Molecular Interactions of DNA-Topoisomerase I and II Inhibitor with DNA and Topoisomerases and in Ternary Complexes: Binding Modes and Biological Effects for Intoplicine Derivatives†

Igor Nabiev,*,‡,§ Igor Chourpa,§ Jean-François Riou,‖ Chi Hung Nguyen,⊥ Francois Lavelle,‖ and Michel Manfait*,§

Laboratoire de Spectroscopie Biomoléculaire, UFR de Pharmacie, Université de Reims, 51096 Reims Cedex, France, Optical Spectroscopy Division, Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 117871 Moscow, Russia, Rhône-Poulenc Rorer, S.A., Département de Biologie, 94403 Vitry sur Seine, France, and Institut Curie, Centre National de la Recherche Scientifique, 91405 Orsay, France

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ABSTRACT: Molecular interactions of intoplicine, dual DNA-topoisomerases (Topo) I and II inhibitor, with topoisomerases, plasmid DNA, in ternary cleavable complexes with enzymes and plasmid DNA, and in the reversed cleavable complexes were examined by means of surface-enhanced Raman scattering (SERS) and CD spectroscopy and by biochemical techniques. Detailed spectral analysis of intoplicine derivatives allowed us to assign SERS vibrational modes of chromophores and to propose the models for these complexes. Intoplicine was found to be able to interact specifically with the Topo II alone, but with Topo I only when in the presence of DNA. It shows at least two modes of binding to the DNA: the first was found to be dominant for its derivative **1c** (most potent Topo I inhibitor), and the second was dominant for derivative **2a** (most potent Topo II inhibitor). The possibility of forming these two types of complexes simultaneously is suggested to be one of the main factors enabling the drug to be a dual Topo I and Topo II inhibitor. The "deep intercalation mode" of the drug from the DNA minor groove with the long axis of the chromophore oriented roughly parallel to the dyad axis has been suggested to be responsible for induction of distortions of the DNA structure by the intercalating drug. Being involved in the formation of Topo I-mediated cleavable ternary complex, the molecules participating in the deep intercalation mode within the DNA do not change their molecular interactions as compared with their complex with the DNA alone. The stabilization of the Topo I-mediated cleavable complex was shown to be followed by the local denaturation of DNA in the AT-rich regions of the helix. When the ternary cleavable complex was reversed, the drug was shown to be in the complex with the plasmid. The "outside binding mode" from the DNA major groove via the hydroxyl group of the A-ring of the chromophore has been suggested to be responsible for Topo II inhibition. These molecules did not induce significant distortions of the DNA structure. Being involved in the formation of Topo II-mediated cleavable ternary complex, the drug changed its molecular interactions as compared with the complex with DNA alone. Drug–Topo II interactions within the ternary complex involve the hydroxyl group of the A-ring of the chromophore, which was found to be easily accessible to the functional groups of enzyme when the outside binding mode of the drug to DNA is realized. Direct interactions between the drug and Topo II can play a critical role in the process of DNA recognition by the enzyme and Topo II inhibition. Intoplicine shows higher affinity to the enzyme than to the DNA: reversion of ternary cleavable complex led to elimination of drug–DNA interactions, whereas interactions between drug and Topo II seem to be unchanged. These results suggest that dual Topo I and II inhibition, being critical for the antitumor activity of intoplicine, should be explained not only by effects induced at the DNA level but also by molecular interactions displayed by the drug with DNA and enzymes, in cleavable ternary complexes and in reversed ternary complexes.

DNA-Topo I and II[1] both are the primary cellular targets for a wide variety of clinically relevant antitumor drugs (Liu, 1989; Wang, 1989; Pommier & Tanizawa, 1993). The chemotherapeutic potentials of these agents correlate with their abilities to stabilize covalent topoisomerase-cleaved DNA complexes, which are reaction intermediates in the enzyme's catalytic cycle (Hsiang et al., 1985; Lock & Ross, 1987). These drug-stabilized cleavable complexes, in contrast to other protein–DNA complexes, can be converted to protein-linked DNA breaks by treatment with a strong protein denaturant such as SDS (Liu, 1989; Bodley & Liu, 1988). Different from other types of DNA damage, drug-induced cleavable complexes are reversible; they readily dissociate when the drug is removed from the reaction, after addition of the excess of salt, or after brief heating of a pre-equilibrated reaction mixture (Liu, 1989; Liu et al., 1983).

The physical basis for cleavable complex formation by topoisomerase-targeting drugs remains to be defined. Most

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* To whom correspondence should be addressed, at Laboratoire de Spectroscopie Biomoléculaire, UFR de Pharmacie, 51 rue Cognacq Jay, 51096 Reims cedex, France.

‡Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences.

§ Université de Reims, Champagne-Ardenne.

‖ Rhône-Poulenc Rorer, S.A.

⊥ Institut Curie, CNRS.

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[1] Abbreviations: Topo I, DNA-topoisomerase I; Topo II, DNA-topoisomerase II; Intoplicine, 7*H*-benzo[*e*]pyrido[4,3-*b*]indole derivative; SERS, surface-enhanced Raman scattering; CD, circular dichroism; CT-DNA, calf thymus DNA; PBS, potassium buffered saline.

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of the anticancer drugs are supposed to induce their effects at the DNA level (Monnot et al., 1991). However, no single known parameter of drug–DNA interaction correlated with drug cytotoxicity or antitumor activity. DNA-intercalating and "outside"-binding antitumor compounds were both shown to be able to be DNA-topoisomerase poisons (Liu, 1989; Wang, 1989; Pommier & Tanizawa, 1993). Moreover, some of the antitumor drugs (e.g., camptothecin), strong topoisomerase inhibitors, have been demonstrated to exhibit lack of any chemical reactivity with purified DNA (Pommier et al., 1991). Therefore, the molecular background of the modes of interaction within the complicated supramolecular complexes of enzymes, DNA, and antitumor drugs should be investigated if the overall patterns of drug structure–activity relationships are to be clarified.

Recently, a new highly potent antitumor compound, intoplicine, became available (Nguyen et al., 1990; Bissery et al., 1993). Intoplicine is a synthetic anticancer agent belonging to the series of 7*H*-benzo[*e*]pyridol[4,3-*b*]indoles. This new compound might be of particular interest for cancer research due to its potential to circumvent Topo I-mediated and Topo II-mediated resistance by poisoning both enzymes simultaneously (Poddevin et al., 1993). Twenty-two analogues of intoplicine were evaluated for their effects on Topo I- and II-mediated DNA cleavage reactions (Riou et al., 1993). A study of the relationships between the *in vivo* antitumor activity and the Topo I- and /or II-mediated DNA cleavage activity revealed that the most highly active antitumor compounds possessed both Topo I- and II-inhibitory properties (Riou et al., 1993). Studies of molecular interactions of antitumor compounds, dual Topo I and II inhibitors, must shed light on the general mechanisms of action of different classes of topoisomerase-targeting drugs.

Unfortunately, there are only very few techniques available for the selective studies of molecular interactions within complicated high-molecular-weight supramolecular complexes. Compared with the others, Raman spectroscopy has excellent fingerprinting capabilities. It can be utilized *in situ* under physiological conditions, and when resonance Raman and/or SERS spectroscopy can be used, it is extremely sensitive (Nabiev et al., 1993, 1994a,b). Structural models of molecular interactions of new (Nabiev et al., 1994b) and well-known (Smulevich & Feis, 1986; Nonaka et al., 1990; Nabiev et al., 1993, 1994a) antitumor drugs with the DNA have been proposed on the basis of SERS data and were found to be consistent with the NMR and X-ray diffraction data. Recently, the SERS study of intoplicine and those of its complexes with DNA and Topo II *in vitro* and in K562 cancer cells have been published (Morjani et al., 1993). SERS analysis showed that the drug is able to interact specifically in the internal regions of Topo II in the absence of DNA—it was the first experimental evidence of such a complex formed by an inhibitor of topoisomerase.

In the present paper we report a comparative SERS and CD spectroscopic and biochemical study of molecular interactions of a dual Topo I and II inhibitor, intoplicine, with plasmid DNA, with topoisomerases I and II, within cleavable ternary complexes (drug–Topo I/Topo II–plasmid DNA), and within reversed ternary complexes. The incidence of the intoplicine binding modes to the DNA and within the ternary complexes on the biological effects of the drug is discussed and compared with the results obtained for different intoplicine chemical derivatives.

