DNA Binding by Fagaronine and Ethoxidine, Inhibitors of Human DNA Topoisomerases I and II, Probed by SERS and Flow Linear Dichroism Spectroscopy†

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Raman, surface-enhanced Raman scattering (SERS), and flow linear dichroism (FLD) spectroscopies were employed to study the potent anticancer agent fagaronine (FGR, NSC 157995) and its derivative ethoxidine (ETX)—inhibitors of DNA topoisomerases (topos) I and II (Figure 1)—and their complexes with DNA. The FLD data obtained suggest that both compounds are strong major groove intercalators with stoichiometries 1 FGR/2.0 DNA bp and 1 ETX/4.0 DNA bp The SERS spectra of both compounds were recorded at the concentrations down to 10^{-8} M for FGR and 10^{-6} M for ETX, and the SERS-active modes were assigned by comparison of Raman and SERS spectra of the drugs following the changes induced by deuteration and pH environment. The SERS-active surface was proved not to affect the drug/DNA interactions, since the DNA binding constants calculated from the SERS experiments were found to be practically the same as those determined previously by viscosimetric measurements. The SERS study of the FGR/DNA complex showed that the OH group of FGR plays a key role in DNA binding, most probably because of formation of the H bond with DNA. Cooperative use of Raman, SERS, and FLD techniques enabled us to propose a molecular model for drug/DNA interactions. The differences in DNA binding by FGR and ETX are discussed in terms of different topoisomerases inhibitory activities of these drugs.

Introduction

Elucidation of the structure−function relationships and search of the key structural determinants mediating the mechanisms of action of antitumor drugs seem to be one of the most important problems in anticancer research.[1] Selective, sensitive, and nondestructive physicochemical and particularly optical spectroscopic approaches enable us to determine molecular groups of drugs and their targets (DNA, proteins, membranes) and to propose the ways for design of new molecules with desired functions. Among the novel optical spectroscopic techniques available up to date, surface-enhanced Raman scattering (SERS) spectroscopy and flow linear dichroism (FLD) techniques are characterized by high sensitivity and selectivity of analysis of individual components within the supramolecular complexes. SERS spectroscopy proved to be a powerful technique for selective analysis of the structure of low-molecular-weight ligands (e.g., antitumor drug) in complexes with a high-molecular-weight (DNA, protein) target.[2,3] Compared to the other techniques, SERS spectroscopy allows us to detect signals from the individual molecular groups at extremely low (down to 10^{-8} M) concentrations.[4−6] Molecular models of interactions of some antitumor drugs and DNA have been proposed and were found to be consistent with NMR and/or X-ray data.[4−7]

The linear dichroism technique enables us to determine the drug's orientation relative to the axis of oriented in the flow (FLD) or with the electric field (ELD) DNA or protein matrix and was found to be extremely sensitive to the changes of degree of DNA molecule orientation induced by intercalated drugs. So both drug and DNA alterations induced by their interactions may be followed by linear dichroism techniques.[8, 9]

Most of the antitumor drugs are presumed to induce their effects at the DNA level.[10] Some of them are known to be effective inhibitors of the topoisomerases, intranuclear enzymes participating in all aspects of cellular replication and transcription machinery.[1,11] The enzymes' poisons kill cells by trapping so-called cleavable complexes between the DNA and topoisomerases. The chemical structure of poisons and mode of their interactions with DNA determine its ability to interfere with topoisomerases in the cleavage complexes. Structural analysis and identification of functional molecular determinants of individual partners of these supramolecular complexes are quite complicated and require careful selection of appropriate and self-complementary physicochemical techniques but present an

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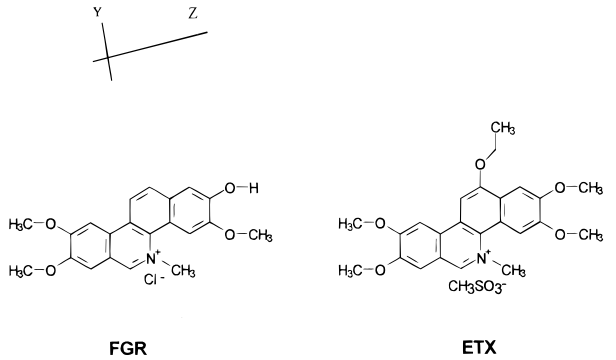

Figure 1. Chemical structures of fagaronine (FGR) and ethoxidine (ETX).

