase I and supercoiled pBR322 DNA, KT6006 induced topoisomerase I mediated DNA cleavage in a dose-dependent manner at drug concentrations up to 50 μM, while DNA cleavage induced by KT6528 was saturated at 5 μM. The maximal amount of nicked DNA produced by KT6006 was more than 50% of substrate DNA, which was comparable to that of camptothecin. Heat treatment (65 °C) of the reaction mixture containing these compounds and topoisomerase I resulted in a substantial reduction in DNA cleavage, suggesting that topoisomerase I mediated DNA cleavage induced by KT6006 and KT6528 is through the mechanism of stabilizing the reversible enzyme–DNA "cleavable complex". Both KT6006 and KT6528 did not induce topoisomerase II mediated DNA cleavage in vitro. KT6006 and KT6528 were found to induce nearly identical topoisomerase I mediated DNA cleavage patterns, which was distinctly different from that with camptothecin. In contrast to the similarity between KT6006 and KT6528 in their structures and the nature of their cleavable complex with topoisomerase I, these drugs have different properties with repect to their interaction with DNA: KT6006 is a very weak intercalator whereas KT6528 is a strong intercalator with potentials comparable to that of adriamycin. These results indicate that KT6006 and KT6528 represent a new distinct class of mammalian DNA topoisomerase I active antitumor drugs.

DNA topoisomerases are nuclear enzymes that catalyze the concerted breaking and rejoining of DNA strands, thereby controlling the topological states of DNA. Topoisomerase I catalyzes the passage of a DNA strand through a transient single-strand break, while topoisomerase II catalyzes the passage of DNA double strands through a transient double-strand break. These topoisomerases have been known to be involved in many processes of DNA metabolism including replication, recombination, transcription, and chromosome segregation at mitosis (Wang, 1985). In addition to their normal cellular functions, both topoisomerase I and topoisomerase II have generated extensive clinical interest in chemotherapy. Eukaryotic topoisomerase II is the target for intercalative antitumor agents such as 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA),[1] adriamycin, and ellipticine, as well as nonintercalative agents like etoposide and teniposide (Liu, 1989; D'Arpa & Liu, 1989). These drugs have the common properties of stabilizing a key covalent reaction intermediate of topoisomerase II, termed the cleavable complex, which can be detected as DNA double-strand breaks upon protein–denaturant treatments. A number of studies have shown that the clinical efficacies of these drugs correlate with their abilities to induce enzyme-mediated DNA cleavage (Rowe et al., 1986; Yamato et al., 1989; Long et al., 1987). On the basis of understanding the link between the drug-induced DNA cleavable complex with topoisomerase II and antitumor activity, we have screened cultures of actinomycetes and fungi for their ability to induce topoisomerase II mediated DNA cleavage in vitro. As a result of this screening, we have found that antitumor antibiotics, streptonigrin (Yamashita et al., 1990a), terpentecin and clerocidin (Kawada et al., 1991), and saintopin (Yamashita et al., 1990b), are potent inducers of topoisomerase II mediated DNA cleavage.

Eukaryotic topoisomerase I is the target for the antitumor plant alkaloid camptothecin (Liu, 1989; D'Arpa & Liu, 1989; Hsiang et al., 1985) and its synthetic derivatives such as CPT-11 (Kaneda et al., 1990) and topotecans (Kingsbury et al., 1991). In parallel with the results of topoisomerase II targeting drugs, the studies on several new camptothecin derivatives indicate that antitumor activity is correlated with their abilities to induce topoisomerase I mediated DNA cleavage in vitro (Hsiang et al., 1989; Jaxel et al., 1989). For topoisomerase II targeting drugs, high levels of the enzyme in proliferating cells and very low levels in quiescent cells appear to explain the selective sensitivity of proliferative tumor cells to the cytotoxic effects of these drugs (D'Arpa & Liu, 1989). In contrast, the intracellular levels of topoisomerase I have been reported to be largely unaffected by growth conditions of culture cells. However, the findings by Giovanella et al. (1989) that topoisomerase I was elevated in advanced-stage human colon cancer as compared to normal colon tissues would support the possibility that topoisomerase I is also an important target of antitumor drugs. In agreement with these results, recent clinical trials of camptothecin derivatives have shown that these drugs have promising potentials as a new antitumor agent (Taguchi, 1991). Thus, the identification of new drugs which induce cleavable complex formation with topoisomerase

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[1] Abbreviations: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; VP-16, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); SDS, sodium dodecyl sulfate.

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I is now viewed as a promising approach to find clinically effective antitumor agents. We have reported that a new antitumor antibiotic, saintopin, produced by *Paecilomyces* sp. is a new class of DNA intercalator targeting both topoisomerase I and topoisomerase II (Yamashita et al., 1991). In the course of continuous screening, we have now found that KT6006 and KT6528, synthetic derivatives of indolocarbazole antibiotic K252a (Kase et al., 1986; Yasuzawa et al., 1986), are potent inducers of the DNA cleavable complex with topoisomerase I. In this report, we describe the effects of indolocarbazole derivatives on calf thymus topoisomerase I and discuss the relation of topoisomerase I targeting activity to their antitumor activities.

