DNA Cleavage by Topoisomerase I in the Presence of Indolocarbazole Derivatives of Rebeccamycin†

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ABSTRACT: DNA topoisomerase I has been shown to be an important therapeutic target in cancer chemotherapy for the camptothecins as well as for indolocarbazole antibiotics such as BE-13793C and its synthetic derivatives NB-506 and ED-110 [Yoshinari et al. (1993) *Cancer Res. 53,* 490−494]. To investigate the mechanism of topoisomerase I inhibition by indolocarbazoles, we have studied the induction of DNA cleavage by purified mammalian topoisomerase I mediated by the antitumor antibiotic rebeccamycin and a series of 20 indolocarbazole derivatives. The compounds tested bear (i) various functional groups on the non-indolic moiety (X = CO, CH_2, CHOH), (ii) a hydrogen or a chlorine atom at positions 1 and 11 (R_2), and (iii) different substituents on the maleimido function (R_1 = H, OH, NH_2, NHCHO). Half of the ligands have the same carbohydrate moiety as rebeccamycin whereas the other ligands have no sugar residue. The inhibitory potency of the test compounds was assessed *in vitro* by comparing the cleavage of [^{32}P]-labeled restriction fragments by the enzyme in the absence and presence of the drug. In addition, the DNA-binding properties of these compounds were investigated by means of complementary spectroscopic techniques including electric linear dichroism, and the DNA sequence selectivity was probed by DNase I footprinting. The study shows that the sugar residue attached to the indolocarbazole chromophore is critical for the drug ability to interfere with topoisomerase I as well as for the formation of intercalation complexes. Structure−activity relationships indicate that the presence of chlorine atoms significantly reduces the effects on topoisomerase I whereas the substituents on the maleimido function and the functional group on the non-indolic moiety can be varied without reduction of activity. The results suggest that the inhibition of topoisomerase I by indolocarbazoles arises in part from their ability to interact with DNA. Analysis of the base preferences around topoisomerase I cleavage sites in various restriction fragments indicated that, in a manner similar to camptothecin, the rebeccamycin analogue R-**3** stabilized topoisomerase I preferentially at sites having a T and a G on the 5′ and 3′ sides of the cleaved bond, respectively. By analogy with models previously proposed for camptothecin and numerous topoisomerase II inhibitors which intercalate into DNA, a stacking model for the interaction between DNA, topoisomerase I and indolocarbazoles is proposed. These findings provide guidance for the development of new topoisomerase I-targeted antitumor indolocarbazole derivatives.

An increasing number of drugs which prevent the action of the enzymes topoisomerase I and topoisomerase II are used clinically in the treatment of a variety of cancers (Ralph *et al.*, 1993; Capranico & Zunino, 1995). The drugs include (i) many topoisomerase II inhibitors such as daunomycin, amsacrine, mitoxantrone and the epipodophyllotoxins etoposide and teniposide, (ii) a few topoisomerase I inhibitors such as the camptothecin derivatives irinotecan (CPT-11) and topotecan, and (iii) dual topoisomerase I−topoisomerase II poisons such as actinomycin D. Other potent topoisomerase inhibitors (e.g., CI-921, intoplicine, merbarone) are currently undergoing clinical trials. Both the limited number of topoisomerase I inhibitors available and the broad spectrum of potent antineoplastic activity observed for camptothecin call for the identification of new compounds which can efficiently poison mammalian topoisomerase I. Although camptothecin derivatives continue to be the subject of intensive efforts aimed at developing more efficient analogues (Pommier & Tanizawa, 1993; Tanizawa *et al.*, 1994, 1995; Luzzio *et al.*, 1995; Sawada *et al.*, 1995), many groups are searching for structurally distinct compounds. Recently, three promising categories of drugs which stabilize the DNA−topoisomerase I covalent intermediate (termed cleavable complex) have been identified: (i) benzophenanthridine alkaloids such as fagaronine, coralyne, and berberine (Fujii *et al.*, 1993; Larsen *et al.*, 1993; Makhey *et al.*, 1994; Gatto

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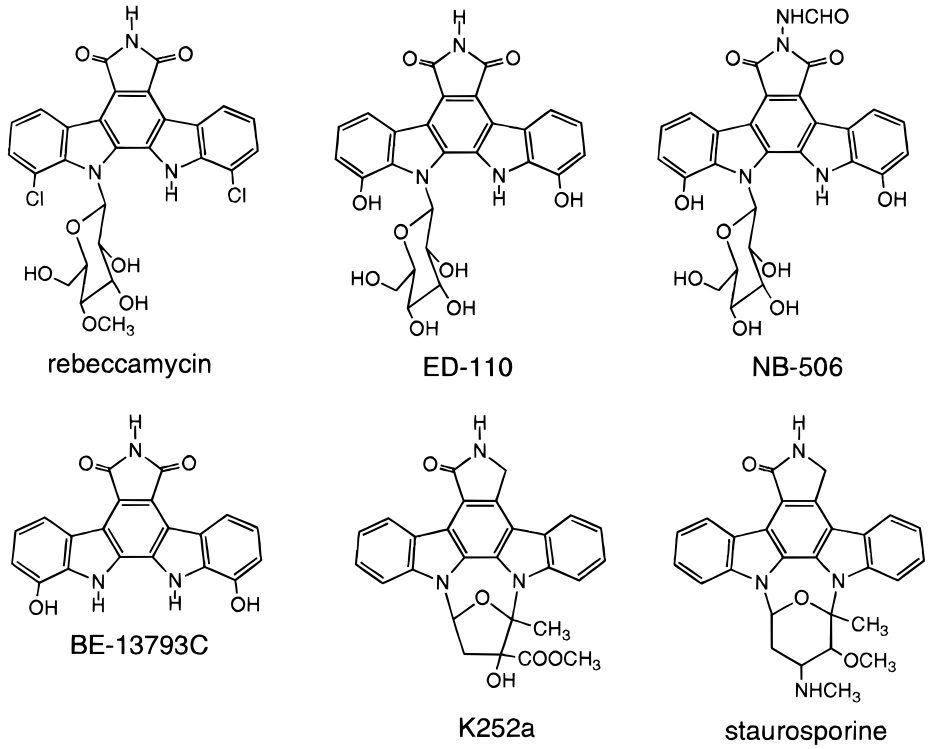


FIGURE 1: Chemical structures of rebeccamycin and related compounds.

et al., 1996), (ii) compounds isolated from a Chinese plant *Erodium stephanianum* such as corilagin and chebulagic acid (Berry *et al.*, 1992) and (iii) indolocarbazoles related to the antibiotics K252a and BE-13793C (Yamashita *et al.*, 1992; Yoshinari *et al.*, 1993, 1995). The latter class of compounds appears particularly interesting for at least two reasons. First, in contrast to the camptothecins, indolocarbazoles bind to DNA in the absence of topoisomerase I. This property has immediate implication for the design of sequence-specific topoisomerase I-targeted drugs (Xie *et al.*, 1996). Second, a direct correlation has been found between their *in vivo* anticancer activity and the abilities of indolocarbazoles to induce topoisomerase I-mediated DNA cleavage *in vitro* (Yamashita *et al.*, 1992). These two observations prompted us to investigate the DNA-binding properties and effects on topoisomerase I of a series of indolocarbazoles structurally related to the antitumor antibiotic rebeccamycin (Figure 1).

Rebeccamycin was isolated ten years ago from a culture of *Saccharotrix aerocolonigenes* (Nettleton *et al.*, 1985; Bush *et al.*, 1987). This indolocarbazole derivative exhibits significant antitumor properties, but its toxicity proscribes its use in cancer chemotherapy. Rebeccamycin bears a structural resemblance to the aforementionned topoisomerase I inhibitors K252a and BE-13793C (Figure 1) because they all contain the indolocarbazole chromophore. It is also structurally close to the antibiotic staurosporine (Figure 1) which, together with K252a, is a potent inhibitor *in vitro* of a wide range of protein kinases including protein kinase C (PKC) (Tamaoki *et al.*, 1986; Kase *et al.*, 1986). Despite these structural analogies, rebeccamycin has no inhibitory activity on protein kinase C and only weakly interferes with topoisomerase I. Different series of rebeccamycin analogues have been developed, principally in an effort to find potent PKC and/or topoisomerase I inhibitors and to confer higher antitumor activity. Among the numerous indolocarbazole derivatives synthesized, compound NB-506 (Figure 1) which is a 13-*N*-glucopyranosyl and 6-*N*-formylamino derivative of BE-13793C, was found to be a topoisomerase I inhibitor endowed with remarkable antitumor effects in transplanted tumors in mice (Yoshinari *et al.*, 1995; Arakawa *et al.*, 1995). These different considerations together with our continuing synthesis of indolocarbazole-based PKC inhibitors prompted us to search for topoisomerase I poisons among the rebeccamycin analogues recently synthesized (Fabre *et al.*, 1992, 1993a,b, 1994; Sancelme *et al.*, 1994; Rodrigues-Pereira *et al.*, 1995). In an effort to identify the structural features of the drugs responsible for topoisomerase I inhibition, we have investigated a series of 21 compounds, hereafter referred to as R-**1** to R-**21** (Table 1), which differ from rebeccamycin (R-**1**) in four respects: (i) by the functional groups on the non-indolic heterocyclic moiety (X = CO, CH_2, CHOH), (ii) by the presence or not of chloro groups at positions 1 and 11 (R_2 = H or Cl), (iii) by the substituent at position 6 on the maleimido function (R_1 = H, OH, NH_2 or NHCHO) and (iv) by the presence or absence of the sugar residue (Table 1).