extremely important domain of research, being vital for an adequate new drug's design.[1]

Recently, we have applied a combined SERS, Raman, CD, and biochemical approach to study molecular mechanisms involved in the formation of binary and ternary complexes between antitumor drugs and their targets (DNA and topoisomerases). Molecular interactions within the complexes of well-known (doxorubicine, aclacinomycin, saintopin, netropsins) and new (intoplicine, camptothecin derivatives) compounds that are just in clinics or have potential antitumor activities have been studied.[2,4−6,12]

This paper describes the SERS and FLD analysis of DNA binding by fagaronine (FGR), a natural antileukemic alkaloid and potent differentiation inducer of various hematopoietic cell lines, and by its even more biologically active derivative ethoxidine (ETX). FGR is known to be a potent double inhibitor of DNA topoisomerases I and II, stabilizing in vitro cleavable ternary complexes between the enzymes and their DNA substrates.[13,14] It was shown that the drug is a DNA intercalator,[15] but the mode of its DNA binding is not known and its structural determinants playing a key role in interactions remain to be defined. Cooperative use of SERS and FLD techniques for the study of FGR/DNA and ETX/DNA complexes enabled us to address the following questions. (i) What is the orientation of the drug's chromophores within the drug/DNA complexes? (ii) What are the molecular groups responsible for the drug/DNA recognition and formation of complexes?

We have found that the OH group of FGR participates directly in the drug/DNA binding. According to our model, FGR intercalates within the DNA bases so that its OH group is directed toward the DNA minor groove and so that quaternary N^+ (Figure 1) plays the role of anchor for stabilizing this interaction. ETX is proposed to intercalate within the DNA in a way that its OCH_2CH_3 moiety (Figure 1) extends into the DNA minor groove and probably plays a key role in the disturbance of DNA recognition by the topoisomerases that attack their DNA substrates from the side of the minor groove.

Materials and Methods

Chemicals. FGR (NSC 157995), provided by Dr. E. Couillerot, was extracted from the roots of *Fagara xanthoxyloides* Lam. (Rutaceae) as described in ref 16. ETX was synthesized by one of us, and the details of the protocol will be published elsewhere. Both compounds were prepared in a 1 mM stock solution in methanol.

CT DNA was purchased from Sigma and dissolved in potassium-buffered saline (PBS) to 5 mg/mL.

Drug/DNA complexes were prepared by mixing the drug stock solutions with the DNA solution in PBS in order to obtain a final drug/DNA bp ratio (r) from 0.005 to 0.8.

Raman and SERS Spectroscopy. Raman and SERS spectra were recorded with a PHO (CODERG) spectrometer with a double monochromator in the frequency range 300−1800 cm^{-1}. An Ar^+ ion laser (Coherent Radiation, model Innova 2020) operating at 80 mW power at 457.9 nm (for FGR) or at 488 nm (for ETX) wavelength was used for spectra excitation. For Raman spectra the accumulation of 30 independent scans with time averaging was used to improve the signal-to-noise ratio. SERS spectra were recorded for 1 scan with a 1 s time constant. Silver hydrosol was prepared according to the protocols published before.[4−6] The concentrations of the drugs used for Raman spectroscopy were 5 mM, and the SERS spectra were recorded with the 10^{-5}−10^{-8} M concentrations for free drugs and 10^{-4}−10^{-5} M for drug/DNA complexes.

UV/Visible and Flow Linear Spectroscopy. UV/vis spectra were recorded with a Philips PU8720 UV/vis scanning spectrophotometer.

FLD spectra of DNA and drug/DNA complexes in the region 220−450 nm were recorded with a Jobin Yvon, Mark III dichrograph equipped with a self-made achromatic $\lambda/4$ device to transform circular polarized light to a linear one. The self-made flow cell with an optical length of ca. 0.5 mm and a volume of 200 μL was used for orientation of DNA in the flow. The details of the FLD measurements were published elsewhere.[17]

The linear dichroism ΔA is defined as the difference at a given wavelength between the absorbance for light polarized parallel ($A_{||}$) and perpendicular ($A_{\perp}$) to the flow. The reduced linear dichroism is

$$LD_r = \Delta A/A = (A_{||} - A_{\perp})/A$$

where A is the isotropic absorbance of the sample.