MATERIALS AND METHODS

Enzymes, Nucleic Acids, and Chemicals. DNA topoisomerases I and II were isolated from calf thymus gland as described by Halligan et al. (1985) and partially purified with Bio-Rex70, hydroxylapatite, and P-11 phosphocellulose column chromatography. Topoisomerase I and II activities were monitored throughout the purification steps by DNA relaxation assay in the absence (topoisomerase I) or presence (topoisomerase II) of ATP and $MgCl_2$. To rule out contamination by each enzyme, the DNA cleavage activities with purified topoisomerases I and II were assayed in the presence of the topoisomerase I specific drug camptothecin or the topoisomerase II specific drug *m*-AMSA. Thus, the enzymes used in this study were free from contamination of another topoisomerase or endonucleases which was proved by data showing no production of nicked or linear DNA in the relaxation assay. Topoisomerases were kept at −20 °C in a storage buffer containing 50% (v/v) glycerol, 0.5 mM dithiothreitol, 0.1 mM EDTA, and 30 mM potassium phosphate, pH 7.5. One unit of activity was the amount of topoisomerase that relaxes half of the 0.4 μg of supercoiled DNA in the standard conditions described below. Supercoiled pBR322 and pUL402 DNAs were purified from *Escherichia coli* as described (Maniatis et al., 1982). Proteinase K was from Sigma Chemical Co. Bacteriophage T4 DNA ligase, S1 nuclease, and the restriction endonuclease *Hin*dIII were from Takara Shuzo Co. VP-16[1] was obtained from the National Cancer Institute, and *m*-AMSA and rebeccamycin were a gift from the Warner-Lambert Co. and Bristol-Myers Co., respectively. Camptothecin was isolated from wood of *Camptotheca accuminata* according to the method described previously (Wall et al., 1966). K252a was isolated from the culture broth of *Nocardiopsis* sp. as reported previously (Kase et al., 1986), and KT compounds such as KT6006 and KT6528 were synthesized on the basis of K252a as described by Murakata et al. (unpublished results). Stock solutions of these drugs were dissolved in dimethyl sulfoxide at 50 mM, stored at −20 °C, and diluted in methanol containing 40% dimethyl sulfoxide before use. The solvent used as diluent (dimethyl sulfoxide/methanol) did not affect topoisomerase I activities over the concentration ranges included in each reactions.

DNA Relaxation and DNA Cleavage Reactions with Topoisomerases. Reaction buffer contained 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 30 μg/mL bovine serum albumin. In the relaxation and cleavage reaction with topoisomerase I, reaction buffer (17 μL), DNA (0.48 μg in 1 μL of Tris–EDTA buffer), drug dissolved in 1 μL of dimethyl sulfoxide/methanol, and 1 μL of topoisomerase I in storage buffer were mixed in this order in ice/water. Reactions were incubated at 37 °C for 30 min, terminated by adding 6× loading buffer (0.25% bromo phenol blue, 0.25% xylenecyanol, and 15% Ficoll), and analyzed by agarose gel electrophoresis as described below (DNA relaxation assay). For DNA cleavage, reactions were terminated by the addition of 2 μL of a solution containing 5% SDS and 2.5 mg/mL proteinase K and incubated for an additional 30 min at 37 °C. After an appropriate volume of 6× loading buffer was added, samples were run onto an 1.2% agarose gel in 89 mM Tris–borate (pH 8.3)/2 mM EDTA buffer containing 0.1% SDS at 2 V/cm overnight. In the DNA cleavage assay with topoisomerase I, agarose gel electrophoresis was carried out in the presence of 0.5 μg/mL ethidium bromide. Gels were stained with ethidium bromide and washed in large amounts of water. The DNA band was visualized over UV light and photographed with Polaroid type 665 positive/negative film. The amount of DNA was quantitated by scanning negatives with a Shimazu scanning densitometer. The increase of nicked DNA was estimated as drug-induced topoisomerase I mediated DNA cleavage.

DNA Unwinding Measurements. DNA unwinding effects of drugs were assayed according to the method described by Camilloni et al. (1986) with minor modifications. Plasmid DNA was linearized with *Hin*dIII restriction endonuclease and recovered by phenol extraction and ethanol precipitation. Reaction mixtures (200 μL) containing 66 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 10 mM dithiothreitol, 0.7 mM ATP, 0.6 μg of linearized DNA, and drugs were equilibrated at 15 °C for 10 min and then incubated with excess amounts of T4 DNA ligase at 15 °C (controlled within ±0.5 °C) for 60 min. The reactions were stopped by addition of EDTA at 20 mM final concentration. DNA was analyzed by agarose gel electrophoresis after treatment to remove the drugs from the reaction mixture; extraction with phenol and ether and precipitation with ethanol.