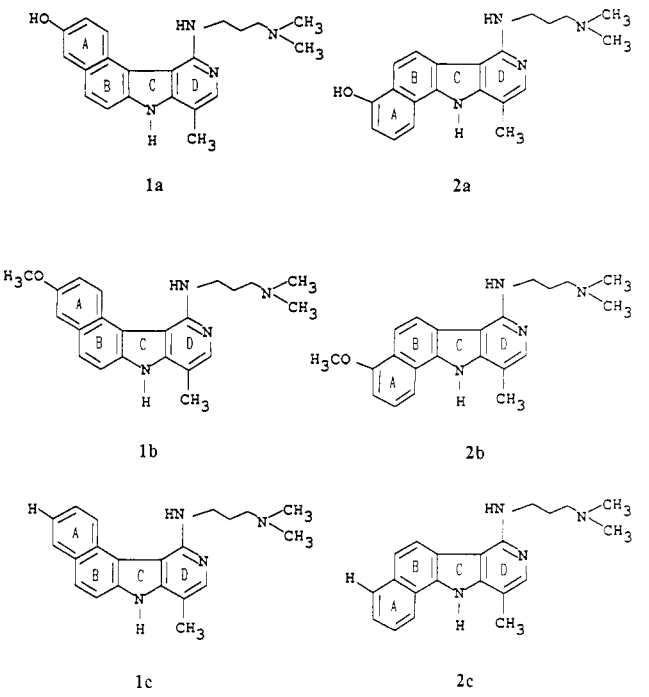

FIGURE 1: Structures of intoplicine derivatives of the 7*H*-benzo[*e*]pyrido[4,3-*b*]indole series (**1a–c**) and of the 11*H*-benzo[*g*]pyrido[4,3-*b*]indole series (**2a–c**), modified on the A ring of the chromophore. (**1a**) Intoplicine.

MATERIALS AND METHODS

Enzymes, Nucleic Acids, and Chemicals. Intoplicine and its derivatives (Figure 1) were synthesized as described by Nguyen et al. (1990) and were dissolved at a concentration of 10 mM in distilled water.

Calf thymus DNA (Sigma) was dissolved in PBS. The concentration of DNA (phosphate) was estimated on the basis of a molar absorption coefficient of 6600 M^{-1} (bp) cm^{-1} at 260 nm. Drug–DNA complexes were prepared by mixing the components in PBS. Drug/DNA ratios (DNA in base pairs) are indicated in the figure legends. pBR322 DNA was purchased from Boehringer Mannheim. Calf thymus Topo I (10 units/μL) was purchased from GIBCO BRL.

Topo II was purified to homogeneity from calf thymus according to previously published procedures (Halligan et al., 1985; Riou et al., 1986). Topoisomerases were aliquoted and stored, without detectable loss of activity, for 12 months at −70 °C, in a conservation buffer containing 10 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 0.5 mM EDTA, 100 μg/mL bovine serum albumin, and 50% glycerol. The specific activities of the Topo I and II preparations were 2.6×10^6 units/mg and 0.18×10^6 units/mg, respectively. One unit of Topo II (or Topo I) corresponds to the amount of enzyme necessary to relax (or decatenate) 50% of 0.5 μg of pBR322 DNA (or kinetoplast DNA), when incubated for 30 min at 37 °C under the assay conditions.

Preparation of the Complexes for SERS Measurements. The assay for the SERS experiments with Topo I and II was performed with various concentrations of enzymes (0.1–20 units) in a 20-μL final reaction volume containing 0.25 μg of supercoiled pBR322 DNA, 20 mM Tris-HCl (pH 7.5), 60 mM KCl, 10 mM $MgCl_2$, 30 μg/mL bovine serum albumin, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 1 μM drug or water. The final nucleotide concentration is 20.8 μM. The reactions were performed on ice, and the reaction mixture was then incubated at 37 °C for 5 min. Then, samples were

mixed at room temperature with 20 μL of preaggregated silver hydrosol and immediately analyzed by SERS. Control experiments consisting of measurement of the SERS spectra of buffer alone, Topo II alone, Topo I alone, drug alone, Topo I or II + drug, and plasmid DNA + drug were performed under the same conditions, except that distilled water was used to adjust the reaction volume to 20 μL. The reversal of cleavable ternary complexes was performed as described (Liu, 1989) by brief (ca. 2 min) exposure of ternary complexes to 65 °C followed by their storage on ice until SERS measurements were carried out (ca. 15 min). Deactivation of Topo I or II for the control experiments with nonactive enzymes was performed by prolonged (ca. 20 min) heating of the enzyme solution to ca. 90 °C followed by its storage on ice for SERS measurements.

UV Absorption and Circular Dichroism. Absorption spectra were recorded on a Philips PU 8720 UV–vis scanning spectrophotometer, and a Jasco-500C dichrograph was used to measure the CD spectra. Samples were prepared in a potassium buffered saline (PBS), pH 7, at a weak ionic strength ($I = 0.02$). The complexes were prepared by mixing the drug solutions and DNA (the ratios are indicated in the figure legends). Measurements were performed using quartz cells of 1-cm path length containing 2.5 mL of 65 μM DNA. For titration, small aliquots of 2 mM aqueous drug solutions were added incrementally.

SERS. The SERS spectra were measured on a computer-controlled DILOR Omars-89 Raman spectrometer. Samples were excited by the 514.5-nm line of a Spectra-Physics 2020-03 argon laser. The experimental conditions are described in the figure legends. Band intensities were corrected by the monochromator/detector response. The high-frequency bands at ca. 3400 cm^{-1} of water or at ca. 2500 cm^{-1} of deuterium oxide were used as references for the measures in intensities. The bands of ring-breathing vibrations of the chromophores (ca. 990 cm^{-1}) were used for normalization of the SERS spectra of free drug and drug–target complex (when indicated in the figure legend as "normalized spectra") in order to obtain the difference spectra. These bands do not change upon interaction of the drugs with targets as has been demonstrated before in the SERS studies of related compounds (Morjani et al., 1993; Nabiev et al., 1994a,b).

All spectra were reproduced at least three times for different preparations of drug–DNA, drug–enzyme, and ternary complexes. The spectral differences were reproducible from one preparation to the next and over multiple experiments.

Hydrosol Preparation. Aqueous silver hydrosol was prepared by reduction of silver nitrate with trisodium citrate as described by Hildebrandt and Stockburger (1984) and preaggregated by the addition of sodium perchlorate up to the final concentration of 0.06 M. The concentration of trisodium citrate corresponds to a silver particle density of 3.2×10^{14} L^{-1} at the formation of spherical (80%) and ellipsoidal (20%) particles, with the mean diameter of the spheres ca. 30 nm and the average length of the rods ca. 60 nm (Hildebrandt & Stockburger, 1984). As was noted by the different research teams (Smulevich & Feis, 1986; Nonaka et al., 1990; Nabiev et al., 1993), an interaction of drug–target complexes with the colloid is very smooth and does not disturb the structure of these complexes. D_2O colloids were made in exactly the same fashion, substituting D_2O (100%, Sigma) for water.