The elucidation of the mode of binding to DNA and the effects of these indolocarbazoles on topoisomerase I *in vitro* may provide useful information concerning the likely mechanism of action of structurally related antibiotics such as K252a and BE-13793C and may ultimately facilitate the design of new therapeutic agents with improved antitumor properties. This report demonstrates that some of the drugs tested are potent topoisomerase I inhibitors and presents data which imply that the inhibition of topoisomerase I arises, in part, from their ability to interact with DNA. A hypothetical model for the interaction between DNA, topoisomerase I, and indolocarbazoles is presented. The results provide guidelines for the design of indolocarbazole topoisomerase I inhibitors.

MATERIALS AND METHODS

Drugs and Chemicals. Camptothecin was purchased from Sigma Chemical Co. (La Verpillière, France). The synthesis of the rebeccamycin derivatives and indolocarbazoles used in this study has been described previously (Fabre *et al.*, 1992, 1993a,b, 1994; Sancelme *et al.*, 1994; Rodrigues-Pereira *et al.*, 1995). Drugs were dissolved in dimethyl sulfoxide (DMSO) at 3 mg/mL and then further diluted with water. The final DMSO concentration never exceeded 0.3% (v/v) in the cleavage reactions. Under these conditions DMSO, which is also used in the controls, does not affect the topoisomerase activity. The stock solutions of drugs were kept at −20 °C and freshly diluted to the desired concentration immediately prior to use (diluted solutions tend to precipitate when stored at 4 °C). All other chemicals were analytical grade reagents.

Biochemicals. DNA from calf thymus and the double-stranded polynucleotides poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC) were from Sigma. Their concentrations were determined applying molar extinction coefficients of 6600, 6600, and 8400 M^{-1} cm^{-1}, respectively (Wells *et al.*, 1970). Calf thymus DNA was deproteinized with sodium dodecyl sulfate (protein content <0.2%) and all nucleic acids were dialyzed against 1 mM sodium cacodylate buffer pH 6.5. Plasmid DNA were isolated from *E. coli* by a standard sodium dodecyl sulfate−sodium hydroxide lysis procedure and purified by banding in CsCl-ethidium bromide gradients. The 160 base pair *tyr T* DNA was obtained by digestion of plasmid pKM27 (Drew et al., 1985) with *Eco*RI and *Ava*I and was labeled at the *Eco*RI site on the lower strand with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I (Boehringer). The 117-mer and 265-mer were obtained from the plasmid pBS (Stratagene, La Jolla, CA) digested with *Pvu*II and *Eco*RI. These digestions also yielded fragments suitable for 3′-end labeling by the Klenow polymerase. The detailed procedures for isolation, purification, and labeling of these duplex DNA fragments have been described previously (Bailly *et al.*, 1990, 1992a). Electrophoresis on a non-denaturing 6.5% (w/v) polyacrylamide gel removed excess radioactive nucleotide. The desired 3′-end-labeled product was cut out of the gel and eluted overnight in 500 mM ammonium acetate, 10 mM magnesium acetate. The purifed DNA was then precipitated twice with 70% ethanol prior to being resuspended in 10 mM Tris buffer, pH 7.0, containing 10 mM NaCl.

Circular dichroism (CD) measurements were recorded on a Jobin-Yvon CD6 dichrograph. Solutions of drugs and/or nucleic acids in 1 mM sodium cacodylate buffer, pH 6.5 were scanned in 1 cm quartz cuvettes. Measurements were made by progressive dilution of a drug−DNA complex at a high P/D ratio with a pure ligand solution to yield the desired drug/DNA ratios. Three scans were accumulated and automatically averaged.

Electric linear dichroism (ELD) measurements were performed using a computerized optical measurement system built by C. Houssier (Houssier & O'Konski, 1981). The procedures outlined previously were followed (Houssier, 1981). The optical set-up incorporating a high sensitivity *T*-jump instrument equipped with a Glan polarizer was used under the following conditions: bandwidth 3 nm, sensitivity limit 0.001 in $\Delta A/A$, response time 3 μs. Equations used for the calculation of the different parameters have been reported (Houssier, 1981; Bailly *et al.*, 1992b). All experiments were conducted at 20 °C with a 10 mm pathlength Kerr cell having 1.5 mm electrode separation, in 1 mM sodium cacodylate buffer, pH 6.5. The DNA samples were oriented under an electric field strength of 13 kV/cm, and the drug under test was present at 10 μM together with the DNA or polynucleotide at 100 μM unless otherwise stated. This electro-optical method has proved most useful as a means of determining the orientation of drugs bound to DNA, and has the additional advantage that it senses only the orientation of the polymer-bound ligand: free ligand is isotropic and does not contribute to the signal (Bailly *et al.*, 1992b; Colson *et al.*, 1996).

To investigate the geometry of drug binding to DNA by ELD, the reduced dichroism $\Delta A/A$ of a ligand−DNA complex measured in the ligand absorption band must be analyzed with respect to the reduced dichroism measured for the same DNA or polynucleotide at 260 nm in the absence of drug, $(\Delta A/A)^{DNA}$. The reduced dichroism ratio DR is defined as follows: $DR = [(\Delta A/A)^{ligand-DNA}]/[(\Delta A/A)^{DNA}]$. The numerator refers to the reduced dichroism of the drug−DNA complex measured at the absorption maximum of the ligand bound to DNA. The denominator is always negative under the experimental conditions used. The dichroism ratio is expected to be +1 if the transition moment of the drug chromophore is parallel to the DNA bases, as in the case of complete intercalative binding. For groove binders, the angle between the double helical axis and the long axis of the chromophore lies below 54° which gives rise to positive dichroism and thus to a negative DR value. Under these conditions, the reduced dichroism ratios for any given drug−DNA and drug−polynucleotide complexes can be mutually compared with good relative accuracy, independent of the polymer size (Bailly *et al.*, 1992d; Colson *et al.*, 1996).

Topoisomerase I DNA Cleavage Reactions

Experiments with Linear Plasmid DNA on Agarose Gels. Topoisomerase I was purified from calf thymus using a previously described procedure (Riou *et al.*, 1986). The stock solution of purified topoisomerase I was at 450 units/μL with a protein content of 105 mg/mL. pBR322 DNA (Boehringer Mannheim, Germany) was linearized with *Eco*RI and labeled with [α-^{32}P]dATP in the presence of the Klenow fragment of DNA polymerase I. The labeled DNA was then digested to completion with *Hin*dIII. The cleavage reaction mixture contained 20 mM Tris-HCl, pH 7.4, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 2×10^4 dpm of [α-^{32}P]pBR322 DNA and the indicated drug concentrations. The reaction was initiated by the addition of topoisomerase I (40 units in 20 μL reaction volume) and allowed to proceed for 10 min at 37 °C. Reactions were stopped by adding SDS to a final concentration of 0.25% and proteinase K to 250 μg/mL, followed by incubation for 30 min at 50 °C. Samples were denatured by the addition of 10 μL of denaturing loading buffer consisting of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, 0.1% bromocresol green prior to loading on to a 1% agarose gel in TBE buffer containing 0.1% SDS. Electrophoresis was conducted at 2 V/cm for 18 h.

Sequencing of Topoisomerase I-Mediated DNA Cleavage Sites. Each reaction mixture contained 2 μL of 3′-end [^{32}P]-labeled DNA (∼1 μM), 5 μL of water, 2 μL of 10×

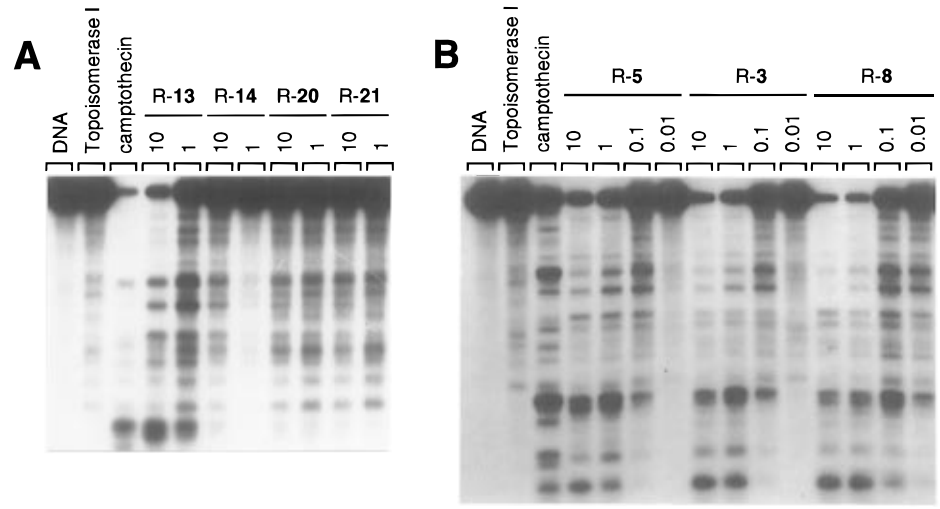
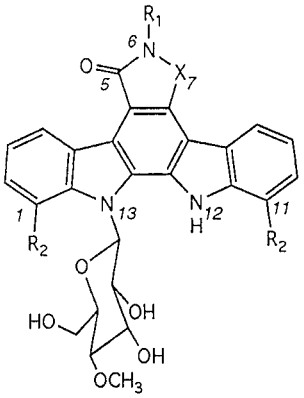
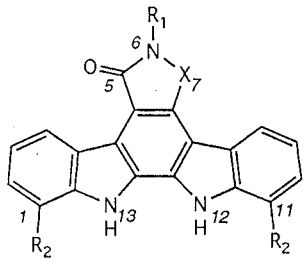