Comparison of the Major Cleavage Sites. In the DNA cleavage reaction with DNA topoisomerase I, linearized pUL402 DNA was used as substrate. Topoisomerase I mediated DNA cleavage patterns (single-strand break) induced by the drugs were detected as a result of a double-strand break in combination with S1 nuclease reactions which can cut DNA single strand at nicks preinduced by topoisomerase I. After topoisomerase I mediated DNA cleavage in the presence or absence of drugs, the DNA was extracted with phenol and ether, and precipitated with ethanol. The thus obtained DNA (about 0.6 μg) was dissolved in 20 μL of reaction buffer containing 30 mM sodium acetate buffer (pH 4.6), 10 mM NaCl, and 1 mM $ZnSO_4$ and then incubated with 1 unit of S1 nuclease at 37 °C for 60 min. The reactions were stopped by addition of EDTA at 25 mM. DNA fragments produced by the successive reactions with topoisomerase I and S1 nuclease were analyzed by 1.2% agarose gel electrophoresis under the same conditions described for DNA cleavage.

RESULTS

Induction of Topoisomerase I Mediated DNA Cleavage by Indolocarbazole Derivatives. KT6006 and KT6528 (Figure 1) are semisynthetic derivatives of the indolocarbazole antibiotic K252a, which was isolated from *Nocardiopsis* sp. and found to inhibit protein kinases as well as calmodulin (Kase et al., 1986). In the course of screening for topoisomerase I targeting antitumor drugs, we found that these compounds induced topoisomerase I mediated DNA cleavage in vitro (Figure 2). In order to gain better understanding about the interaction with topoisomerase I, KT6006, KT6528, and their analogs, KT6661 and also the structurally related

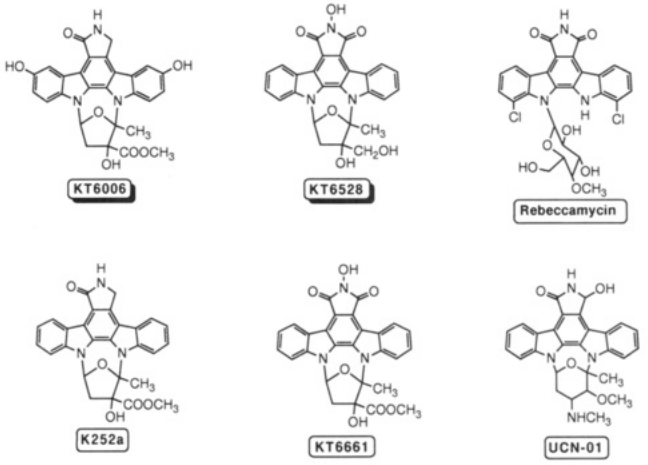

FIGURE 1: Structures of KT6006, KT6528, and related indolocarbazole compounds.

antibiotic rebeccamycin (Bush et al., 1987) were compared for their effects on calf thymus topoisomerase I. In the DNA cleavage assay using purified topoisomerase I and supercoiled plasmid pBR322 DNA, topoisomerase I mediated DNA cleavage is represented by a conversion of closed circular DNA into slower migrating nicked DNA on an agarose gel containing 0.5 μg/mL ethidium bromide. As shown in Figure 2, KT6006 and KT6528 produced the nicked DNA in a similar manner with camptothecin, while other analogues, K252a and KT6661, could not produce a significant amount of nicked DNA. The induction of DNA cleavage by K252a and KT6661 was not detected even if drug concentrations were increased up to 250 μM or higher amounts of topoisomerase I (>200 units) were used in the DNA cleavage assay (data not shown). Indolocarbazole compounds tested in this study did not induce topoisomerase II mediated DNA cleavage in vitro (data not shown). KT6528 induced a shift of the DNA band in a gel (Figure 2, lane n) because of the alteration in DNA conformation by strong intercalation as described below. To estimate the potency of topoisomerase I mediated DNA cleavage, the amount of nicked DNA was measured by scanning negatives with a densitometer. KT6006 induced topoisomerase I mediated DNA cleavage in a dose-dependent manner at drug concentrations up to 50 μM. The maximal amount of nicked DNA produced by KT6006 was more than 50% of substrate DNA, which was comparable to that of camptothecin. DNA cleavage induced by KT6528 was slightly suppressed at a high drug concentration of 50 μM. At higher concentrations, 100 μM, DNA cleavage by KT6528 was markedly suppressed whereas no suppression of DNA cleavage was observed for KT6006 (data not shown). Rebeccamycin was a week inducer of topoisomerase I mediated DNA cleavage under the same condition.