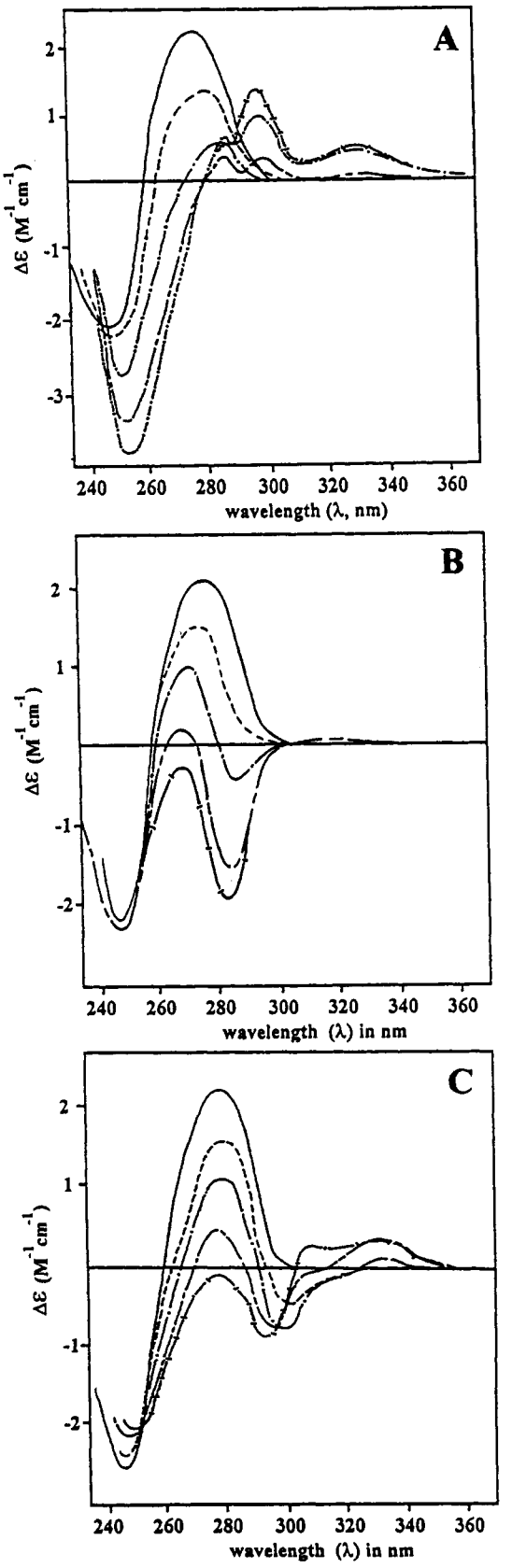

FIGURE 2: Titration of pBR322 plasmid DNA with intoplicine derivatives **1c** (A) and **2a** (B) and with intoplicine (C) in phosphate buffer at pH 7, I = ca. 0.02, at 20 °C. (A) CD spectra for r (drug to DNA ratios): 0 (—), 0.02 (- - -), 0.04 (– ·), 0.05 (– -), 0.07 (– · –), 0.1 (– ′). (B and C) 0 (—), 0.02 (- - -), 0.05 (– ·), 0.1 (– -), 0.21 (- ′).

RESULTS

CD Spectroscopy. Figure 2 shows the three families of CD spectra that resulted when plasmid DNA was titrated with

intoplicine and its derivatives **1c** and **2a** (Figure 1). These three compounds demonstrate very different modes of interaction with the DNA (see below).

CD spectra of drug-free plasmid DNA at pH 7, $I = 0.02$, are those expected for B-DNAs (Figure 2). With drug addition, new signals are generated in both UV absorption and CD at wavelengths above 260 nm, and more or less pronounced changes are observed in the overall DNA spectrum.

At the drug to DNA ratios (r) in the range from 0.02 to 0.1, the induced CD spectra of intoplicine derivative **1c** bound to plasmid DNA consist of a strong positive band centered at ca. 300 nm and medium positive bands at ca. 285 and 330 nm, approximately where the red-shifted UV absorption bands (compared to the UV-absorption band of the free molecule) of derivative **1c** bound to DNA contribute (Figure 2A). It should be noted that this drug induces dramatic changes in the overall CD spectrum of DNA in the region below 260 nm even at a very low ($r = 0.02$) drug to DNA ratio.

Derivative **2a** (Figure 2B) shows appearance of a strong negative CD band at ca. 285 nm (at exactly the same position when the positive band of the derivative **1c** was detected). The spectra of derivatives **2a** and **1c** were also found to be very different in the region higher than 300 nm: the positive CD signal at ca. 330 nm was not induced by derivative **2a** across the entire range of drug to DNA ratios used. Derivative **2a** does not induce any pronounced perturbations in the CD signals of the DNA below 260 nm even at the very high (r = ca. 0.2) drug to DNA ratios.

The induced CD spectra of intoplicine bound to plasmid DNA (Figure 2C) show a superposition of CD spectral features found to be characteristic for its derivatives **1c** and **2a**. The spectrum consist of a medium negative band (which was found to be strong for its derivative **2a**) and a medium positive band at ca. 330 nm (as for its derivative **1c**), both approximately where the red-shifted UV absorption bands (compared to the UV-absorption band of the free molecule) of intoplicine bound to DNA contribute (ca. 298 and 338 nm, respectively). At the high drug to DNA ratios ($r > 0.1$) an additional positive band centered at ca. 310 nm appeared. This band also corresponds to the one of the UV absorption bands of the intoplicine bound to DNA. The absolute intensity of the induced CD of intoplicine at ca. 330 nm is nearly 2 times less than that of its analogue **1c** at the same drug to DNA ratios, whereas absolute intensity of the induced negative CD signal of intoplicine at ca. 290 nm is nearly 2 times less than that of its analogue **2a** at the same drug to DNA ratios. Intoplicine induces small perturbations in the overall CD spectrum of the DNA—more pronounced than those induced by derivative **2a**, but much less than those of derivative **1c**. Moreover, intoplicine induces a slight decrease of the absolute intensity of the negative CD signal of DNA in the region below 260 nm, whereas derivative **1c** induces a strong increase of absolute intensity and red-shift of the negative CD band of DNA.

Intoplicine derivatives **1b**, **2b**, and **2c** (Figure 1) showed CD spectra similar to those for intoplicine in the region from 235 to 305 nm (data not shown), a strong negative band was at ca. 290 nm, but were different from intoplicine in the region higher than 310 nm. The derivatives did not induce a pronounced positive band in this region of CD spectra. They also did not induce significant perturbations in the overall DNA spectrum as has been shown for derivative **1c**.

SERS Spectra of Free Drugs. The preliminary vibrational assignments of the Raman and SERS active modes in the spectrum of intoplicine have been described by us (Morjani et al., 1993). In the present study the SERS spectra of intoplicine and its derivatives (Figure 1) have been recorded both in aqueous and in deuterium oxide solutions and at different pH and used for more precise spectral assignments.

General characteristic SERS spectral features for free drugs are as follows (Figure 3): (i) The bands at ca. 1651, 1617, 1566, and 1504 cm^{-1} have nearly the same frequencies and relative intensities for all derivatives of intoplicine. These bands are not affected by going from the derivatives of the benzo[*e*]pyridol[4,3-*b*]indole series to benzo[*g*]pyridol[4,3-*b*]indole derivatives. So, they must be strongly coupled with the vibrations of groups existing in the all intoplicine derivatives: rings B, C, or D of the chromophore. The bands at ca. 1651, 1617, and 1504 cm^{-1} are sensitive to the deuteration of the molecule since they disappear completely or have a pronounced shift on going from H_2O to D_2O. These vibrations must be localized on the D ring (being strongly coupled with the vibrations of the NH group of the six-membered ring) and (or) C ring (with coupling with the motions of NH group of the five-membered ring) of intoplicine molecule. These bands are not affected by going from pH 7 to 9. The NH group of ring C has a pK_a in this region, whereas the pK_a of other protonated groups were found to be in the other regions (Bisagni, 1993). It is compatible with the preferential localization of these vibrations to the motions of ring D. The band at 1566 cm^{-1} is not very sensitive to the deuteration of the chromophores and does not change on going from pH 7 to 9. We attribute this band to the pure ring-stretching vibrations of the chromophore.

(ii) The bands in the region from 1420 to 1500 cm^{-1} (1433 and 1472 cm^{-1}) are not affected on going from pH 7 to 9 and can be assigned to the vibrations of rings A, B, and/or D. On the other hand, they exhibit differences from each other depending on the substitutions of the ring A and on the stereometric geometry of the chromophore. The band at 1472 cm^{-1} in the spectrum of intoplicine is not very sensitive to the deuteration but strongly depends on the C–O substitution in ring A and on the geometry of the chromophore. The band at 1433 cm^{-1} disappears or exhibits a significant shift on going from aqueous to deuterium oxide solution but is not strongly dependent on the geometry of the chromophore. Hence, the former band is attributed to the motions of ring A (and, probably, B), coupled with the C–O motions, whereas the latter, to ring D, is coupled with the vibrations of the out-of-ring NH group.