FIGURE 2: Topoisomerase I-mediated cleavage of DNA in the presence of (A) indolocarbazoles R-**13**, R-**14**, R-**20**, and R-**21** and (B) rebeccamycin analogues R-**3**, R-**5**, and R-**8** at concentrations ranging from 0.01 to 10 μg/mL. Purified calf thymus topoisomerase I (40 units) was incubated with the *Eco*RI-*Hin*dIII restriction fragment from pBR322 (^{32}P-labeled at the *Eco*RI site) in the presence and absence of the test ligands. Reactions were carried out for 10 min at 37 °C and then stopped with SDS−proteinase K treatment. Single-strand DNA fragments were analyzed on a 1% alkaline agarose gel in TBE buffer. Lanes DNA and topoisomerase I refer to the radiolabeled 4330 base pair DNA substrate incubated without and with enzyme respectively. Camptothecin was used at 0.3 and 0.03 μg/mL in panels A and B, respectively.

Table 1: Structure of the Indolocarbazole Derivatives Used in This Study





compound	R_1	R_2	X	MIC
R-1[a]	H	Cl	CO	1[b]
R-18	H	H	CO	1
R-19	H	Cl	CH_2	1
R-8	H	H	CH_2	0.01
R-6	OH	Cl	CO	0.3
R-3	OH	H	CO	0.1
R-5	NH_2	Cl	CO	0.1
R-7	NH_2	H	CO	0.1
R-9	NHCHO	Cl	CO	1
R-17	NHCHO	H	CO	0.1

compound	R_1	R_2	X	MIC
R-2	H	Cl	CO	>10
R-11	H	H	CO	10
R-21	H	Cl	CH_2	>10
R-10	H	H	CH_2	10
c				
R-4	OH	H	CO	>10
R-20	NH_2	Cl	CO	>10
R-14	NH_2	H	CO	10
R-16	NHCHO	Cl	CO	>10
R-15	NHCHO	H	CO	1
R-12	H	Cl	CHOH	>10
R-13	H	H	CHOH	1

[a] Rebeccamycin. [b] MIC corresponds to the minimum drug concentration at which topoisomerase I-mediated DNA cleavage was detected (μg/mL). [c] The indolocarbazole with R_1 = OH, R_2 = Cl, and X = CO could not be synthesized.

topoisomerase I buffer, and 10 μL of drug solution at the desired concentration (5−25 μg/mL). After at least 30 min incubation to ensure equilibration, the reaction was initiated by addition of 2 μL (40 units) purified topoisomerase I from calf thymus (as above). Samples were incubated for 40 min at 37 °C prior to adding SDS to 0.25% and proteinase K to 250 μg/mL to dissociate the drug−DNA−topoisomerase I cleavable complexes. The DNA was precipitated with

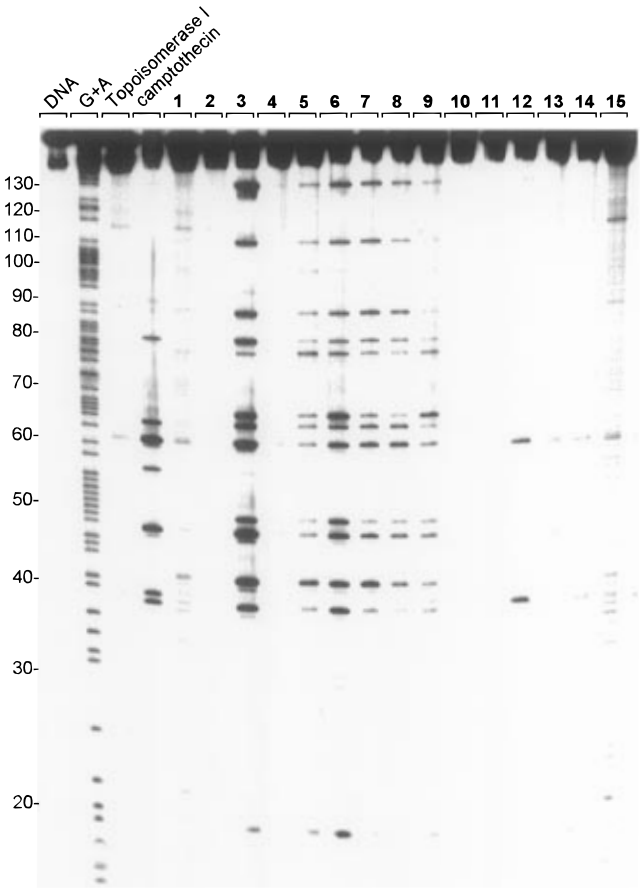


FIGURE 3: Phosphorimage comparing the susceptibility of the 160 bp *tyr*T fragment to cutting by topoisomerase I in the presence of compounds R-**1** to R-**15**. The 5′-end labeled fragment (DNA lane) was incubated in the absence (topoisomerase I lane) or presence of the indolocarbazole derivatives at 25 μg/mL or camptothecin at 5 μg/mL (camptothecin lanes). Topoisomerase I cleavage reactions were analyzed on an 8% denaturing polyacrylamide gel as described in Materials and Methods. Numbers at the left of the gel show the nucleotide position, determined with reference to the purine nucleotide tracks labeled G+A.

ethanol and then resuspended in 5 μL of formamide−TBE loading buffer, denatured at 90 °C for 4 min, and then chilled in ice for 4 min prior to loading on to the sequencing gel.

DNase I footprinting experiments were performed essentially according to the protocols previously described (Bailly & Waring, 1995). Briefly, samples of the labeled DNA fragment were incubated with a buffered solution containing the desired drug concentration. After 30−60 min incubation at 37 °C to ensure equilibration, the digestion was initiated by addition of the DNase I solution. The extent of digestion was limited to less than 30% of the starting material so as to minimize the incidence of multiple cuts in any strand. After 3 min incubation at room temperature, the reaction was stopped by freeze drying and samples were lyophilized. The DNA in each tube was resuspended in 5 μL of formamide−TBE loading buffer, denatured at 90 °C for 4 min then chilled in ice for 4 min prior to loading on to the sequencing gel.

Electrophoresis and Quantitation by Storage Phosphor Technology Autoradiography. DNA cleavage products were resolved by electrophoresis under denaturing conditions in polyacrylamide gels (0.3 mm thick, 8% acrylamide containing 8 M urea). Electrophoresis was performed for about 2 h at 60 watts in TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM Na_2 EDTA, pH 8.3). Gels were soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80 °C, and then analyzed on the phosphorimager. Photostimulable phosphor imaging plates (Kodak storage phosphor screens obtained from Molecular Dynamics) were pressed flat against dried sequencing gels and exposed overnight at room temperature. A Molecular Dynamics 445SI PhosphorImager was used to collect all data which were analyzed using the ImageQuant version 4.1 software. Each resolved band on the autoradiograph was assigned to a particular bond within the DNA fragment by comparison of its position relative to sequencing standards (Maxam & Gilbert, 1980).

Statistical Tests and Probability Calculations. The χ^2 one-sample test was used to determine the deviation from the random distribution of base at each position of the aligned sequences. The expected occurrence of each nucleotide was calculated considering the nucleotide average frequencies of the three restriction fragments tested (A = T= 25.7; G = C = 24.3). The procedure used to calculate probabilities (p) of deviations from expected base frequency was based on previous methods (Capranico *et al.*, 1990, 1993, 1994a,b; Pommier *et al.*, 1991; Jaxel *et al.*, 1991). The negative values of the decimal logarithm of p, $-\log p$, are reported for each base at each position around the cleavage site. The statistical analysis was performed using the software package StatGraphics Plus 6.0.