The experiment of Figure 3 was designed to prove that all the DNA cleavage detected in the presence of KT6006 and KT6528 was dependent on topoisomerase I. Neither KT6006 nor KT6528 produced any DNA cleavage in the absence of topoisomerase I (Figure 3, lanes e and h, respectively). Nicked products induced by KT6006 and KT6528 were released from the enzyme–DNA complex after digestion with proteinase K (lanes c and f) as observed for camptothecin (lanes i–k). Without proteinase K treatment, all cleaved DNA in the presence of KT6006 or KT6528 was covalently attached to the enzyme (lanes d and g), which is one of the hallmarks of the cleavable complex with topoisomerase I (Hsiang et al., 1985; Edwards et al., 1982). The shift of relaxed DNA bands

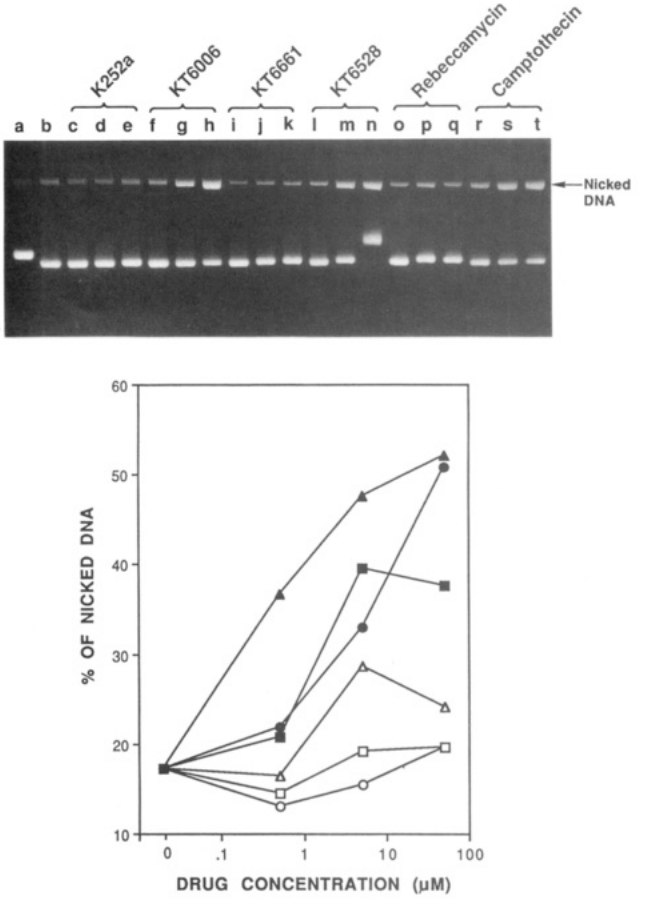

FIGURE 2: Mammalian DNA topoisomerase I mediated DNA cleavage activities of indolocarbazole derivatives. In the upper panel, 0.48 μg of supercoiled pBR322 DNA was incubated with 100 units of topoisomerase I in the presence of drugs (lanes c–t) followed by SDS/proteinase K treatment and then analyzed on an agarose gel containing 0.5 μg/mL ethidium bromide. Lane a, CCC-DNA control; lane b, no drug; lanes c–e, K252a; lanes f–h, KT6006; lanes i–k, KT6661; lanes l–n, KT6528; lanes o–q, rebeccamycin; lanes r–t, camptothecin. Drug concentrations were (lanes c, f, i, l, o, and r) 0.5, (lanes d, g, j, m, p, and s) 5, and (lanes e, h, k, n, q, and t) 50 μM. The bottom panel illustrates the appearance of nicked DNA as a function of drug concentration by densitometric analysis of lanes b–t from the gel; K252a (○); KT6006 (●); KT6661 (□); KT6528 (■); rebeccamycin (△); camptothecin (▲). Assay conditions are described under Materials and Methods.

in lanes f and g was due to drug-induced DNA unwinding by intercalation.

Reversibility of Topoisomerase I Mediated DNA Cleavage Induced by KT6006 and KT6528. The formation of cleavable complex by antitumor drugs has been shown to be reversed by serveral treatments such as dilution, increased salt concentration, or elevated temperature for a reaction mixture (Liu, 1989; D'Arpa & Liu, 1989; Hsiang et al., 1985; Hsiang & Liu, 1989). To test whether the DNA cleavage with topoisomerase I induced by indolocarbazole derivatives is reversible, a reaction mixture was briefly heated (65 °C) before termination with SDS and proteinase K. The reversibility of DNA cleavage induced by KT6006 and KT6528 was described as the disappearance of nicked DNA by the heat treatment (Figure 4). The same treatment for the complex with camptothecin was included for comparison. Heat treatment (65 °C) could reverse topoisomerase I mediated DNA cleavage by both KT6006 and KT6528, suggesting that the mechanism of DNA cleavage induced by indolocarbazole derivatives is through the formation of a reversible cleavable complex which has been reported for other known topoisomerase targeting drugs. The reverse rate of the complex with KT6006 and