The angle β between the transition moment of the dye chromophore and the orientation axis of the DNA molecules was calculated from the measured ratios of the reduced linear dichroism for the bases and for the drugs:

$$(\Delta A/A)_{drug}/(\Delta A/A)_{DNA} = (3\cos^2\beta - 1)/(3\cos^2\alpha - 1) \quad (1)$$

where $\alpha = 86°$ is the angle between transition moment of the bases and the orientation axis of the DNA molecule.[8]

Results

UV/Visible Spectra of FGR and ETX and Their Complexes with DNA. UV/vis spectra of FGR and ETX show two groups of bands (Figure 2): one in the region 380−420 nm and the second, which is more intense, in the region 270−350 nm. Both groups of the bands correspond to the $\pi \rightarrow \pi^*$ electronic transitions of the conjugated chromophore rings system of types L_b and L_a, respectively.[18] The L_a transition moment is directed along the Z-axis, whereas L_b lies along the Y-axis of the chromophore molecule (Figure 1). Complexation of the drugs with the DNA induces an increase of intensity of the L_b group of bands in the absorption spectra of FGR as well as ETX (Figure 2).

FLD of FGR/DNA and ETX/DNA Complexes. Figure 3 shows reduced linear dichroism as a function of [DNA]/[drug] ratios for FGR and ETX molecules. Both curves were found to possess a maximum. For FGR the highest value of the reduced linear dichroism is observed for a [DNA]/[drug] ratio of about 2.0, whereas for ETX this value corresponds to [DNA]/[drug]

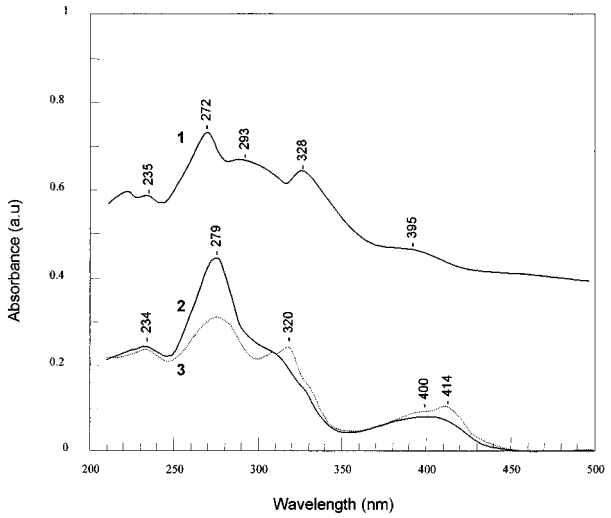

Figure 2. UV/vis spectra of FGR (1), ETX (2), and ETX/DNA complex (3). Drugs concentrations are 10 μM. DNA concentration is 100 μM (bp).

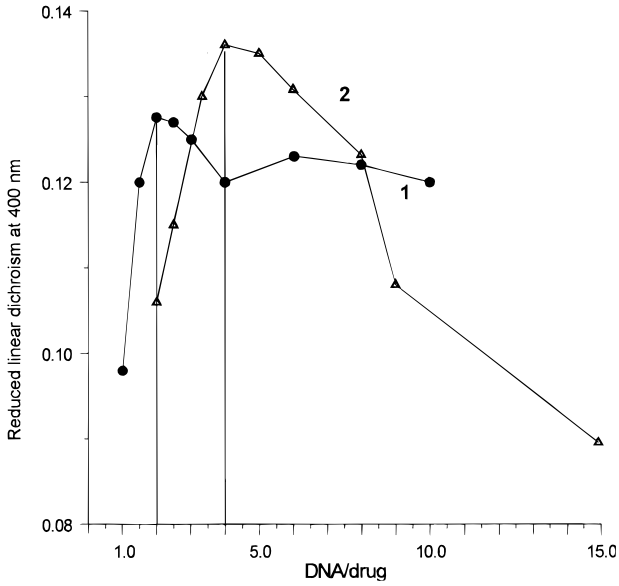

Figure 3. Dependence of the reduced linear dichroism (LD_r) for FGR (1) and ETX (2) on the [DNA]/[drug] ratio. LD_r was measured at 400 nm. DNA concentration (bp) is 10 μM.

ratio at ca. 4.0. These positions of maxima on the reduced linear dichroism curves indicate the points of DNA "saturation" with the drugs. So FGR and ETX interact with the DNA in a binding stoichiometry of 1 drug per ca. 2.0 bp of DNA and 1 drug per 4.0 bp of DNA, respectively.