RESULTS

Topoisomerase-Mediated DNA Cleavage

It is well-established that in the presence of purified topoisomerase I and DNA, camptothecin stabilizes the enzyme−DNA interaction in the form of a covalent intermediate called "cleavable complex" (Hsiang *et al.*, 1985; Hertzberg *et al.*, 1989, 1990; Pommier & Tanizawa, 1993). In this complex the enzyme is covalently linked to the 3′-end of the DNA and treatment with a detergent (e.g., SDS) results in the formation of DNA single-strand breaks which can be revealed by gel electrophoresis of the DNA fragments. This well-established methodology was employed to evaluate the capacity of compounds R-**1** to R-**21** to stabilize topoisomerase I−DNA complexes. The effects of the indolocarbazole derivatives were initially tested on purified calf thymus topoisomerase I using a [^{32}P]-labeled *Eco*RI-*Hin*dIII restriction fragment of pBR322 as a substrate. The DNA cleavage products were analyzed by agarose gel electrophoresis under alkaline conditions. A typical autoradiograph of a gel obtained after treatment of the 4330 base pair DNA fragment with topoisomerase I in the presence and absence of the indolocarbazoles R-**13**, R-**14**, R-**20**, and R-**21** and the rebeccamycin analogues R-**3**, R-**5**, and R-**8** at concentrations ranging from 0.01 to 10 μg/mL is shown in Figure 2.

The inhibitory potency of the test compounds was assessed by comparing the cleavage of DNA by the enzyme in the absence and presence of the drug. The relative efficacy of the drugs to stimulate DNA cleavage varies considerably from one congener to another (Table 1). A detailed comparison between the 21 compounds tested allows us to make four significant observations. Firstly, compounds lacking the sugar moiety are poor inhibitors of topoisomerase I suggesting that the glucose residue is necessary for poisoning the enzyme. Only a weak effect was detected with

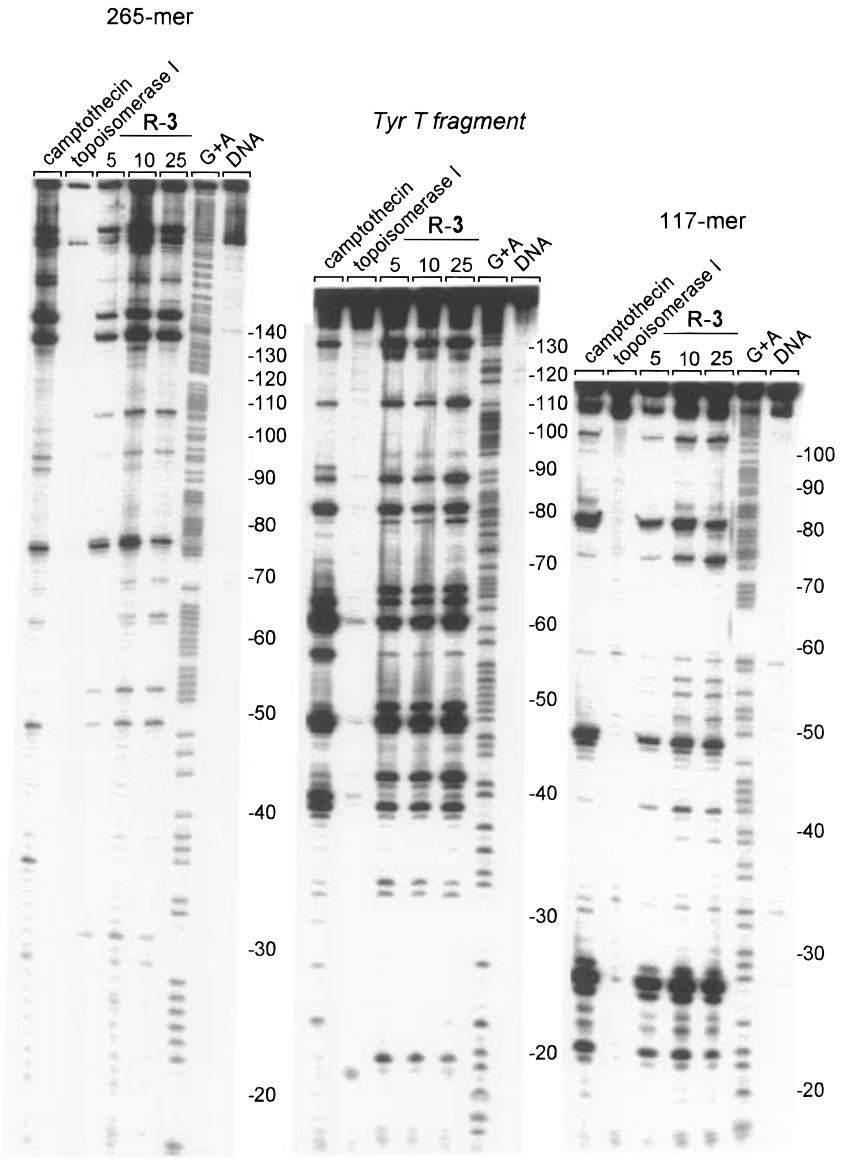


FIGURE 4: Sequence analysis of the cleavage sites stimulated by R-**3** and camptothecin. The 5′-end labeled 117-mer, 265-mer, and *tyr*T fragments (DNA lanes) were incubated in the absence (topoisomerase I lanes) or presence of 5, 10, or 25 μg/mL of R-**3** or 5 μg/mL of camptothecin. Other details as for Figure 3.

R-**15** but the effect is enhanced 10-fold in the presence of R-**17** which is the corresponding analogue possessing the glucose residue. Secondly, the presence of the chlorine atoms at positions 1 and 11 on the indolocarbazole ring diminishes and sometimes abolishes the capacity of the drugs to interfere with topoisomerase I. For example, compounds R-**3**, R-**8**, and R-**15** are noticeably more efficient in promoting topoisomerase I-mediated DNA cleavage than the chlorinated analogues R-**6**, R-**19**, and R-**16**, respectively. It is likely that the weak activity of rebeccamycin against topoisomerase I is due to a large extent to the bulky chlorine atoms at the 1 and 11 positions. It is worth noting here that these chloro groups can with profit be deleted but they can also be replaced with hydroxyl groups as found in the potent topoisomerase I inhibitors ED-110 and NB-506 (Figure 1). Thirdly, the substituent R_1 on the nitrogen of the maleimido function plays a significant role on the interference with the enzyme. By comparing the intensities of the bands in each autoradiogram, we note that with respect to the nature of the R_1 substituent, the compounds rank in the order NHCHO, NH_2 > OH > H. Thus, the maleimide nitrogen can be substituted with functional groups bearing a labile hydrogen. Fourthly, the X functional group on the non-indolic heterocyclic moiety can be varied without preventing the effect on topoisomerase I. Apparently, the replacement of the carbonyl group (X = CO) with a methylene group (X = CH_2) at position 7 does not significantly hinder the drug from interfering with topoisomerase I. The activity of the dechlorinated analogue of rebeccamycin (R-**18**) is considerably improved when the methylene group is substituted for the carbonyl group (compare the results R-**18** and R-**8** in Table 1). This is unsurprising since it is known that the antibiotic K252a (Figure 1) as well as other synthetic derivatives lacking one carbonyl group [e.g., KT6006, Yamashita *et al.* (1992)], function as topoisomerase I inhibitors. In other words, the maleimido function is not absolutely required for poisoning the enzyme.

Sequencing of Drug-Stimulated DNA Cleavage by Topoisomerase I

The next question was whether the differently substituted indolocarbazoles that inhibit topoisomerase I do so by

stabilizing enzyme−DNA complexes at similar or different sites along the DNA. To begin with, experiments were carried out on the 160 base pair long *tyr*T fragment (Drew & Travers, 1984) obtained by digestion of the plasmid pKM27 with *Eco*RI and *Ava*I. A typical autoradiogram of a topoisomerase I-mediated cleavage assay using the *Eco*RI 3′-end labeled strand of this DNA fragment is shown in Figure 3. The DNA cleavage patterns resulting from topoisomerase I-mediated single-strand breaks stimulated by the 20 derivatives were initially studied at a fixed concentration of 25 μg/mL in order to compare the potency of the different analogues in this sequencing gel system. As shown in Figure 3, practically no bands can be seen in the absence of drugs (lane topoisomerase I). The cleavage sites are almost unperceptible under the conditions used because the enzyme continually nicks and closes the DNA. Only a few discrete bands can be seen with rebeccamycin (R-**1**) and R-**15**, in agreement with the results obtained with the 4330 base pair fragment from pBR322 (Table 1). In sharp contrast, much stronger bands are produced at several defined positions in the presence of compounds R-**3**, R-**5**, R-**6**, R-**7**, R-**8**, and R-**9** which all bear the glucose residue. Therefore, it is clear that the carbohydrate moiety plays a decisive role in the interference with the enzyme. The results are totally consistent with those reported above, and the same conclusions can be drawn. Manifestly, despite their close structural homology R-**3** and R-**6** modulate the catalytic activity of the enzyme differently. The introduction of the chlorine atoms is detrimental to the effect on topoisomerase I. It can also be observed that the cleavage patterns observed with R-**3**, R-**7**, and R-**8** are slightly different confirming the belief that the R_1 substituent on the nitrogen maleimido group is involved in the interaction with the enzyme (see Discussion). Under the experimental conditions used in Figure 3, the most active analogue is R-**3**, but all 10 analogues having the carbohydrate moiety stimulate the same topoisomerase I cutting sites although the extent of stabilization of DNA−topoisomerase I cleavable complexes varies significantly depending on the nature of the R_1, R_2, and X substituents.