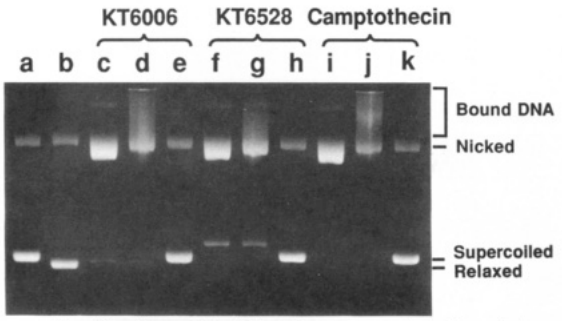

FIGURE 3: Requirement of topoisomerase I and proteinase K treatment for detection of single-stranded DNA cleavage by KT6006 and KT6528. Conditions used in the topoisomerase I mediated DNA cleavage assay are described under Materials and Methods, and the reaction was analyzed on an agarose gel containing 0.5 μg/mL ethidium bromide. DNA cleavage assays were carried out in the absence (lane b) or presence of 50 μM drugs, KT6006 (lanes c–e), KT6528 (lanes f–h), and camptothecin (lanes i–k). Lane a, substrate supercoiled DNA; lanes b, c, f, and i, standard topoisomerase I mediated DNA cleavage assay with SDS/proteinase K treatment; lanes d, g, and j, after the incubation with topoisomerase I and drugs, reactions were treated with SDS but not proteinase K; lanes e, h, and k, assays were carried out in the absence of topoisomerase I. The portions of topoisomerase I bound DNA as well as those of supercoiled, relaxed, and nicked DNA are indicated.

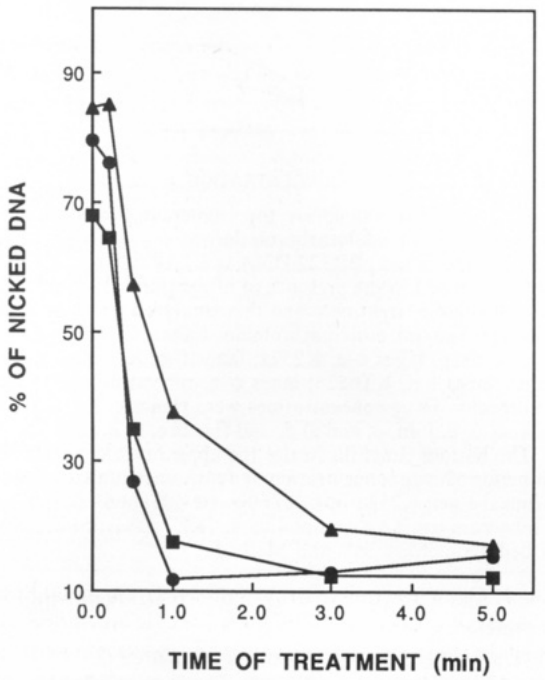

FIGURE 4: Heat reversal of drug-induced DNA cleavable complex. A large reaction mixture (120 μL) containing 50 μM KT6006 (●), KT6528 (■), or camptothecin (▲) was incubated with 200 units of topoisomerase I at 37 °C for 30 min. The reaction mixture was then heated to 65 °C, and aliquots (20 μL) were withdrawn at various times (0, 0.2, 0.5, 1, 3, and 5 min) during the treatment. SDS/proteinase K treatments and analysis with agarose gel electrophoresis were done as described under Materials and Methods. The amount of nicked DNA was quantified by scanning the agarose gels with a densitometer.

KT6528 was more rapid than that observed with camptothecin: Within 1 min, DNA cleavage induced by both KT6006 and KT6528 was suppressed to a background level, while the complete suppression of DNA cleavage by camptothecin required heat treatment for more than 3 min.

Effects of KT6006 and KT6528 on the DNA Relaxation Reaction Catalyzed by Topoisomerase. It has been shown that cleavable complex formation with antitumor drugs results in inhibition of the catalytic activity of topoisomerase. The

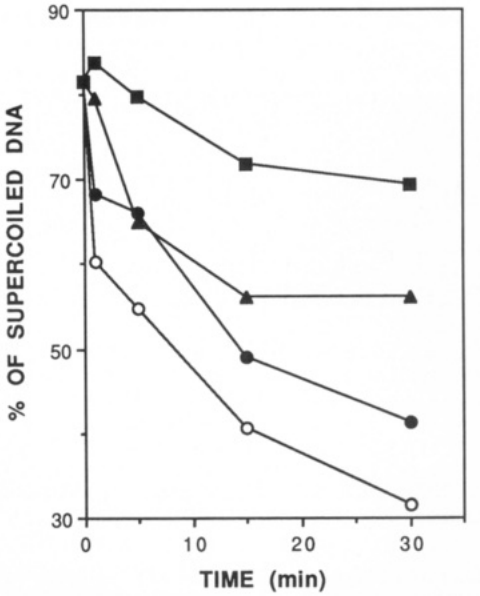

FIGURE 5: Inhibition of the catalytic activity of DNA topoisomerase I by KT6006 (●), KT6528 (■), and camptothecin (▲). Supercoiled pBR322 DNA was reacted with 2.0 units of topoisomerase I in the absence (○, control) or presence of the drugs at a concentration of 12.5 μM. The amount of supercoiled DNA was quantified from the band intensity of the gels by densitometric analysis.