The FLD technique enables us to determine the relative orientation of the plane of the drug chromophore to the plane of DNA bases; the linear dichroism of intercalators is known to be negative, whereas that of the minor groove binders is positive.[9] FLD signals from FGR and ETX bound to DNA at "saturation" ratios ([DNA]/[drug] = 2.0 and 4.0, respectively) were found to be negative in both L_a and L_b regions of electronic transitions. In other words, both compounds were found to be typical major groove intercalators with their chromophore's planes rather perpendicular to the flow.

Then eq 1 was employed to calculate the angle between the L_b electronic transition of the drug's chromophore and the axis of orientation of the DNA molecule. These angles were found to be ca. 73 $\pm$ 5° for FGR and 79 $\pm$ 5° for ETX. Owing to the strong absorption of the DNA base pairs in the region of the L_a electronic transitions of the drugs, we failed to determine the exact angle between this electronic transition moment of the drug's chromophore and the DNA orientation axis. By subtracting the FLD spectrum of DNA from the FLD spectrum of the bound drug, we obtained an approximate value of 80−85°. Anyway, the plane of the drug's chromophore was found to be nearly perpendicular to the DNA orientation axis, that is, almost parallel to the plane of the DNA bases.

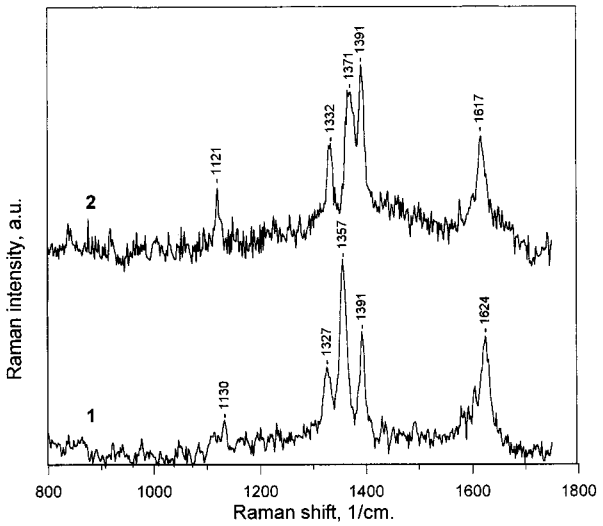

Figure 4. Raman spectra of FGR (1) and ETX (2) in H_2O. λ_{exc} is 457.9 nm for FGR and 488 nm for ETX. Laser power is 50 mW. Drugs concentrations are 5 mM.

Raman Spectra of FGR and ETX. Raman spectra of FGR and ETX are characterized as a "preresonance" recorded with excitation in the region of the drug absorption (Figure 2). The spectra of FGR and ETX recorded at pH 7.5 appeared to be quite similar (Figure 4) with the four intense bands at ca. 1620, 1391, 1360−1370, and 1330 cm^{-1} and a band at 1120−1130 cm^{-1} with moderate intensity. The position and relative intensities of the bands were found to be unchanged at pH 4.0−11.0, and no spectral effects of deuteration was observed (data not shown). So these bands represent skeletal vibrations of the conjugated ring system with probable contribution of $\delta(CH_3)$ motions.

SERS Spectra of FGR and ETX. *General Characteristics*. When the region of chromophore absorption is excited, the SERS spectra of the drugs arise because of surface enhancement itself coupled with the resonance enhancement.[2,3] As a result, an extremely high sensitivity for SERS of FGR and ETX was observed, so we managed to record spectra at very low limits (down to 10^{-9}−10^{-10} M). At the same time, the SERS spectrum of FGR was found to be about 100-fold more intense than that of ETX. There is no direct evidence that the resonance contribution to the overall enhancement must be different for each of these compounds because electronic structures of FGR and ETX are very much the same as seen from their UV/vis spectra (Figure 2). Therefore, the peculiarities in surface enhancement of the Raman signal must account for the differences observed for the SERS spectra of FGR and ETX. It is known that the "chemical" (or short- range) enhancement plays the major role in SERS on silver hydrosols.[19] In other words, only compounds with a high ability to be adsorbed by the hydrosol may exhibit strong Raman enhancement. Adsorptivity is affected by the presence of charged and/or hydrophobic (hydrophilic) groups.[3] Therefore, we propose that the difference in the chemical structures of FGR and ETX should explain the higher adsorption ability of FGR and, consequently, higher