Additional topoisomerase I-mediated DNA cleavage experiments were performed with a 117-mer and 265-mer *Eco*RI-*Pvu*II restriction fragments obtained from the plasmid pBS to provide an assessment of the sequence selectivity of the indolocarbazole derivatives with respect to a wide variety of potential cutting sites. Strong topoisomerase I-mediated cutting sites were detected with compounds R-**3**, R-**6**, and weaker sites were visible with the other sugar-containing analogues R-**9**, R-**6**, R-**7**, R-**5**, and R-**8** but not with the sugar-free derivatives (gels not shown). In each case, the results were totally consistent with those obtained with the *tyr*T fragment. Rebeccamycin analogues differ in potency but produce a unique cleavage pattern.

Another key question is whether the location of DNA cleavage sites is similar for the rebeccamycin analogues and the reference inhibitor camptothecin. To answer that question, the patterns of DNA cleavage induced by topoisomerase I in the presence of camptothecin and the most active indolocarbazole derivative R-**3** were compared. Figure 4 shows sequencing gels produced by reacting the *tyr*T and the 117-mer and 265-mer fragments from pBS (all 3′-labeled) with topoisomerase I in the presence of camptothecin and increasing concentrations of R-**3**. From these gels and many others, it was possible to localize with accuracy 36 cutting sites (Table 2). Although 72% of the sites broken in the presence of R-**3** were also detected in the presence of camptothecin, it seems that the two drugs modulate the catalytic activity of the enzyme slightly differently (Table 3). Some topoisomerase I cleavage sites are trapped by camptothecin but not by R-**3** (such as at positions 39 and 56 and a weak site at position 92 on the *tyr*T fragment) whereas other sites are trapped by R-**3** but not by camptothecin such as at positions 41, 49, 66, and 80 on the *tyr*T fragment.

Figure 5 compares the base distribution from positions −6 to +6 for the 36 and 31 topoisomerase I-mediated cleavage sites stimulated by R-**3** and camptothecin, respectively. In both cases, the same drug-preferred bases are observed at positions immediately flanking the strand cut. Nearly all cutting sites detected with camptothecin have a T at position −1 (3′-terminus of the breaks) and most of the strong sites have a G at position +1 (5′-terminus of the breaks), in agreement with the base preferences at camptothecin-induced cleavage sites previously deduced from *in vitro* (Thomsen *et al.*, 1987; Jaxel *et al.*, 1988, 1991; Porter & Champoux, 1989a; Pommier *et al.*, 1993) and *in vivo* studies (Porter & Champoux, 1989b). A similar preference is observed with R-**3** although the requirement for T at −1 is less pronounced with R-**3** compared to camptothecin (Figure 6). Conversely, the requirement for G at +1 is slightly more pronounced with R-**3** than with camptothecin. The data in Table 3 suggest also that the two drugs exhibit a weak preference for a A or T at position −2 and to a lesser extent at position +2. In addition, with R-**3** positions −3 and −5 show a slight preference for a A. All other positions display quasi-random base distributions, but, overall, the base requirement determined for R-**3** is not greatly different from that of camptothecin (Figure 6 and Table 3). Apparently, despite their structural differences and their distinct DNA binding properties, the rebeccamycin analogue R-**3** and camptothecin modulate the catalytic activity of topoisomerase I in roughly comparable fashion.

Geometry of Intercalated Drug−DNA Complexes

The mode of binding of R-**3**, which is one of the most potent topoisomerase I inhibitors in the series, was investigated by circular and linear dichroism measurements. These two complementary techniques provide useful information on the geometry of the drug−DNA complexation.

Electric Linear Dichroism (ELD). Figure 7 shows a typical set of experimental data for the dependence of the reduced dichroism $\Delta A/A$ on (A) the DNA concentration, (B) the electric field strength, and (C) the wavelength. The mode of binding of R-**3** can be determined only on the basis of the highest ELD values, obtained when the drug molecules are fully bound to DNA, i.e., for P/D ratios >20. At lower P/D ratio, the measured ELD values fall significantly due to the appearance of unbound molecules in the solution. The ELD spectrum of the R-**3**−DNA complex indicates that the reduced dichroism is always negative in sign, even in the 300−360 nm region where the indolocarbazole chromophore absorbs the light. This situation differs from that reported previously with minor groove binders such as netropsin and distamycin which exhibited positive reduced dichroism signals around 320 nm (Bailly *et al.*, 1990, 1992a). The intensity of the ELD signal is a function of the degree of alignment of the DNA molecules in the electric field. There

Table 2: Sequences of the Cleavage Sites Stimulated by R-**3** and Camptothecin

position[a]	camptothecin	intensity[b]	R-**3**	intensity
	*tyr*T Fragment from pKM-27			
(5'→3')	−6 −1 +1 +6		−6 −1 +1 +6	
38	CTGGT**T**↓**G**CGTAA	++	CTGGT**T**↓**G**CGTAA	++
39	ACTGG**T**↓TGCGTA	++		
41			GAACTG↓GTTGCG	++
47	AAAAA**T**↓**G**TAACT	+++	AAAAA**T**↓**G**TAACT	+++
49			AGAAAA↓ATGAAC	++
56	TACGT**T**↓**G**AGAAA	++		
61	AGTGT**T**↓ACGTTG	+++	AGTGT**T**↓ACGTTG	+++
64	TAAAG**T**↓**G**TTACG	+++	TAAAG**T**↓**G**TTACG	++
66			TGTAAA↓GTGTTA	++
80			AAATGA↓CGCGCC	–
82	TCAAA**T**↓**G**ACGCG	++	TCAAA**T**↓**G**ACGCG	++
89	CTTCA**T**↓ATCAAA	+	CTTCA**T**↓ATCAAA	++
92	GCGCT**T**↓CTATAT	–		
111	TCCCT**T**↓ATCGGG	+	TCCCT**T**↓ATCGGG	++
134	GGTAA**T**↓**G**CTTTT	++	GGTAA**T**↓**G**CTTTT	+++
	117-mer Fragment from pBS			
(5'→3')	−6 −1 +1 +6		−6 −1 +1 +6	
22	TGTAA**T**↓ACGACT	+	TTGTAA↓TACGAC	+
26	TGAAT**T**↓**G**TAATA	+++	TGAAT**T**↓**G**TAATA	+++
31	GCCAG**T**↓**G**AATTG	–	GCCAG**T**↓**G**AATTG	–
41	TAAAAC↓**G**ACGGC	–	TAAAAC↓**G**ACGGC	+
48	GACGT**T**↓**G**TAAAA	+++	GACGT**T**↓**G**TAAAA	++
51			CACGAC↓**G**TTGTA	+
54			AGTCAC↓**G**ACGTT	+
56			CCAGTC↓ACGACG	+
59	TTCCCA↓**G**TCACG	–	TTCCCA↓**G**TCACG	+
73	GGTAAC↓**G**CCAGG	–	GGTAAC↓**G**CCAGG	++
81	TAAGT**T**↓**G**GGTAA	+++	TAAGT**T**↓**G**GGTAA	++
86	GCGAT**T**↓AAGTTG	+	GCGAT**T**↓AAGTTG	+
102	GGGGA**T**↓**G**TGCTG	++	GGGGA**T**↓**G**TGCTG	++
	265-mer Fragment from pBS			
(5'→3')	−6 −1 +1 +6		−6 −1 +1 +6	
28	GACTC**T**↓AGAGGA	–	GACTC**T**↓AGAGGA	–
30			TCGAC**T**↓CTAGAG	–
35	GCAGG**T**↓CGACTC	+		
49	AAGCT**T**↓**G**CATGC	++	AAGCT**T**↓**G**CATGC	++
54			AACAAA↓AGCTTG	+
64	CACTAA↓AGGGAA	–	CACTAA↓AGGGAA	+
70	AACCC**T**↓CACTAA	–	AACCC**T**↓CACTAA	+
76	GAAAT**T**↓AACCCT	++	GAAAT**T**↓AACCCT	++
94	CATGA**T**↓TACGCC	+		
97	AGCCA**T**↓**G**ATTAC	+	AGCCA**T**↓**G**ATTAC	+
109			GGAAAC↓AGCTAT	++
137	AATTG**T**↓**G**AGCGG	+++	AATTG**T**↓**G**AGCGG	+++
145	TTGTG**T**↓**G**GAATT	+++	TTGTG**T**↓**G**GAATT	+++

[a] Positions of the drug-stimulated cleavage sites within three DNA fragments examined. [b] −, +, ++, and +++ correspond to very weak, weak, medium, and strong cleavage, respectively. Arrows point out the cut which occurs between positions −1 and +1. The thymine and guanine residues in −1 and +1 position, respectively, are printed in bold type.