time course of DNA relaxation by topoisomerase I in the presence or absence of drugs is shown in Figure 5. Under the condition used in this study, 2 units of topoisomerase I relaxed supercoiled DNA in a time-dependent manner, and about 30% of supercoiled DNA remained unchanged after 30 min. The presence of KT6006, KT6528, or camptothecin, at 12.5 μM, resulted in a decrease of the velocity of the relaxation. KT6006 was slightly less efficient than camptothecin with respect to the inhibition of DNA relaxation, which correlates with their ability to induce enzyme-mediated DNA cleavage (see Figure 2). KT6528 inhibited DNA relaxation of topoisomerase I more efficiently than camptothecin: about 70% of the supercoiled DNA remained unchanged after 30 min. The lack of DNA relaxation observed for KT6528 was due to the inhibition of topoisomerase I but not due to drug-induced DNA unwinding, since supercoiled DNA was relaxed in the presence of KT6528 at 12.5 μM when higher amounts of topoisomerase I (>10 units) were used. Considering the saturation of topoisomerase I mediated DNA cleavage activity at 5 μM (see Figure 2), it is likely that KT6528 inhibits the catalytic activity of topoisomerase I not only due to a stabilization of cleavable complexes but also because of an intercalation into DNA.

Effects of KT6006 and KT6528 on a DNA Unwinding Assay. Although camptothecin, VP-16, and terpentecin (Kawada et al., 1991) are classified as nonintercalative drugs, most of the antitumor drugs which can induce the cleavable complex with topoisomerase II are DNA intercalators, such as *m*-AMSA, adriamycin, ellipticines, and saintopin (Yamashita et al., 1991). To investigate whether KT6006 and KT6528 intercalate into DNA, an unwinding assay was performed using linearized pBR322 DNA and T4 DNA ligase (Yamashita et al., 1991; Camilloni et al., 1986). In this assay, DNA intercalator first unwinds linearized DNA, which results in a change of the twist of the duplex helix. Circularization of drug-bound DNA by T4 ligase freezes the linking number of the unwound DNA. Upon drug removal, the twist changes back to normal while the linking number remains constant, which causes the introduction of negative superhelicity into

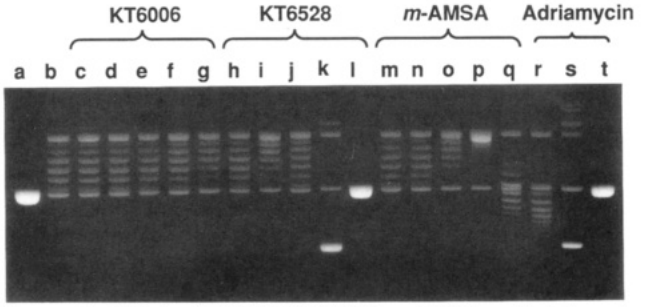

FIGURE 6: Effects of KT6006, KT6528, *m*-AMSA, and adriamycin on the DNA unwinding assay with T4 ligase. Unwinding measurements were done as described under Materials and Methods. In this assay, linearized plasmid DNA was incubated with T4 DNA ligase in the presence of the drugs at various concentrations. Drug-induced DNA unwinding was detected on an agarose gel containing no ethidium bromide. Lane a, substrate linear DNA; lane b, no drug; lanes c–g, KT6006; lanes h–l, KT6528; lanes m–q, *m*-AMSA; lanes r–t, adriamycin. Drug concentrations were (lanes c, h, m, and r) 0.5, (lanes d, i, and n) 1.5, (lanes e, j, o, and s) 5, (lanes f, k, and p) 15, and (lanes g, l, q, and t) 50 μM.

the DNA. Figure 6 shows the separation on an agarose gel of the products of the DNA unwinding assay with KT6006, KT6528, and the intercalators *m*-AMSA and adriamycin. Control DNA molecules appear as a lightly positively supercoiled population of topoisomers (lane b). Upon ligation in the presence of increasing concentration of intercalator, the resulting population of molecules becomes increasingly negative supercoiled compared to the control. On the gel, the topoisomers initially appear more relaxed and then progressively more negatively supercoiled (lanes m–q for *m*-AMSA and lanes r and s for adriamycin). In addition, the inhibition of ligation by a high level of intercalation is also revealed as an increase of the level of substrate linear DNA remained. This is the case at the highest concentration (50 μM) of adriamycin (lane t), which is known to cause inhibition of T4 ligase due to its strong intercalation activity (Montecucco et al., 1988). Under the assay condition described above, KT6006 induced slight upward shift of topoisomers at the highest concentration of 50 μM (lane g). Thus, the DNA unwinding activity observed for KT6006 was much less than that of *m*-AMSA (lanes m–q) which belongs to the group of a weak intercalator, indicating that KT6006 is a very weak intercalator. In contrast, KT6528 converted topoisomers to more relaxed form at low concentration (1.5 μM, lane i) and then to complete negative supercoiled form at high concentration (15 μM, lane k). Moreover, as found for adriamycin, inhibition of ligation by KT6528 was also detected at 50 μM (lane l), indicating that KT6528 is a strong intercalator with potentials comparable to that of adriamycin. Thus, indolocarbazole derivatives with topoisomerase I mediated DNA cleavage activities are classified into two groups with respect to their potency in DNA intercalation; KT6006 is a very weak intercalator, and KT6528 is a strong intercalator.