(iii) The bands in the region 900–1420 cm^{-1} are very sensitive to the configuration of the chromophore but are quite similar in each of the (benzo[*e*]pyridol[4,3-*b*]indole or benzo[*g*]-pyridol[4,3-*b*]indole) series of derivatives. The band at 1406 cm^{-1} (in intoplicine series) is sensitive to the deuteration, decreases on going from pH 7 to 9, and is affected by the substitutions of the A ring. The motions of rings B and C (with a slight contribution of the ring A) are preferably involved in this vibration. In contrary, the band at 1391 cm^{-1} corresponds to vibrations much more significantly coupled with ring A being very sensitive to substituents in this ring and not affected by going from pH 7 to 9. The band at 1310 cm^{-1} in intoplicine series is not sensitive to deuteration and pH in the range from 7 to 9 (but strongly depends on the configuration of the chromophore), being mainly localized at ring B. The band at 1220 cm^{-1} is the same for all analogues of intoplicine series and exhibits only slight pH dependence (since it involves vibrations of ring B or D) but has a significant (ca. 15 cm^{-1}) shift upon deuteration of the chromophore; it is also coupled with the vibrations of ring C. The bands at

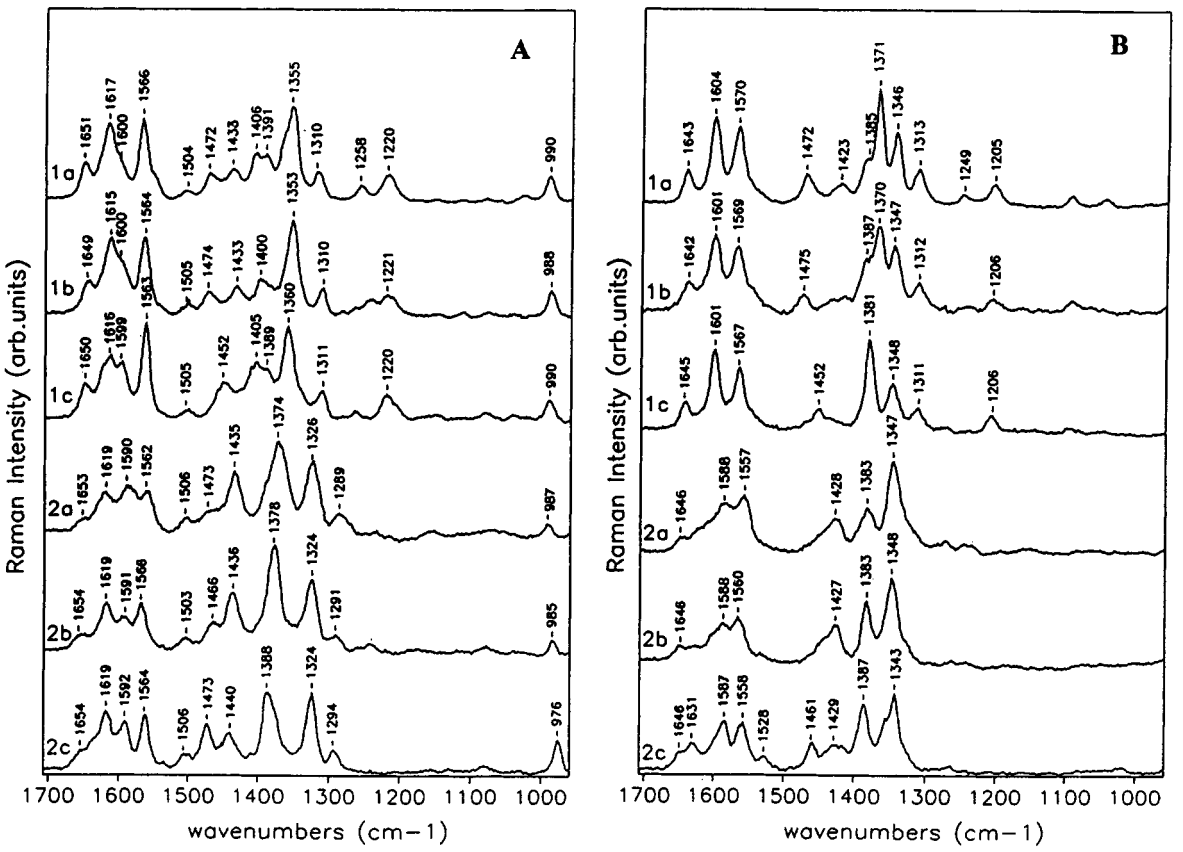

FIGURE 3: SERS spectra of intoplicine derivatives in aqueous (A) and deuterium oxide (B) hydrosols. Excitation line, 514.5 nm; laser power, 50 mW; concentrations, 10^{-7} M; 25 accumulations (1 s each).

Table 1: Comparison of Absolute Intensities of the SERS Spectra of Intoplicine Derivatives in Complexes with DNA and Topoisomerases and in Ternary Complexes (% from the SERS Intensity of Signal from the Free Drugs)[a]

		drug/Topo I		drug/Topo II					
derivative[b]	drug/DNA	active	deactivated[g]	active	deactivated[g]	CTCI[c]	RCI[d]	CTCII[e]	RCII[f]
1a (intoplicine)	96	67	66	0	65	45	66	43	0
1c	0	64	65	65	67	0	0	0	0
2a	98	65	65	0	66	59	66	45	0
2c	97	67	66	67	65	48	64	55	65

[a] Each value is a result of averaging of three independent experiments. All spectra were recorded under identical experimental conditions. Drug-to-DNA ratios were 0.1. The high-frequency band at ca. 3400 cm^{-1} of water was used as reference for intensity measurements. The standard deviation of the measured intensities was within 10% from the absolute value of the signal. [b] The symbols of derivatives (**1a,c**; **2a,c**) correspond to those presented in Figure 1. [c] CTCI: Topo I-mediated cleavable ternary complex. [d] RCI: Reversed (by brief heating at 65 °C) Topo I-mediated ternary complex. [e] CTCII: Topo II-mediated cleavable ternary complex. [f] RCII: Reversed (by brief heating at 65 °C) Topo II-mediated ternary complex. [g] "Active" and "Deactivated": Experiments with active Topo II and with the enzyme deactivated by heating at 90 °C.

1258 and 1355 cm^{-1} are both related to the very complicated group vibrations involving motions of the all rings of the chromophore.

SERS of Drug–DNA Complexes. The SERS signal of the intoplicine derivative **1c** disappears even at quite high ($r \approx 0.2$) drug to DNA ratios (Table 1). The absolute intensity of the SERS signal from intoplicine and its derivatives **1b**, **2a**, **2b**, and **2c** in the complex with both calf thymus DNA or plasmid DNA was nearly the same (within ca. 10%) as compared with the signal from the free drugs, even at very low ($r = 0.02$) drug/DNA ratios. The general features of the spectra, namely, positions of the bands, remain unchanged on going from the free drugs to drug–DNA complexes, whereas some important differences in the relative intensities have been found.

Intoplicine and its analogue **2a** (both including the hydroxyl group in ring A) showed significant SERS spectral differences upon interaction with DNA (Figure 4). The differences become very small on going to analogues with the methoxy group (**1b**, **2b**) or proton (**1c**, **2c**) at the same position. The most pronounced spectral features for intoplicine include a decrease of relative intensity of the bands at 1472, 1391, and 1355 cm^{-1}. These bands involve vibrations localized at the A ring (and partially B) of the chromophore and coupled with the C–O motions. Similar perturbations have been found in the SERS spectra of the complexes of analogue **2a** with DNA. The bands corresponding to the motions mainly localized at the A ring decreased in intensity, whereas the vibrations corresponding to rings C and D (e.g., 1651, 1617 cm^{-1}, etc.) increased or remain unchanged upon drug–DNA interaction.