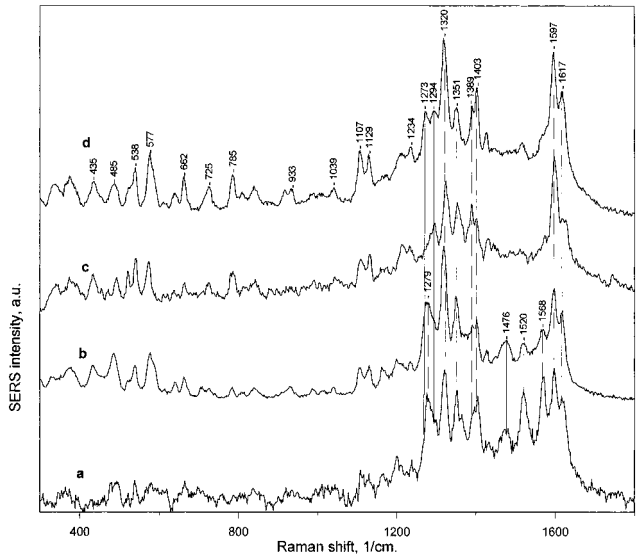

Figure 5. SERS spectra of FGR at pH 11.0 (a), 7.5 (b), 4 (c) and in a complex with DNA (d) at pH 7.5. Drug concentration is 1 μM. DNA concentration (bp) is 0.1 mM. $\lambda_{exc} = 488$ nm. Laser power is 25 mW.

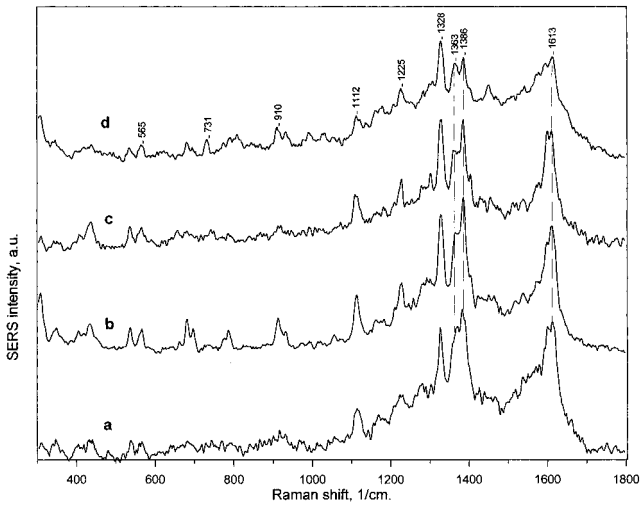

Figure 6. SERS spectra of ETX at pH 11.0 (a), 7.5 (b), 4 (c) and in a complex with DNA (d) at pH 7.5. Drug concentration is 10 μM. DNA concentration (bp) is 0.5 mM. $\lambda_{exc} = 488$ nm. Laser power is 25 mW.

Raman enhancement factor. On the other hand, if the OH group of FGR determines its surface enhancement, any changes in the environment or interactions of this group (e.g., hydrogen bonding) would influence the SERS intensity of FGR.

The SERS spectra contain four strong bands (Figures 5 and 6), which have their counterparts in the Raman spectrum (ca. 1620, 1400, 1350, and 1320 cm^{-1} for FGR and ca. 1610, 1385, 1370, and 1330 cm^{-1} for ETX). These can be attributed to skeletal vibrations of the conjugated ring system. The band at 1380−1400 cm^{-1} may be also contributed from $\delta(CH_3)$ and the band at 1320−1330 cm^{-1} from C−C−H motions.[20] In Wilson's notation of vibration modes of benzene, the bands in the region 1590−1610 cm^{-1} are assigned to ν_{8a} and ν_{8b} vibrations.[21] The relative intensities of these bands are known to be extremely sensitive to the mass of the chromophore substituent group[20] and its interactions with the ions.[6] Figures 5 and 6 show that the SERS band of FGR at ca. 1595 cm^{-1} is more intense than that at ca. 1615 cm^{-1}, whereas for ETX the band at ca. 1611 cm^{-1} is more intense than 1597 cm^{-1}. This difference correlates with the difference in the structures of FGR and ETX; the OH group of FGR is replaced by the heavier OCH_3 group in ETX (Figure 1). Moreover, an additional massive OCH_2CH_3 group is presented in the ETX molecule. Since these structural differences of FGR and ETX may be easily detected by SERS spectroscopy, we may expect that any changes in the microenvironment of the OH group of FGR or any interactions of this group with the other moieties should be elicited in the SERS spectrum as a change in the relative intensity of the bands corresponding to ν_{8a} and ν_{8b} vibrations.