Table 3: Base Frequencies at DNA Cleavage Sites Induced by Mammalian Topoisomerase I in the Presence of R-**3** and Camptothecin[a]

position	R-**3** (36 sites)					camptothecin (31 sites)				
(5′→3′)	A	T	G	C	preference	A	T	G	C	preference
−6	28	30	28	14		23	32	32	13	
−5	41	14	28	17	(A)	42	13	22	23	
−4	33	19	19	28		26	26	19	29	
−3	42	16	19	22	(A)	35	13	29	23	
−2	44	30	14	11	(A)	35	35	19	9	
−1	19	61	3	17	T	6	87	0	6	T
+1	30	3	58	8	G	26	6	58	9	G
+2	28	36	19	16		32	29	19	19	
+3	22	14	28	36		29	10	29	32	
+4	28	33	30	8		32	32	23	13	
+5	30	30	16	22		29	29	19	23	
+6	28	16	39	17		32	19	32	16	

[a] Base frequencies are expressed as percentages.

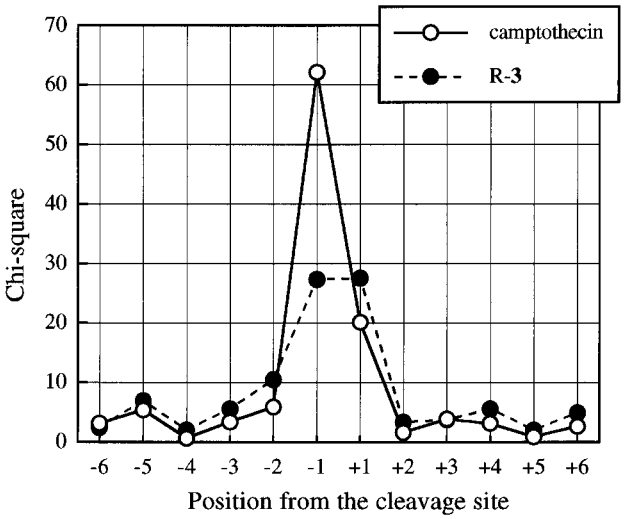


FIGURE 5: Statistical analysis of base preference around cleavage sites of topoisomerase I in the presence of camptothecin or R-**3**. χ^2 values indicate deviation from the expected distribution of base frequencies in the three DNA fragments used to map the cleavage sites. χ^2 values >7.8 and 11.4 correspond to p values <0.05 and 0.01, respectively (3 degrees of freedom). Calculated p values at position −1 and +1 are 2×10^{-13} and 1.6×10^{-4} for camptothecin and 5×10^{-6} and 4.5×10^{-6} for R-**3**, respectively.

is a clear parallelism between the electric field dependence of the reduced dichroism measured at 260 nm for the DNA bases and at 310 nm for R-**3** (Figure 7B). This indicates that the planar indolocarbazole ring is tilted close to the plane of the DNA bases, consistent with an intercalative mode of binding.

Further ELD studies were undertaken to compare the binding of the rebeccamycin derivatives containing the sugar residues with the corresponding indolocarbazole derivatives lacking the glucose. Measurements were carried out at a fixed P/D ratio of 30 and with the same DNA samples and under the same buffer and temperature conditions for each compounds. The results in Figure 8 reveal unambiguously that the glucose moiety plays a decisive role in the interaction with DNA. A reduced dichroism of about −0.48 was measured for compounds R-**3**, R-**7**, R-**9**, and R-**17**, whereas the $\Delta A/A$ values for the corresponding analogues lacking the sugar is 4−5 times smaller in absolute value. Apparently, the R_2 substituent has little effect on the intercalation process since identical $\Delta A/A$ values were measured for compounds

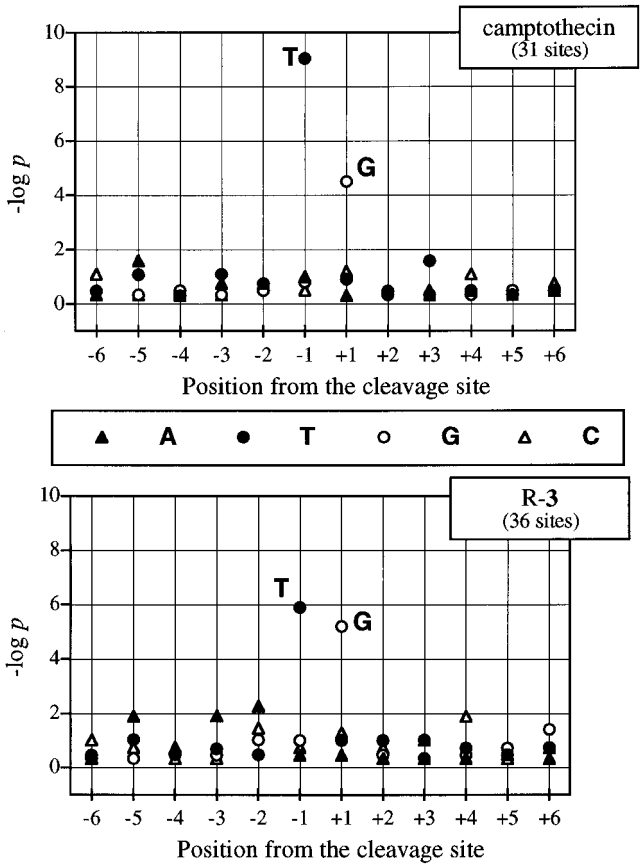


FIGURE 6: Base preferences at the site of topoisomerase I DNA cleavage stimulated by camptothecin and R-**3**. The *p* value is the probability of the observed base frequency deviation at each position. Values above the base line ($p = 0$) indicate excess (preference) relative to the expected frequency of each individual base.

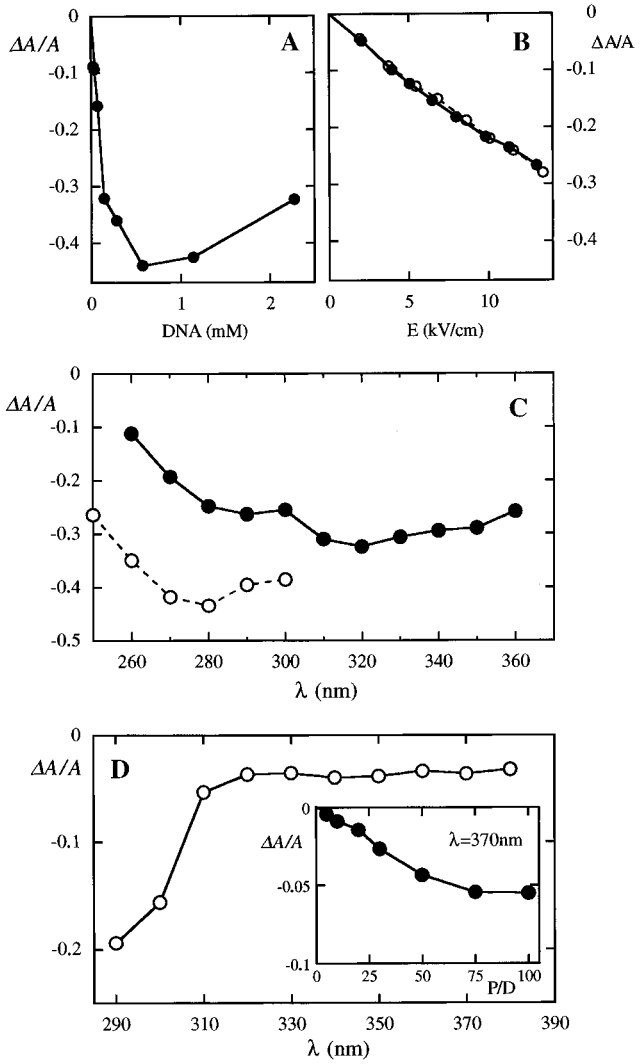


FIGURE 7: Dependence of the reduced dichroism Δ*A*/*A* on (A) DNA concentration, (B) electric field strength, and (C) wavelength. (●) R-**3**, (○) DNA. Conditions: (a) 310 nm, 13.5 kV/cm, (b) 310 nm, P/D = 30, (c) 13.5 kV/cm, P/D = 30 in 1 mM sodium cacodylate buffer, pH 6.5. Panel D shows the ELD spectrum of a camptothecin−DNA complex at a P/D ratio of 50 and inset shows the dependence of reduced dichroism Δ*A*/*A* with the phosphate−DNA/drug (P/D) ratio measured at 370 nm under a field strength of 13.5 kV/cm.

R-**17** and R-**9** which only differ by the presence or absence of the chlorine atoms at positions 1 and 11. The R_1 substituent may have a slight influence on the interaction with the double helix since weaker Δ*A*/*A* values are obtained with R-**1** and R-**8** compared to analogues bearing a OH, NHCHO, or NH_2 group on the nitrogen maleimido group. However, the reduced dichroism values obtained with rebeccamycin (−0.4) is very close to that obtained with DNA alone at 260 nm (Figure 7C) indicating that the chromophore is oriented parallel to the plane of the base pairs, as is the case with an intercalated drug. Both the potent topoisomerase I inhibitor R-**3** and the weak inhibitor rebeccamycin intercalate into DNA, but it is likely that the extent of binding and/or extent of drug-induced stiffening of the double helix differs for the two drugs.