Major DNA Cleavage Sites Induced by Topoisomerase I in the Presence of KT6006 and KT6528: Comparison with Camptothecin. Topoisomerase I mediated DNA cleavage site induced by camptothecin has been shown to be different from a native cleavage site of topoisomerase I without drugs. In the case of topoisomerase II targeting drugs, mapping studies of the DNA cleavage sites have revealed that different DNA cleavage patterns are observed when drugs from the different chemical classes are compared while drugs from the same chemical classes show similar cleavage patterns (Tewey et al., 1984b; Capranico et al., 1990). The major cleavage sites induced by KT6006 and KT6528 were compared with

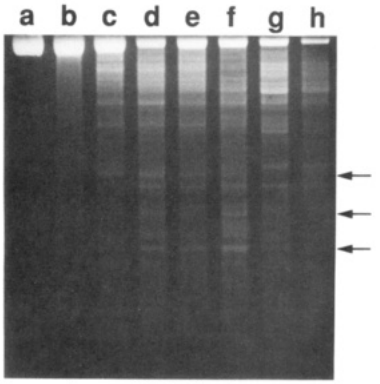

FIGURE 7: Comparison of DNA cleavage patterns induced by KT6006, KT6528, and camptothecin. Plasmid pUL402 DNA linearized with *Hin*dIII was used in this assay. Topoisomerase I mediated DNA cleavage assay in combination with S1 nuclease digestion was done under the conditions described under Materials and Methods. DNA cleavage patterns were analyzed on an agarose gel containing no ethidium bromide. Lane a, DNA alone; lane b, DNA alone plus topoisomerase I/S1 nuclease; lane c, 5 μM KT6006 plus topoisomerase I/S1 nuclease; lane d, 50 μM KT6006 plus topoisomerase I/S1 nuclease; lanes e–f and g–h, same as lanes c–d, respectively, except that KT6528 (lanes e–f) and camptothecin (lanes g–h) were used. Arrows indicate the DNA fragments produced by KT6006 and KT6528 but not by camptothecin.

camptothecin using a DNA cleavage assay in combination with S1 nuclease digestion which cleaved single-strand DNA at nicks (Yamashita et al., 1991). When the major fragments produced by KT6006 (Figure 7, lanes c and d) and KT6528 (lanes e and f) were compared with those produced by camptothecin (lanes g and h), some major DNA fragments produced by both KT6006 and KT6528 (indicated by arrows) were not present in the lanes of camptothecin. Thus, DNA cleavage patterns produced by KT6006 and KT6528 were nearly identical, but strictly different from that of camptothecin.

DISCUSSION

DNA topoisomerases are now viewed as important therapeutic targets in cancer chemotherapy. Although a number of antitumor agents which can interact with topoisomerase II have been used to establish a relationship between drug-induced cleavable complex formation and antitumor activity, topoisomerase I targeting antitumor agent has been known only for camptothecin and its derivatives. Recently, we have reported that the antibiotic saintopin is a new class of antitumor agent which can induce cleavable complex with both topoisomerase I and topoisomerase II (Yamashita et al., 1991). Studies to elucidate which topoisomerase is the principal target of saintopin in tumor cells are currently in progress. The results presented here demonstrate that indolocarbazole derivatives, KT6006 and KT6528, act selectively on topoisomerase I through the mechanism of stabilizing the cleavable complex. Thus, indolocarbazole compounds represent a new class of agents targeting topoisomerase I.

The nature of the cleavable complex with topoisomerase I induced by KT6006 and KT6528 has the following characteristics which are essentially comparable to those of camptothecin. (i) Topoisomerase I mediated DNA cleavage by KT compounds is detected after the digestion of a reaction product with proteinase K, indicating all DNA cleaved is covalently attached to the enzyme. The covalent enzyme-cleaved DNA linkage is a hallmark of both topoisomerase I and topoisomerase II (Liu, 1989; D'Arpa & Liu, 1989; Edwards et al., 1982; Liu et al., 1983). (ii) The topoisomerase

I mediated DNA cleavage by KT6006 and KT6528 is reversed efficiently by heat treatment. Reversal of camptothecin-induced DNA cleavage by heat treatment requires a slightly longer incubation than that of indolocarbazole derivatives. Kjeldsen et al. reported that reversal of topoisomerase I mediated DNA cleavage with camptothecin was slow at certain DNA sequences (Kjeldsen et al., 1987). As found for topoisomerase II targeting drugs, the stability of the cleavable complex against several treatments such as increased salt concentration and elevated temperature has been shown to be varied depending on the drugs (Kawada et al., 1991; Tewey et al., 1984a). At present, it remains unclear whether the difference in the stability of the cleavable complex by antitumor agents is related to their biological activities. (iii) In the assay for topoisomerase I mediated DNA cleavage, KT6006 and KT6528 produce similar DNA fragments which are distinctly different from the fragments generated by camptothecin. This is consistent with a previous study using topoisomerase II targeting drugs, in which DNA cleavage sites are similar for structurally related drugs while chemically unrelated drugs induce DNA cleavage at different sites. In addition, Hsiang et al. demonstrated that a similar cleavage pattern produced by different camptothecin derivatives is an important factor for correlation between antitumor activity in vivo and the frequency of drug-induced topoisomerase I–DNA-cleavable complexes in vitro (Hsiang et al., 1989). Therefore, a similar DNA cleavage pattern detected with indolocarbazole derivatives suggests that the antitumor activity of the structurally related derivatives other than KT6006 and KT6528 could be estimated by the amount of drug-induced topoisomerase I mediated DNA cleavage in vitro.