In addition to the bands mentioned above, there are some features in the region 400−600 cm^{-1} of the SERS spectra of FGR and ETX that may be attributed to δ(C−C−O) and δ-(C−O−C) vibrations: the bands in the region 1100−1140 cm^{-1}, likely interpreted as due to ν(C−O−CH_3) and ν(C−C−O) motion, and the several bands in the region 1200−1600 cm^{-1} typically assigned to the normal vibration of benzene derivatives.[20,21]

Effect of pH on the SERS Spectra. To identify additional spectral indicators for the OH group microenvironment of FGR and to determine the contribution of N^+ (Figure 1) to the vibrational structure of FGR and ETX, we have analyzed the pH dependence of the SERS spectra. At basic pH, the OH group of FGR is expected to be deprotonated, whereas at acidic pH some perturbations in the microenvironment of N^+ are assumed.

First, it should be noted that the overall intensity of the SERS spectra at pH different from neutral is significantly lower than those at pH ca. 7.5. This may be explained by partial degradation of hydrosol at basic and acidic pH that leads to lower SERS enhancement factors.[3] For the ETX molecule (which does not possess the OH group; see Figure 1) the "pure" effect of the N^+ environment on the SERS spectrum may be determined. The SERS spectrum of ETX, recorded at pH 11.0 (Figure 6a), shows slight changes compared to its spectrum at pH ca. 7.5 (Figure 6b). The only detectable difference is a relative decrease of the 1370 cm^{-1} band at neutral pH. At pH close to 4.0 a further loss of this band occurs compared to the band at ca. 1330 cm^{-1}. Moreover, some decrease of the 1380 cm^{-1} band and an increase of the band at ca. 1610 cm^{-1} are also observed. The bands sensitive to pH were attributed to skeletal vibrations of the conjugated ring system with contribution of the $\delta(CH_3)$ motion. Therefore, we may conclude that pH and, subsequently, the environment of N^+ slightly affect the vibrational structure of ETX.

FGR comprises two pH-dependent structural elements: OH group and N^+ (Figure 1). Having established pH-induced spectral changes arising from the variation of the environment of N^+ (in case of ETX), we may interpret all other changes in the SERS spectrum of FGR in terms of pH-dependent modifications of the OH group. Deprotonation of the OH group of FGR at basic pH induces significant changes in its SERS spectrum (curves a and b of Figure 5). First of all, there are two bands at 1520 and 1568 cm^{-1}, relative intensities of which are strongly increased at pH ca. 11.0. In addition, there is a redistribution of the relative intensities of the bands in the region 1250−1450 cm^{-1} compared to the spectrum at neutral pH. At acidic pH we find a total disappearance of the bands 1476, 1520, and 1568 cm^{-1}, a shift of the band at ca. 1279 cm^{-1} to 1294 cm^{-1} with a simultaneous decrease of their relative intensities and increase of the band at ca. 1600 cm^{-1} (Figure 5c). In addition to these changes, all the SERS bands were found to decrease at pH 11.0 versus pH 4.0. We suppose that the bands at 1520 and 1568 cm^{-1} should be assigned to C═C vibrations of the aromatic ring with possible contribution of the N^+═C vibration to 1568 cm^{-1}, whereas the band in the 1270−1295 cm^{-1} region most probably corresponds to the ν(C−O) vibration.[20] Although these

bands cannot be attributed directly to the vibrations of the OH group, they undoubtedly depend on the microenvironment of this group.

The following spectral indicators of the OH group microenvironment in FGR have been characterized. The first is the overall intensity of the SERS spectrum revealed from the comparison of FGR and ETX spectra. The second is the high sensitivity of relative intensities of the ν_{8a} and ν_{8b} vibrations to any interactions of the OH group. Finally, we have revealed some other pH-dependent bands sensitive to the protonation state of the OH group. We can propose therefore the following correlation between the SERS spectrum and the environment of the OH group. Deprotonated at basic pH, the OH group is characterized by strong bands at 1520 and 1568 cm^{-1} and a relatively strong band at ca. 1279 cm^{-1}. At nearly neutral pH 7.5, some fraction of the OH groups are still deprotonated. Therefore, the bands at 1520 and 1568 cm^{-1} as well as at 1279 cm^{-1} are observed but weaker than at basic pH. At acidic pH the 1520 and 1569 cm^{-1} bands completely disappear, the band at ca. 1278 cm^{-1} shifts to 1294 cm^{-1}, and, finally, a significant increase of the band corresponding to ν_{8a} is observed. It should be noted that the latter change might arise not only from interactions of the OH group but also from the $O-CH_3$ group environment, since the same effect was observed for ETX.