Parenthetically, the spectroscopic study with topoisomerase I inhibitors was the occasion for us to examine the interaction of camptothecin with DNA. The question of whether or not camptothecin binds to DNA in the absence of topoisomerase I is still controversial. Previous studies have shown that the drug either does not interact or interacts loosely with DNA (Fukada *et al.*, 1985) but that it is, however, capable of inducing G-specific cleavage upon photoactivation (Leteurtre *et al.*, 1993). We have applied linear dichroism spectroscopy in an attempt to gain insight into mechanism of binding of camptothecin to DNA. Camptothecin gives negative dichroism values in the 320−380 nm region but the Δ*A*/*A* values are considerably less negative for the drug−DNA complex than for the DNA alone at 260 nm (Figure 7D). Therefore it is clear that, unlike what is observed with R-**3**, camptothecin does not intercalate into DNA. The maximum ELD value at 370 nm was found to be −0.055 which corresponds to an orientation of the transition moment at an angle of about 59° (or 57°) with respect to the helix axis assuming a theoretical angle of 90° (or an experimental angle of 72°). The angle is estimated from a comparison of the reduced dichroism for the DNA bases at 260 nm and for camptothecin at 370 nm, respectively, for the DNA bases with respect to the orientation axis of the particles [details in Houssier (1981)]. A 59° tilt of the camptothecin chromophore with respect to the plane of the base pairs is quite different from the angles generally measured with minor groove binders such as netropsin and distamycin ($\alpha = 38°$; Dattagupta *et al.*, 1980; Bailly *et al.*, 1992a), but interestingly it coincides with the angle measured with the antitumor drug bleomycin (Povirk *et al.*, 1979; Bailly *et al.*, 1992b) which also binds into the minor groove (Manderville *et al.*, 1995; Stubbe *et al.*, 1996). Therefore, the possibility that camptothecin fits

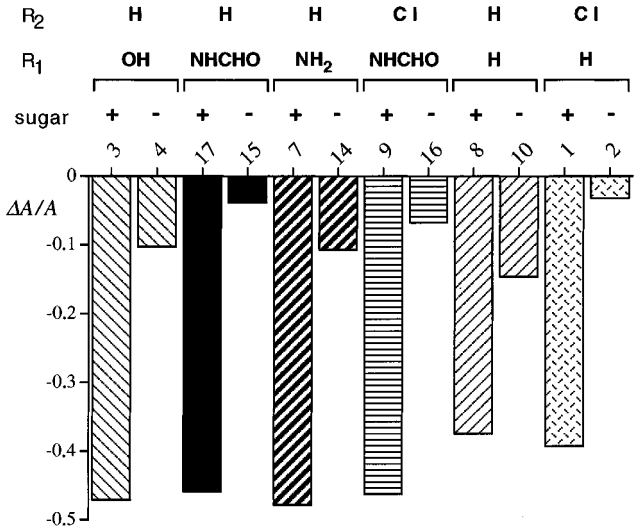


FIGURE 8: Variation of the reduced dichroism ($\Delta A/A$) of the complexes between calf thymus DNA and compounds R-**1**, R-**2**, R-**3**, R-**4**, R-**7**, R-**8**, R-**9**, R-1**0**, R-**14**, R-**15**, R-**16**, and R-**17**. $\Delta A/A$ was measured at 320 nm, 13.5 kV/cm, and at a drug/DNA ratio of 30 in 1 mM sodium cacodylate buffer, pH 6.5.

into the minor groove of DNA has been considered but appears unlikely at first sight, because insertion of a ligand into the minor groove of DNA generally gives rise to large positive ELD signals whereas those observed for the complex between camptothecin and calf thymus DNA are always negative. Moreover, binding in the minor groove of DNA should give rise to large positive CD signals whereas the CD spectra observed with camptothecin−DNA complexes show a weak negative band centered at 355 nm (not shown). Alternatively, we considered the possibility of a binding site within the major groove of DNA which is certainly large enough to accommodate the planar alkaloid chromophore. Insertion of camptothecin between the major groove edges of the bases appears plausible on the basis of the present ELD data and has been previously proposed on the basis of the photocleavage experiments (Leteurtre *et al.*, 1993).

Circular Dichroism (CD). A slight increase in molar dichroism in the 320−340 nm region is observed with R-**3** upon addition of calf thymus DNA or the polynucleotides poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC) (data not shown). The CD data are also compatible with a more or less perpendicular arrangement between the axis of the planar chromophore and the hydrogen bonds of the bases, as previously reported with anthracyclines (Cera *et al.*, 1991).

Sequence Preference

The binding of R-**3** to nucleic acids has also been studied using two synthetic alternating polymers poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC). The reduced electric dichroism values do not vary markedly between the AT and the GC polynucleotide (data not shown) indicating that the indolocarbazole moiety retains much the same orientation whatever the DNA composition. We noted that the complexes of R-**3** with the two polynucleotides exhibit larger reduced electric dichroism amplitudes at 320 nm than the polynucleotides alone at 260 nm, indicating that the drug is likely to induce a local stiffening of the DNA around the intercalation site which enhances the degree of orientation of the DNA molecules in the electric field. This phenomenon has been frequently observed with intercalating drugs such as ethidium, daunomycin, and proflavine for examples (Bailly *et al.*, 1992b).

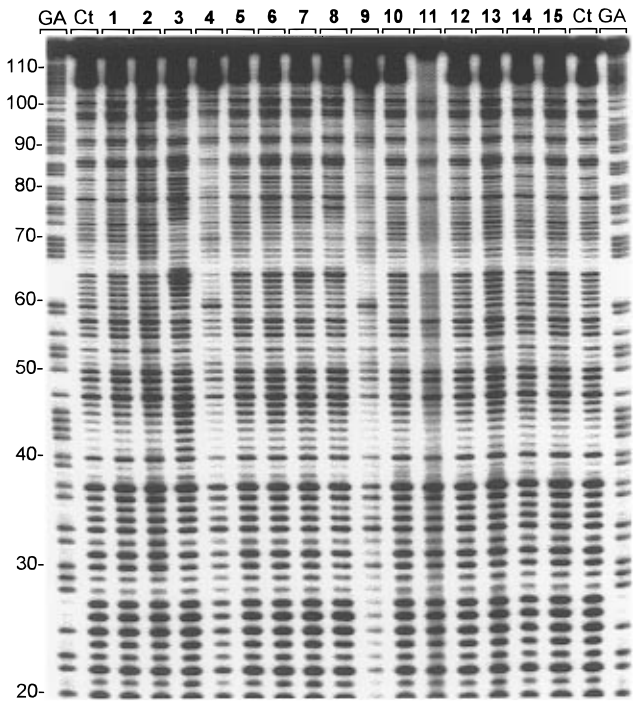


FIGURE 9: DNase I footprinting with the 117-mer *Pvu*II-*Eco*RI restriction fragment of the plasmid pBS in the presence of compounds R-**1** to R-**15** at 25 μg/mL. The DNA was 3′-end labeled at the *Eco*RI site with [α-^{32}P]dATP in the presence of the Klenow fragment of DNA polymerase. The products of nuclease digestion were resolved on an 8% polyacrylamide gel containing 7 M urea. Control tracks (lane Ct) contained no drug. Purine-specific sequence markers obtained by treatment of the DNA with formic acid followed by piperidine were run in the lane marked GA. Numbers on the left side of the gels refer to the standard numbering scheme for the nucleotide sequence of the DNA fragment.

DNase I footprinting was used to probe the nucleotide sequence selectivity of the drugs. Figure 9 shows an autoradiogram resulting from the partial DNase I cleavage of the 3′-end labeled 117 base pair fragment from pBS in the presence and absence of compounds R-**1** to R-**15**. Although with most drugs the DNase I cleavage profile of the DNA is not affected, with R-**3** bound the DNase I cleavage pattern differs significantly from that seen in the control lane. A few bands in the R-**3**-containing lane are weaker than the same bands in the drug-free lane, corresponding to attenuated cleavage, while numerous other bands show relative enhancement of cutting. To define more precisely the effect of R-**3** on the rate of DNA cleavage by the nuclease, band intensities in the control and R-**3** lanes were quantified by densitometry and converted into a differential cleavage plot (not shown). The sequences slightly protected by R-**3** from cleavage by DNase I mostly correspond to G·C-rich sequences (e.g., 5′-CGCCAGGG between positions 67 and 74). By contrast, the susceptibility to DNase I cleavage appears enhanced at A·T-rich sequences, such as around nucleotide positions 65 (5′-TTTT) and 44 (5′-AAAA). Therefore, the binding of this drug to GC sequences is slightly favoured over binding to AT or mixed sequences. Similar effects were previously reported with other intercalating drugs including daunomycin (Chaires *et al.*, 1987, 1990), actinomycin (Fox & Waring, 1984; Bailly *et al.*, 1994), and the alkaloid ascididemin (Bonnard *et al.*, 1995).