Interactions between Intoplicine Derivatives and Topoisomerases I and II. The SERS signal of intoplicine and its derivatives **1c**, **2a**, and **2c** shows some slight decrement in intensity upon interaction with Topo I, ca. 33–36% of the SERS intensity of the free drug (Table 1). The decrement of intensity could either be due to specific interactions of intoplicine within the enzyme or be the result of simple intervention of protein between the drug and the surface of

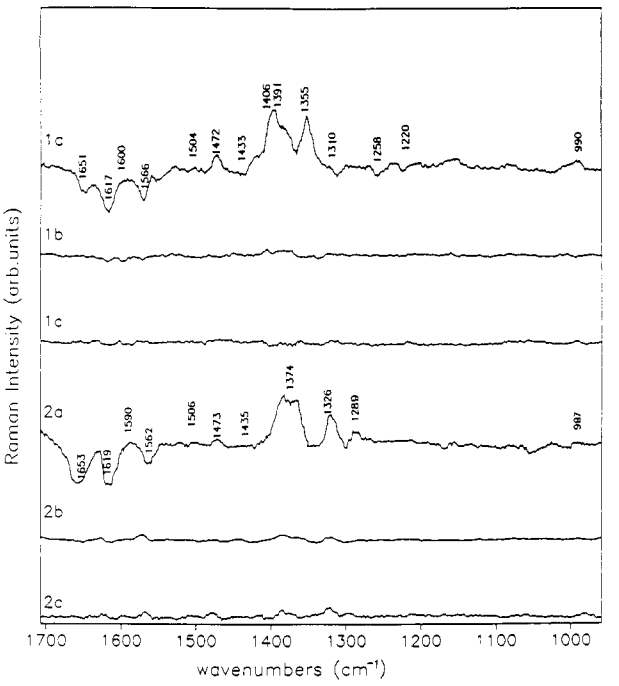

FIGURE 4: Difference spectra between normalized SERS spectra of free intoplicine or its derivatives **1b,c** and **2a,b,c** and SERS spectra of the corresponding complexes of the drugs with plasmid DNA. Original spectra were normalized before substraction as described under Materials and Methods. Drug to DNA (bp) ratios for the SERS spectra of complexes were ca. 0.04. All original spectra were recorded under the same experimental conditions, as given in the legend to Figure 3. Negative peaks correspond to the bands exhibiting an increase of intensity upon interaction of the drug with DNA, and positive peaks correspond to the bands exhibiting a decrease of intensity.

hydrosol. To clarify whether the drug–Topo I interaction is specific, a control experiment has been done. In a control experiment in which intoplicine and nonactive Topo I (deactivated by the 15 min by heating up to 90 °C followed by a 20-min incubation on ice) were used, the intoplicine SERS spectrum showed nearly the same (ca. 35%) decrement in intensity as for the mixture of drug with the active enzyme (Table 1). Hence, a decrease of the drug signal is the result of simple intervention of protein between the drug and the surface of hydrosol. Moreover, the SERS spectrum of complex between intoplicine and Topo I was completely identical in frequencies and relative intensities to the spectrum of free drug.

For intoplicine and derivative **2a**, the formation of a complex between drug and Topo II dramatically decreased the absolute intensity of drug signal (Table 1). The signal desappeared completely at drug/Topo II ratios corresponding to those at which only ca. 33–35% decrement of intensity has been detected for the drug/Topo I mixtures. Even at very high drug/enzyme molar ratios (ca. 10:1), the decrement of intensity of intoplicine signal was ca. 75% from the signal of free drug. A control experiment with intoplicine and nonactive Topo II (deactivated at 90 °C in exactly the same fashion as Topo I) did not show this dramatic loss of intoplicine signal intensity (Table 1). In this case, the level of decrement of intensity was comparable with those for the mixture of intoplicine with the Topo I and was not more than ca. 35% of the SERS intensity of the free drug. This experiment shows that the decreasing of the SERS signal of the drug in the complex with active Topo II is not the result of simple intervention of protein between the drug and the surface of hydrosol (like in the case with Topo I) but is the effect of specific interactions of the drug within the protein.

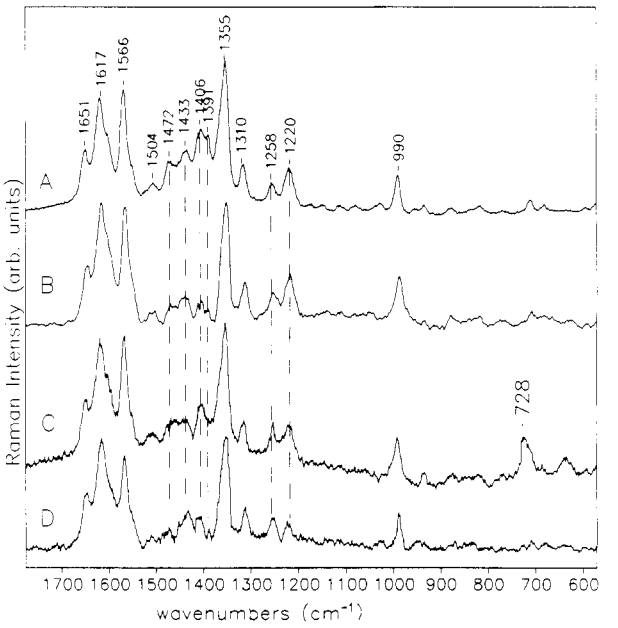

FIGURE 5: SERS spectra of intoplicine (A), intoplicine–plasmid DNA complex (B), intoplicine-stabilized Topo I-mediated ternary cleavable complex (C), and reversed ternary complex (D). Experimental conditions were as given in the legend to Figure 3. Conditions of preparation of the complexes are described under Materials and Methods. Drug to DNA ratios were 0.04.

Other intoplicine derivatives (**1c**, **2c**) did not show this dramatic diminishing of the signal when present in the complex with Topo II (Table 1), but only slight nonspecific decrease of SERS intensity (ca. 33–35% of the level of the SERS signal of the free drug). Their SERS spectra did not show any differences both in frequencies and in relative intensities as compared with the spectra of free drugs.

Induction of Topo I-Mediated Cleavable Ternary Complexes. The following spectral changes have been found upon an interaction of intoplicine in the ternary complex with the plasmid DNA and Topo I. The absolute intensity of the SERS signal from intoplicine seems to decrease to ca. 45% of the level of SERS intensity of the free drug (Table 1). Moreover, no differences in frequencies and only slight differences in the SERS relative intensities of the bands in the spectrum of ternary complex, as compared with the drug–DNA complex, have been found (Figure 5). These results show that the molecular interactions of the drug in the ternary complex were not changed significantly as compared with its complex with the DNA.

One important spectral feature is evident from the SERS spectrum (Figure 5): the band at ca. 730 cm^{-1} appears upon formation of the Topo I-mediated ternary complex. This band does not appear in the control experiment with the Topo I or plasmid DNA alone. Appearance of the ca. 730-cm^{-1} band in the SERS spectrum (Figure 5) will be discussed in terms of local destabilization of the AT-rich region of the DNA (Koglin & Sequaris, 1986; Nabiev et al., 1990, 1993) upon formation of intoplicine-stabilized Topo I-mediated ternary complex (see Discussion).

The formation of a cleavable complex by antitumor drugs has been shown to be reversed by several treatments for a reaction mixture such dilution, increased salt concentration, or elevated temperature (Liu, 1985; Pommier et al., 1991). To test whether the spectral changes observed in the SERS spectra after the formation of complexes between intoplicine, plasmid DNA, and Topo I are reversible, a reaction mixture was briefly (5 min) heated (65 °C) before registration of the

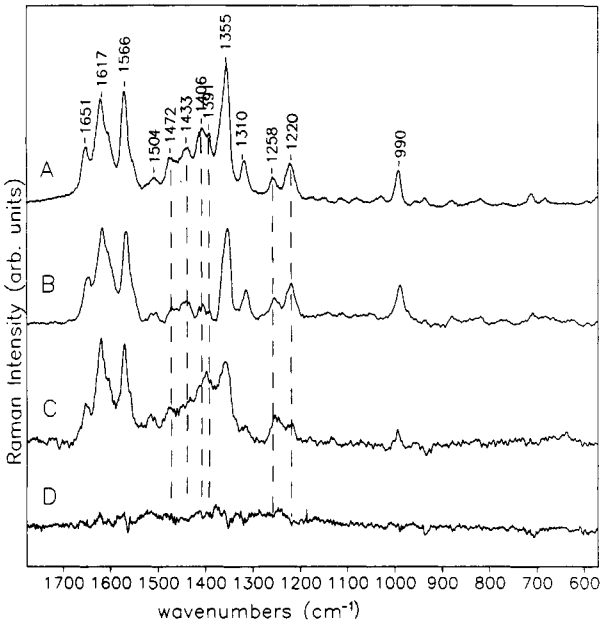

FIGURE 6: SERS spectra of intoplicine (A), intoplicine–plasmid DNA complex (B), intoplicine-stabilized Topo II-mediated ternary cleavable complex (C), and reversed ternary complex (D). Experimental conditions were as given in the legend to Figure 3. Conditions of preparation of the complexes are described under Materials and Methods. Drug to DNA ratios were 0.04.