KT6006 and KT6528 have closely related structures, and the nature of their cleavable complex with topoisomerase I is similar as noted above. With respect to the interaction with DNA, however, these drugs have different properties: KT6006 is a very weak intercalator, and KT6528 is a strong intercalator with a potential comparable to that of adriamycin. Thus, the strength of DNA intercalation of indolocarbazole derivatives is not correlated with their potency in inducing topoisomerase I mediated DNA cleavage. This finding is consistent with the previous observation for topoisomerase II targeting drugs such as anthracyclines and *m*-AMSA derivatives, in which DNA intercalation is not paralleled to the activity to induce topoisomerase II mediated DNA cleavage (Capranico et al., 1990; Pommier et al., 1987). Concerning the effects of DNA intercalation on topoisomerase activities, previous studies with anthracycline or ellipticine derivatives indicated that topoisomerase II mediated DNA cleavage by these strong intercalators was suppressed at higher drug concentrations, due to the change of DNA conformation which blocks topoisomerase II access to the DNA (Capranico et al., 1990; Tewey et al., 1984a; Fosse et al., 1990). Similarly, topoisomerase I mediated DNA cleavage was suppressed with KT6528 at a concentration of 50 μM, but not with the weak intercalator KT6006. In addition, KT6528 was more inhibitory than KT6006 against the DNA relaxation activity of topoisomerase I at a concentration of 12.5 μM (see Figure 5). Thus, these results indicate that the strong DNA intercalation activity of KT6528 causes an inhibitory effect on the catalytic activity of topoisomerase I, presumably due to modifying the DNA structure, which results in the suppression of topoisomerase I mediated DNA cleavage at high concentrations. The knowledge about how the topoisomerase I active drug forms a cleavable complex between enzyme and DNA is now restricted to the data obtained from the study with camptothecin (Herzberg et al., 1990). Therefore, indolocarbazole derivatives with or without topoisomerase I mediated activity, including also both strong and weak DNA intercalators, will provide useful tools for further comparative studies on the precise molecular mechanism of cleavable complex formation with topoisomerase I.

Structure–activity studies on topoisomerase targeting drugs with limited structural modifications suggest that there is a good correlation between the antitumor activity and the ability of drugs to induce the cleavable complex. The evaluation of the antitumor activity of indolocarbazole derivatives against murine leukemia model P388 showed that KT6006 and KT6528 exhibited antitumor activity in vivo while K252a and KT6661, which could not induce topoisomerase I mediated DNA cleavage in vitro, did not exhibit any significant antitumor activity in vivo (data not shown). Thus, the correlation between antitumor activity and DNA cleavage for indolocarbazole derivatives indicates that the cleavable complex formation with topoisomerase I could be responsible for the antitumor activity of KT6006 and KT6528. Besides the effects on mammalian DNA topoisomerase I described in this study, indolocarbazole compounds have been known to show a diversity of biological activities. As previously reported, the microbial alkaloid staurosporine and K252a showed potent inhibitory activities against various protein kinases including both serine/threonine kinases and tyrosine kinases (Tamaoki & Nakano, 1990). In addition, UCN-01 (Figure 1), a selective inhibitor of protein kinase C, showed antitumor activity in vivo (Akinaga et al., 1991). Although the critical protein kinase inhibited in tumor cells by UCN-01 remained unclear, the potent and selective inhibition of protein kinase seems to lead to the antitumor activities of UCN-01, since UCN-01 does not induce topoisomerase I mediated DNA cleavage in vitro (data not shown). In the case of indolocarbazole derivatives tested in this study, however, there is no relevance of protein kinase inhibition with respect to their antitumor activities because K252a and KT6661 inhibit protein kinase C with IC_{50} values of 0.028 and 0.020 μg/mL, respectively, but they do not show any antitumor activities in vivo. Moreover, KT6528 and rebeccamycin, which have a marginal inhibitory activity against protein kinase C with IC_{50} values of 1.8 and 10 μg/mL, respectively, show a significant antitumor activity in vivo (data not shown). Thus, it is evident that the antitumor activities of KT6006 and KT6528 could be due to cleavable complex formation with topoisomerase I. Our present findings provide the important new basis for understanding the mechanism of action of antitumor indolocarbazole compounds as well as for designing new derivatives with superior antitumor activity.

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