DISCUSSION

Recent studies have shown that compounds such as NB-506 and ED-110 which are structurally close to rebeccamycin and the analogues studied here (Figure 1) unwind supercoiled DNA (Yoshinari *et al.*, 1993, 1995). Fluorescence measurements revealed a competitive inhibition for the binding of ethidium bromide to DNA in the presence of ED-110 (Yoshinari *et al.*, 1993). The propensity of the indolocarbazoles to unwind DNA was also clearly established with a series of analogues of K252a (Yamashita *et al.*, 1992). On the basis of these data the authors logically argued that, unlike camptothecin, these drugs intercalate into DNA. The ELD data in Figures 7 and 8 obtained with R-**3** and the other rebeccamycin analogues are identical to those previously reported with anthracyclines, acridines, or anthraquinones (Bailly *et al.*, 1992b), indicating that the indolocarbazole chromophore of the drugs is oriented parallel to the base pair plane. In other words, the electric linear dichroism results confirm that compound R-**3** and its analogues form intercalation complexes with DNA and polynucleotides.

Compound R-**3** behaves as a classical DNA intercalating drug and strongly stimulates topoisomerase I-mediated DNA cleavage, whereas its deglycosylated analogue R-**4** weakly interacts with DNA and has no effect on topoisomerase I. Therefore, at first sight it is tempting to argue that the capacity of the drugs to interfere with topoisomerase I parallels their propensity to intercalate into DNA. However, at least two lines of evidence contradict this idea. First, compounds R-**8** and R-**1** adopt a similar orientation upon binding to DNA ($\Delta A/A = -0.4$ for the two drug−DNA complexes) whereas R-**8** is a much better topoisomerase I inhibitor than R-**1**. Second, R-**13** has little effect on topoisomerase I whereas it also behaves as an intercalating agent. Therefore, intercalation into DNA is not sufficient to confer topoisomerase I inhibition. There exists a large variety of intercalating drugs but only a few are topoisomerase inhibitors. The studies of Yamashita *et al.* (1992) also led to the conclusion that DNA intercalation of the indolocarbazoles does not correlate with their potency in inducing topoisomerase I-mediated DNA cleavage. However, it seems that the interaction with DNA is required for the drug to interfere with topoisomerase I.

The base preference analysis indicates that camptothecin and R-**3** display comparable sequence selectivity of topoisomerase I-mediated DNA cleavage: for the two drugs, the −1 and +1 bases are preferentially T and G, respectively. The preference for a T at −1 was previously attributed to topoisomerase I itself rather than to a drug effect (Jaxel *et al.*, 1991). Conversely, it is thought that the G+1 is essential for a camptothecin−enzyme−DNA cleavable complex to form. Recent studies have shown that camptothecin binds at the interface of the topoisomerase I−DNA complex and interacts with the G+1 residue (Pommier *et al.*, 1995). By analogy, it is tempting to postulate that the planar indolocarbazole chromophore of R-**3** binds by stacking with the G at the 5′ terminus of the breaks. The schematic model in Figure 10A is attractive because it is consistent with the general model proposed for a number of DNA-intercalating topoisomerase II inhibitors such as doxorubicin and amsacrine (Capranico *et al.*, 1990; Pommier *et al.*, 1991).

The peculiar binding behavior of the indolocarbazole drugs reported here prompts the following question: What may

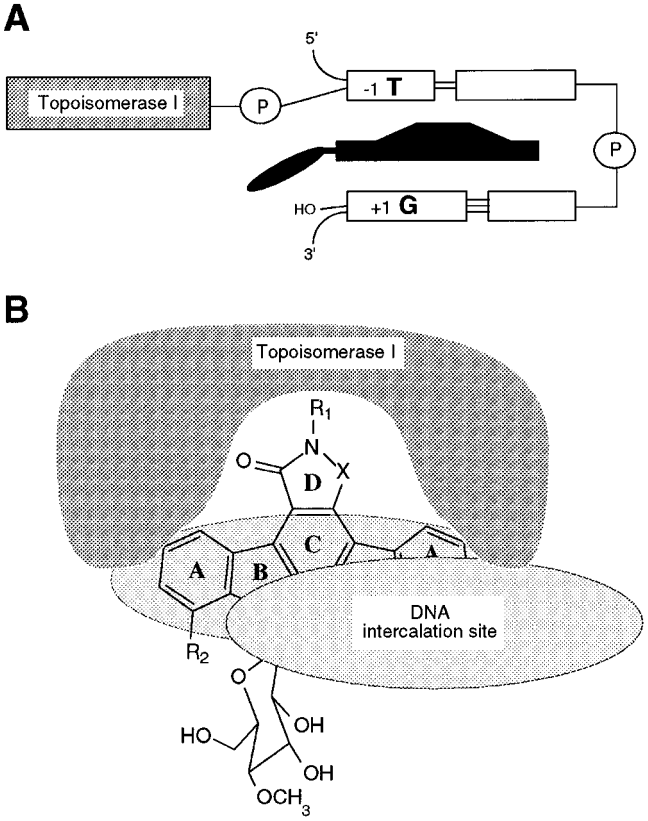


FIGURE 10: Schematic representation of the proposed drug−topoisomerase I−DNA ternary complex. A shows the stacking the drug (in black) with the base pairs flanking the cleavage site. R-**3** binds preferentially between the T (−1) and G (+1) residues and stabilizes the covalent topoisomerase I−DNA complex. B illustrates the intercalation of the indolocarbazole chromophore and the interaction of the drug within the proposed topoisomerase I binding pocket (see Discussion).

be the critical determinants in their structures for the interference with topoisomerase I? Our observations provide direct indication regarding functionalization of the indolocarbazole chromophore. Substitution at positions 1 and 11 (A ring) by chlorine atoms are detrimental to activity, while positions 6 and 7 (D ring) are acceptable sites for functionalization. Positions 1 and 11 require either no substituent or a substituent of minimal bulk (e.g., OH groups). The carbonyl group at position 7 can be replaced by a methylene group without major effect on the capacity of the drug to inhibit the enzyme. Finally, the maleimide nitrogen at position 6 can be substituted with more bulky functional groups bearing a labile hydrogen. Analysis of these experimental observations allows for some speculation on the nature of the drug binding pocket on the enzyme−DNA complex. A schematic model of a ternary complex between topoisomerase I, DNA and the drug is presented in Figure 10B. This hypothetical model accounts for the observed results. Given that bulky substituents on position 6 modulate the capacity of the drug to interfere with topoisomerase I, one can assume that the D ring and the attached R_1 group are on the "inside" of the complex, i.e., the region where the drug recognizes a surface on the topoisomerase I−DNA binary complex. Conversely, the fact that the chlorine atoms at positions 1 and 11 provide steric hindrance for the formation of the intercalated complex but do not totally abolish the interference with the enzyme suggests that they are localized on the "outside" of the complex together with the carbohydrate residue which likely resides in one of the

helical grooves (probably the minor groove as for all antibiotics substituted with carbohydrates, e.g., daunomycin, calicheamicin, mithramycin, and bleomycin). Such structural arrangement would also account for the results reported with other indolocarbazole derivatives (Yamashita *et al.*, 1992; Yoshinari *et al.*, 1993). The fact that substitutions at positions 6 and 7 are tolerated suggests that the D ring does not fit into a narrow cavity on the enzyme−DNA complex and/or that a certain degree of freedom of movement within the binding pocket is permitted. It may be possible to directly test this hypothesis by introducing an alkylating group (e.g., a bromoacetyl) on the D ring so as to determine whether the drug can covalently bind to the enzyme or the DNA. The results reported here also indicate that the sugar moiety constitutes an essential molecular determinant for drug stimulation of topoisomerase I-mediated DNA cleavage. Therefore, it will be interesting to modify the carbohydrate moiety to test whether the OH and OCH_3 groups on the glucose residue can be removed or replaced by an amino group, for example. New rebeccamycin analogues incorporating these modification are currently being synthesized.

The last point to be examined is the relationship between interference with topoisomerase I and biological activity of the indolocarbazoles tested in this study. As recently discussed (Rodrigues-Pereira *et al.*, 1996), the indolocarbazole derivatives bearing a carbohydrate moiety are generally more active than the analogues lacking the sugar. In other words, the drugs which stimulate DNA cleavage by topoisomerase I show better antitumor activity than the drugs which are inactive against topoisomerase I. Therefore, at first sight the results support the conclusion previously drawn by Yamashita *et al.* (1992) that there exists a correlation between topoisomerase I inhibition and antitumor activity. However, the chlorinated compounds were found to be more active than the corresponding dechlorinated analogues against B16 melanoma and P388 leukemia, and all compounds tested here are less active than rebeccamycin both *in vitro* and *in vivo*. Although the derivatives tested are too few to draw general conclusions, it is clear that stimulation of DNA cleavage by topoisomerase I cannot guarantee antitumor activity. One should always keep in mind that, in addition to considering the interaction between the drug and its potential targets, the design of tumor-active drugs demands consideration of a wide range of interdependent parameters, including cellular uptake/efflux, drug metabolism, and drug distribution.

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