SERS spectra. The absolute SERS intensity of the intoplicine signal has been increased in the reversed complex as compared with the ternary complex (Table 1). Moreover, the level of the SERS signal became nearly the same as for the SERS intensity in the drug–Topo I system.

Induction of Topo II-Mediated Cleavable Ternary Complexes. The spectral features induced by an interaction of intoplicine in the ternary complex with the plasmid DNA and Topo II have been found to be quite different from the drug–DNA–Topo I ternary cleavable complex. First, the absolute intensity of the signal from the drug in the ternary complex seems to be increased as compared with its intensity in the drug–Topo II complex (Table 1). Second, no signal from the adenine nucleotide (ca. 730 cm^{-1}), similar to that detected for the ternary complex mediated by Topo I, has been found for the Topo II-mediated ternary cleavable complex (Figure 6).

When the SERS relative intensities are considered, interactions of intoplicine in the Topo II-mediated cleavable ternary complex induced pronounced spectral changes (Figure 6). The bands at ca. 1406 and 1258 cm^{-1} (complicated vibration involving motions of rings B and D) have been found to be strongly increased in relative intensity, whereas the bands corresponding to the motions related to rings A and C of the chromophore (1391, 1472 cm^{-1}, etc.) showed a decrement. The bands at 1220 and 1258 cm^{-1} undergo a change in their relative intensities. These results show that the mode of molecular interactions of the drug in the Topo II-mediated ternary complex is very different from the drug–DNA interactions.

To test whether the spectral changes observed in the SERS spectra after formation of ternary complex between intoplicine, plasmid DNA, and Topo II are reversible, a reaction mixture was briefly heated (65 °C) before recording the SERS spectra. The absolute SERS intensity of the signal from bound intoplicine was found to decrease to zero in the reversed complex as compared with the ternary complex and became similar to the intoplicine signal intensity in the drug–active Topo II complex (Table 1).

DISCUSSION

SERS Spectral Parameters. There are two sets of SERS spectral parameters that enable the analysis both of the topology of high-molecular-weight complexes and of inter- and intramolecular interactions. The first is the SERS absolute intensity. The effect of loss of SERS absolute intensity after the intercalation of the drug into the DNA or upon its penetration in the interior of the protein as well as dependence of SERS intensity on the distance between the molecule and surface of hydrosol have been well documented in terms of short-range character of Raman enhancement (Cotton, 1988; Koglin & Sequaris, 1986; Nabiev et al., 1990, 1993). This enables us to clarify whether the low-molecular-weight substrate is bound on the surface of supramolecular complex (being accessible to the SERS-active surface, e.g., hydrosol) or within its interior. The SERS spectra of typical deep intercalators (e.g., doxorubicin, THP-doxorubicin, aclacinomycin, ethidium bromide) show almost complete disappearance of the signal of drug upon its intercalation within DNA, whereas the level of absolute intensities in the spectra of some minor-groove binders, as well as of a wide variety of noninteracting model compounds seems to be unchanged (Nabiev et al., 1993, 1994a,b). The strong dependence of the SERS signal on the distance of the chromophore from the surface of the hydrosol has been shown also for the reconstructed complexes of bacteriorhodopsin with the analogues of retinal of different length and for the complexes of Na^+,K^+-ATPase with the nonhydrolyzed analogues of ATP (Abdulaev et al., 1987; Nabiev et al., 1988; Nabiev & Efremov 1989). Hence, this effect seems to be of a general character.

The second set of parameters includes SERS frequencies and relative intensities. The SERS frequencies can be analyzed in exactly the same fashion as for normal Raman spectra. SERS relative intensities reflect the mode of drug–target interaction, i.e., the bands corresponding to vibrations of functional groups of drug involved in the interaction with the target are preferably decreased in intensity. This finding enables the construction of the molecular models of drug–target complexes. The models for the complexes of some well-known compounds (doxorubicin, aclacinomycin) were found to be consistent with NMR and X-ray diffraction data (Smulevich & Feis, 1988; Nonaka et al., 1990; Nabiev et al., 1993, 1994 a,b).

Drug–DNA Interactions and Models of Binding. The SERS signal of intoplicine derivative **1c** almost completely disappears upon its interaction with the DNA. So, the drug shows the typical deep intercalating mode of binding being recently described for aclacinomycin, doxorubicin, ethidium bromide, *m*- and *o*-AMSA, and many other compounds (Nabiev et al., 1993, 1994a,b; Nabiev & Efremov, 1989). Since it is deeply intercalated within the DNA double-stranded helix, the molecule becomes undetectable by means of SERS spectroscopy. Structural information concerning orientation of the chromophore has been obtained from the analysis of CD spectra of drug–DNA complexes.

The induced CD signals (Figure 2) are due to the drug-bound complexes, since all analogues of intoplicine are achiral molecules; optical activity is generated in the asymmetric environment of the DNA double helix (Lyng et al., 1987; Norden & Tjerneld, 1982; Monnot et al., 1991). The diversity of the spectra yielded by the titration of plasmid DNA with intoplicine derivatives reflects the strong influence of the chemical factor on the binding process in that series.

The observed appearance of induced CD signals above 280 nm (Figure 2), subsequent to drug addition to plasmid DNA,

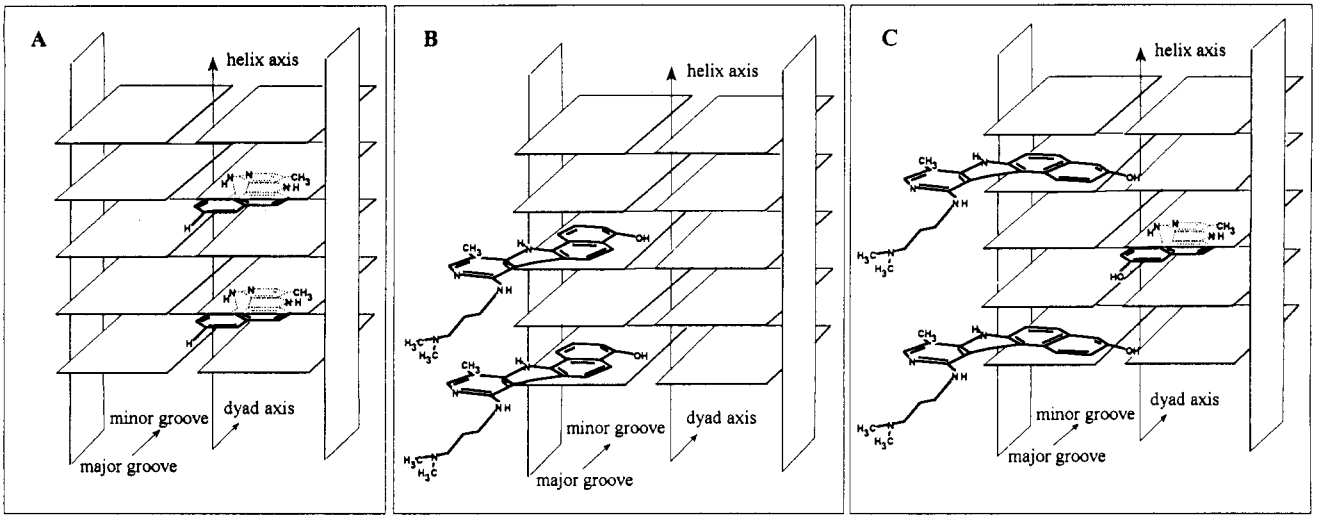

FIGURE 7: Proposed models of the interactions between intoplicine derivatives and DNA. (A) Intercalative model found to be dominant for derivative **1c**, the most potent Topo I inhibitor. (B) Model of external binding from the DNA major groove found to be dominant for derivative **2a**, the most potent Topo II inhibitor. (C) Superposition of two different modes of binding (intercalation and external binding from the major groove) for intoplicine, a dual Topo I and II inhibitor. See the text for details.

is definitive evidence of chromophore interaction with the base pairs (Lyng et al., 1987; Wang et al., 1987; Frederick et al., 1990). The induced CD is considered to arise from interactions of the chromophore transition moment with (nondegenerate) transition moments of the chirally disposed, surrounding DNA bases. The CD signal is strongly dependent on the angular orientation of the drug chromophore and only moderately dependent on its location within the intercalation pocket (Lyng et al., 1987). Monnet et al. (1991) explained the observed appearance of positive induced CD signals at high wavelengths as definitive evidence of the intercalation of 1-methyl-9-hydroxyellipticine derivatives in poly[d(A-T)] with the long axis of the chromophore oriented roughly parallel to the dyad axis of the DNA helix, with γ, the "azimuthal" angle defining the orientation of the intercalator transition moment in the plane of intercalation, approaching 0. In this case, the drug must be placed in the intercalation plane in the minor groove of DNA. The experimental observation that the DNA-induced CD of the intercalating dye methylene blue changes sign with increasing NaCl concentration or upon addition of Mg^{2+} was explained in terms of different preferred orientations of the dye within the plane of intercalation: at low salt the two positively charged ends of the dye molecule tend to lie close to the negative DNA phosphates of the opposite strands (corresponding to γ = ca. 90°, negative CD signal), whereas at high ionic strength the phosphates are partially screened and other orientations of intercalator predominate (positive induced CD signal). Finally, it was concluded that if the dye is placed in the intercalation plane and in the minor groove of DNA, it would exhibit positive CD. If, on the other hand, it is placed in the major groove, the CD would be negative (Lyng et al., 1987; Norden & Tjerneld, 1982).