SERS Spectra of FGR/DNA and ETX/DNA Complexes. The SERS spectra of FGR/DNA (Figure 5d) and ETX/DNA (Figure 6d) complexes were recorded at different [drug]/[DNA] ratios (from 0.001 to 0.5). It is known[2−7] that the intensity of the SERS signal for intercalating agents (such as adriamycin, saintopin, intoplicine) decreases significantly under complexation with DNA. In drug/DNA complexes the drug is buried within the DNA duplex so that it is kept away from the surface, and thereby, the SERS sufficiently decreases.

The same effect was observed for FGR and ETX. In the complex with DNA, the SERS signal was found to be 10−20 times weaker than for the free drug. Using this fact, we have determined the DNA/drug association constant for FGR to be ca. 3×10^5 M^{-1}. This value is close to that determined previously by viscosimetric measurements.[13] Therefore, we conclude that interactions between the drug and DNA are poorly, if at all, affected by the surface and that the information obtained with SERS spectroscopy may be compared and correlated with that obtained with other techniques (UV/vis, CD, FLD). The same conclusion has been drawn before for a number of other anticancer agents.[2−7]

To deduce spectral changes induced by complexation with DNA, the spectra of free drugs were compared to those of the [DNA]/[drug] complex at a 200:1 ratio. Under these conditions all the drug molecules are ensured to participate in the complex and no contribution of the free drugs is present. Comparing the SERS spectrum of the ETX/DNA complex with that of the free drug (Figure 6), we find the following spectral differences. The bands in the region 1370−1400 cm^{-1} decrease in relation to the 1328 cm^{-1} band; the intensity of the band at 1113 cm^{-1} is relatively lower for the complex than for the free drug, and finally, some intensity alterations were observed in the region 1580−1620 cm^{-1}.

As was indicated above, the bands in the region 1370−1400 cm^{-1} correspond to skeletal vibrations of the conjugated ring with some contribution of $\delta(CH_3)$ motions. Therefore, these bands hardly provide particular information concerning interactions of individual molecular groups of ETX. Any change in the state of these groups may lead to an alteration of the intensity of skeletal vibrations. Nevertheless, this spectral range was found to be sensitive to the modification of the N^+ environment. Thus, we have indications that N^+ is involved in the interaction with DNA. Moreover, the band at 1113 cm^{-1} (assigned to the $\nu(C-O-CH_3)$ vibration) clearly indicates that the exterior group $O-CH_3$ is involved in the interaction with DNA. Finally, as was shown above, bands in the 1580−1620 cm^{-1} region may also serve as indicators of interaction of the $O-CH_3$ group.

The spectral changes observed for SERS of FGR/DNA complexes (Figure 5) are more significant than those for ETX. The differences include a decrease or even disappearance of the bands at 1476, 1520, and 1570 cm^{-1}, several modifications of band intensities in the 1330−1420 and 1590−1610 cm^{-1} regions, a relative increase of the bands at 1100−1130 cm^{-1}, and finally, a splitting of the 1278 cm^{-1} band into two bands at 1292 and 1272 cm^{-1}. These changes are very similar to those observed for SERS spectra of the free drug at acidic and neutral pH and may be attributed to the changes of the environment of N^+ and interactions of OH and $O-CH_3$ groups. Therefore, we suppose that the effect of DNA on the structure of the drugs occurs through interactions between the OH, $O-CH_3$, and N^+ groups of the drug with the DNA.

The band at 1278 cm^{-1} corresponds to the $\nu(C-O)$ vibration and was shown to be sensitive to the formation of the H bond with participation of OH groups of various chromophores.[7,22] The presence of the two peaks at the expected position of this band in the spectrum of the drug/DNA complex may be explained as follows. As we have established, a significant number of OH groups at neutral pH still exist in deprotonated form. Therefore, we may observe the same position of this band at neutral as at basic pH. Upon complexation with DNA some bond(s) between DNA and the OH group appeared. These bonds are most likely H bonds, but we are not able to distinguish if the oxygen serves as a donor or as an acceptor of proton. It is reasonable to suppose that the oxygen in the deprotonated OH group is an acceptor of a proton, while the oxygen of the protonated OH group may be either an acceptor or a donor. Therefore, we propose that in the SERS spectrum of the drug/DNA complex the bands at 1272 and 1292 cm^{-1} correspond to vibrations of the OH group participating in H-bonding as an acceptor and/or a donor of proton.