The CD data confirmed the conclusion based on the SERS spectroscopic analysis of complex between intoplicine derivative **1c** and the DNA. Induced CD signals of the drug are typical for chromophores exhibiting the deep intercalation mode from the minor groove and oriented with the long axis roughly parallel to the dyad axis of the DNA helix (Figure 7A). This derivative induces strong changes in the overall CD spectrum of DNA below 260 nm (Figure 2), similar to those induced by typical intercalators (Lyng et al., 1987; Monnot et al., 1991). In terms of SERS spectral data, the chromophoric system of the drug interacting with the DNA in this fashion becomes completely unaccessible to the surface of the hydrosol and the SERS signal becomes undetectable, as was seen from our SERS experiments with derivative **1c**.

SERS spectra of the complexes of intoplicine and its derivatives **1b**, **2a**, **2b**, and **2c** (Figure 1) with the plasmid DNA do not show a significant (but only about 10%) decrement in absolute intensity as compared with the SERS signals of free drugs. Hence, the mode of deep intercalation within DNA cannot be considered as dominant for these compounds. On the other hand, their molecular interactions with DNA seem to be evident (Figure 4). The strongest spectral changes induced by interaction with DNA have been found for intoplicine and its analogue **2a**, both with the OH group in the A ring of the chromophore. The changes in relative intensities of corresponding SERS bands undoubtedly indicate direct participation of the A ring in drug–DNA interaction. So, only a portion of the overall chromophore could be involved in the interaction. This portion includes ring A and, probably, ring B (Figure 1). Rings C and D of the chromophore are not buried inside DNA double helix since they are easily accessible to the surface of hydrosol. What could be a preferable orientation of chromophores involved in the mode of outside binding to the DNA? Analysis of CD spectra of drug–DNA complexes can provide an answer to this question.

The induced negative CD signal for derivative **2a** at the nearly the same position where the strong positive band of the derivative **1c** was detected (Figure 2) shows that the orientations of these two chromophores in their dominant modes of binding with DNA are very different. Derivative **2a** has the dominant outside binding mode in the major groove of DNA and is oriented with γ = ca. 90°. These data also correlate with the results of SERS analysis. The outside binding mode was found to be dominant for this analogue, and the hydroxyl group of the A ring of the chromophore was found to be involved in the interactions with DNA. Any group belonging to the backbone, bases, or sugars can participate in interactions with drugs as illustrated tentatively in Figure 7B.

On the basis of the CD data (Figure 2), intoplicine exhibits at least two different modes of binding to the DNA: (i) induced negative CD signal from the outside bound molecules preferably placed in the major groove of DNA; and (ii) an induced positive CD signal at ca. 330 nm, also showing the possibility for intoplicine to be deeply intercalated within DNA from the

minor groove of the target. So, the binding mode of intoplicine seems to be superposition of the binding modes of its derivative **1c** (most potent inhibitor of Topo I) and derivative **2a** (most potent inhibitor of Topo II, see below). Intoplicine does not induce strong changes in the CD signals of DNA below 260 nm, indicating that there are very few deeply intercalated molecules as compared with its analogue **1c** (Figure 2). The SERS spectrum of intoplicine has been found to be easily detectable upon interaction with DNA confirming that its partial intercalating or outside binding mode is dominant. Moreover, the hydroxyl group of the A ring plays a critical role in the process of intoplicine binding from the major groove of the DNA: SERS spectral differences induced by intoplicine–DNA interaction were attributed preferably to the vibrations coupled with rings A and B of the chromophore, whereas the vibrations localized on rings C and D were unchanged (Figure 4). As was mentioned above, SERS spectroscopy gave information only about the outside bound molecules since deeply intercalated chromophoric systems become undetectable by this technique. Nevertheless, it is possible to conclude that there are not more than 10% of deeply intercalating intoplicine molecules, because the SERS absolute intensity of drug in the corresponding spectra of drug–DNA complexes were found to be unchanged (within ca. 10%) as compared with the spectra of free drugs (Table 1). The possibility for intoplicine molecules to participate in two different modes of binding with DNA is illustrated tentatively in Figure 7C.

It is interesting to note that intoplicine analogues **1b**, **2b**, and **2c** showed a negative induced CD signal above 280 nm (similar to the mode of intoplicine derivative **2a** major groove binding), whereas pronounced positive CD signals were not induced for these compounds for the entire range of drug to DNA ratios. The SERS intensities of these drugs were found to be unchanged (within 10% of intensity) in the complex with DNA. These facts demonstrate the preferable "outside" position of the chromophores in the major groove of DNA.

Interactions between Drug and Topoisomerases. The SERS signal of intoplicine in the complex with active Topo II is completely diminished (Table 1). The signal is not totally diminished when the drug is added to the preparation of deactivated enzyme. We propose that intoplicine is able to interact within the specific site in the interior of Topo II. This site was found to be nonstereospecific: the same effect has been found for intoplicine derivative **2a**, belonging to the benzo[*g*]pyrido[4,3-*b*]indole series, but it was not the case for intoplicine derivatives without hydroxyl group in the A ring. The same slight (ca. 30%) nonspecific decrease of SERS intensity has been detected when derivatives without hydroxyl group in the A ring (**1c**, **2c**) were mixed with the active or with deactivated enzyme. So, a specific (but not stereospecific) binding site of intoplicine (and its derivative **2a**) exists in the interior of Topo II. The binding of intoplicine to this site is achieved via the hydroxyl group of ring A of the chromophore. The outside binding mode in the major groove of the DNA with the hydroxyl group of ring A, easily accessible to the enzyme (which was found to be dominant for intoplicine and its analogue **2a**, the most potent inhibitors of Topo II), can play a critical role for enzyme–DNA recognition and also for inhibition of Topo II. The role of this mode of binding in the process of DNA recognition by the enzyme, induction and suppression of ternary cleavable complex, will be discussed below.

Intoplicine and its derivatives were shown to be unable to interact specifically with the Topo I in the absence of DNA (Table 1). In this case, recognition by the enzyme of specific sites on DNA and stabilization of the cleavable ternary complex could be most probably induced by the drug on the level of DNA. Our spectral data provide some experimental evidences confirming this hypothesis.

Molecular Interactions of Drugs and Biological Effects. Intoplicine is a dual Topo I and Topo II inhibitor, with DNA sites of enzyme inhibition being different for these two enzymes. Site-specific DNA cleavage mediated by Topo I was observed with benzo[*e*]pyridol[4,3-*b*]indole derivatives but not with benzo[*g*]pyridol[4,3-*b*]indole derivatives. Site-specific DNA cleavage mediated by Topo II occurred with both derivatives with a hydroxyl group at the A ring of the chromophore (Figure 1). Study of the relationships between the *in vivo* antitumor activity on P388 leukemia and the Topo I- and/or II-mediated DNA cleavage activity (Riou et al., 1993) revealed that the most highly active antitumor compounds possessed both Topo I- and II-inhibitory properties (intoplicine). Compounds selectively inhibiting either Topo I (derivative **1c**) or II (derivative **2a**) were less active. Intoplicine appears to exhibit a broad spectrum of activity and to be cytotoxic in cells that are resistant to either Topo II or Topo I inhibitors. Data derived from resistant cell lines indicated that multidrug-resistant cells were cross-resistant to intoplicine but that *m*-AMSA- and camptothecin-resistant cells were sensitive to intoplicine. Hence, intoplicine might circumvent Topo I-mediated and Topo II-mediated resistance by poisoning both enzymes simultaneously (Poddevin et al., 1993).

As it follows from the spectral data, intoplicine shows at least two very different modes of binding to the DNA. These modes have been found to be well correlated with the dominant modes of binding of intoplicine derivatives **1c** and **2a**. The first one corresponds to the derivative with the maximum potency to inhibit Topo I, whereas the second corresponds to the derivative with the maximum potency to inhibit Topo II (Riou et al., 1993). We speculate that the possibility of forming these two types of complexes is the main (but not single) factor enabling the drug to be a dual Topo I and Topo II inhibitor. The following experimental results and the data published recently led us to this conclusion.

(i) The first mode, corresponding to the deep intercalation of intoplicine or its derivative **1c** from the minor groove of the DNA, induces local and medium-range distortions which could be responsible for the recognition of the specific sites on the DNA by the Topo I (Monnot et al., 1991). This mode involves not more than 10% of intoplicine molecules at drug to DNA ratios from 0.02 to 0.2 but becomes dominant for intoplicine derivative **1c** (most potent Topo I inhibitor). The process of intercalation was found to be stereospecific: similar derivatives, but belonging to the benzo[*g*]pyridol[4,3-*b*]indole series, exhibited pure outside binding mode in the major groove of DNA. The following biological effects can be mediated by the drug molecules exhibiting this mode of binding.

Intoplicine induces Topo I-mediated cleavages at the very low concentration (0.01 μM) and also suppresses them at quite low (3 μM) concentration (Riou et al., 1993). A very few molecules of the deep intercalator can induce significant distortions of the DNA structure, which can be used by the enzyme for specific site recognition (Saenger, 1984; Schleif, 1988). On the other hand, according to the nearest-neighbor exclusion principle, which seems to apply to most good intercalators (Saenger, 1984), only about half of all potential sites are filled by the drug molecules. An increase of drug concentration must lead to the suppression of the Topo I-mediated cleavable complex due to the consecutive occupa-

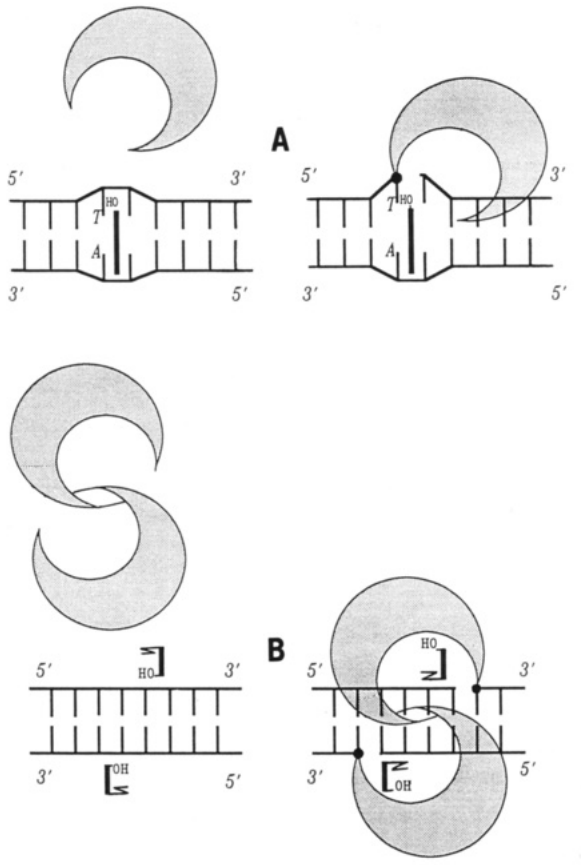

FIGURE 8: Hypothetical models of molecular interactions between intoplicine, plasmid DNA, and topoisomerases I (A) and II (B). The model is modified from the models of initial steps in the strand-passing reactions of topoisomerases I and II and for the formation of enzyme-linked strand breaks, proposed by Liu (1989).

tion, by the drug molecules, of external sites on the surface of DNA (Monnot et al., 1991).

Appearance of the SERS signal of adenine after formation of the Topo I-mediated ternary cleavable ternary complex shows that the stiffening of DNA preferably occurs in the AT-riched regions of DNA sequence. The band at ca. 731 cm^{-1} is characteristic of the adenine nucleotide ring breathing vibration. In the SERS spectra of double-stranded DNA, this vibration could not be detected due to the short-range character of SERS effect (Koglin & Sequaris, 1986; Nabiev et al., 1990). On the other hand, it appears to be very intensive when the adenine is localized in the regions of destabilization of helix, being well accessible to the surface of hydrosol. This signal disappeared when the ternary complex was reversed by brief heating of the reaction mixture and did not appear in the control experiments with Topo I or plasmid alone or in the complex of plasmid with Topo I. Hence, formation of Topo I-mediated cleavable ternary complex led to the appearance of regions of local destabilization in the AT-rich regions of the DNA double-stranded helix (Figure 8A). It is known that DNA cleavage and relaxation by eukaryotic Topo I probably occur at many sequences but most frequently at sites bearing a thymine at the 3′-DNA terminus of cleavage sites (Pommier et al., 1991). It is possible that the local denaturation of DNA in the surroundings of the intercalation site in the AT-rich regions of DNA is the necessary step before DNA cleavage by the enzyme in the intoplicine-stabilized ternary cleavable complex (Figure 8A). Sites of DNA–intoplicine–Topo I interaction have been found to be buried in the protein interior but are released from the protein interior when the ternary cleavable complex has been reversed. In the reversed ternary complex, intoplicine was found to be bound to DNA and did not show any interactions with the enzyme (Figure 8A).

(ii) Intoplicine and its derivative **2a** (most potent Topo II inhibitor) both exhibit an unusual dominant mode of interaction with the DNA (Figure 7). An interaction involves ring A and, probably, ring B of the chromophore. The dominant part of the chromophore is easily accessible to the surface of the hydrosol. So, this mode is found to be outside binding. This mode could be responsible for the recognition of the DNA by the Topo II. Intoplicine and derivative **2a** have been found to have significant specific affinity to the enzyme alone, and the orientation of the chromophores in the complex with the DNA enables it to interact with the functional groups of Topo II via the hydroxyl group of the A ring and the B ring. Recognition of the DNA by Topo II, as illustrated by studies with other DNA-binding proteins, mostly concerns the major groove (Saenger, 1984). In this case, drug bound to DNA in the major groove and having high affinity to Topo II can play an important role in enzyme–DNA recognition. The stabilization of the DNA–Topo II cleavable complex can then result from the direct interaction of the drug via its hydroxyl group with the functional group of the enzyme. Direct drug–Topo II interactions in the ternary cleavable complex were suggested before for daunomycin on the basis of X-ray studies (Wang et al., 1987).

Spectral changes induced by formation of Topo II-mediated ternary complex were reversible, and intoplicine was found to be bound to Topo II in the reversed Topo II-mediated ternary complex. Hence, reversion of the ternary complex led to elimination of drug–DNA interactions whereas interaction between drug and Topo II seems to be unchanged. These results are illustrated tentatively in Figure 8B.

Most anticancer drugs are supposed to induce their effects at the DNA level. Effects are conveyed according to the size and shape of molecules depending on the electron density distribution on atoms and bond polarizabilities within the partner molecules. Our results demonstrated that the mode of DNA binding is one of the most important but not single factor in the formation of topoisomerase-mediated ternary cleavable complexes. Therefore, the molecular background of interactions within the supramolecular complexes of enzymes, DNA, and antitumor drugs should be investigated if the overall patterns of drug structure–activity relationships are to be clarified. The approach developed in the present paper seems to be well applicable to these studies. Comparative investigations of molecular interactions of other antitumor drugs, dual Topo I and II inhibitors (e.g., saintopin, actinomycins) or classical Topo I (camptothecin derivatives) and Topo II (e.g., anthracyclines, ellipticines, acridins, etc.) inhibitors could shed light on the general mechanisms of action of topoisomerase-targeting antitumor drugs. Molecular interactions of these compounds in the ternary cleavable complexes with topoisomerases are presently under studies in our laboratories.

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