Discussion

There are two principal "extreme" modes of anticancer drug interactions with DNA: minor groove binding and intercalation. The only exception is specific DNA topoisomerase I inhibitor camptothecin, exhibiting the extremely low affinity to DNA or topoisomerase I alone but exhibiting its anticancer activity by stabilizing the ternary cleavage complex between DNA and topoisomerase I enzyme and thus preventing the substrate's religation.[23,24]

These two "extreme" modes of DNA binding by drugs were found to be distinguishable using linear dichroism spectroscopy.[9] The intercalators exhibit a negative signal in the LD spectrum, whereas the minor groove binders give rise to a positive signal. The most usual "mixed mode" of DNA interaction of agents induces normally the superposition of the features characteristic for the two "extreme" modes.

In the present study we have demonstrated that FGR and ETX induce negative LD signals. Moreover, precise calculations of the angle between the transition moment of the drug and the DNA orientation axis show that the planes of the drug's chromophores are nearly parallel to the plane of the nucleotide bases. Therefore, we may conclude that both compounds, FGR and ETX, are intercalators. The conclusion concerning FGR

supports the result obtained before by measurements of the DNA length increase induced by FGR/DNA interactions. [13]

In addition to traditional methods providing information on DNA/drug interactions, SERS spectroscopy allows the selective detection and study of molecular interactions of individual molecular groups of drugs. To date, this method was applied to the analysis of a number of anticancer drugs and their interactions with DNA.[2−7] In the case of FGR and ETX, SERS appeared to be an extremely sensitive and selective technique, owing to tremendous enhancement of Raman scattering from these molecules. The SERS spectroscopy reveals pronounced differences in molecular interactions of FGR and ETX with DNA. These differences were explained in terms of the chemical nature of the substituent groups of the drugs. The OH group of FGR was found to play a key role in the drug's interactions with DNA. In addition, the N^+ and OCH_3 moieties (Figure 1) should be involved in DNA binding. Replacement of the OH group by OCH_3 and the presence of an additional OCH_2CH_3 group in an ETX molecule should lead to redistribution of electric charge in the chromophore conjugated system. As a result, the orientation of the total dipole moment of the molecule should be different for FGR and ETX. Therefore, one may suggest that, upon intercalation, orientation of the ETX molecule with respect to DNA bases is quite different from that of FGR.

In the previous study of FGR and its complexes with DNA by UV/vis spectroscopy it was proposed that the quaternary cation (N^+) may serve as an anchor that binds the negatively charged phosphate group of the backbone of double-stranded nucleic acids.[15] Hereupon initial interaction a more hydrophobic region of the molecule could intercalate between the stacked base pairs.

In the studies of protoberberine analogues whose chemical structures are rather similar to those of FGR and ETX, the so-called "mixed-mode" DNA binding model was presented.[25] This model suggests that a portion of the ligand intercalates into the double helix, while the nonintercalated portion of the molecule protrudes into the minor groove of the host duplex, where it becomes available for interactions with the atoms lining the floor and/or walls of the minor groove.

This "mixed-mode" DNA-binding model seems very suitable for FGR and ETX molecules. Indeed, the quaternary cation may serve as an anchor that binds the negatively charged phosphate backbone of the double-stranded nucleic acids, and the molecule could intercalate between the stacked base pairs. However, a significant part of the molecule enters the DNA minor groove. We suppose that FGR is oriented so that its OH group is rather directed to minor groove, whereas the OCH_2CH_3 group of ETX protrudes into the minor groove where it becomes accessible for interactions with the DNA-binding intracellular enzymes.

FGR is known to be a potent inhibitor of DNA topos I and II, stabilizing the cleavable complex between the enzyme and its DNA substrate.[13,14] ETX, a synthetic derivative of FGR, seems to be also an effective double inhibitor of topos[26] with the even higher cytotoxicity on the cellular level. This paper presents the first comparative study of the molecular interactions of these two antitumor agents with the DNA. The differences of the modes of DNA binding by FGR and ETX, especially in the terms of their molecular interactions within the DNA minor group (minor-groove-directed position of the OH group of FGR and minor-groove occupation with the spacious OCH_2CH_3 group of ETX), should obviously modulate the topos inhibitory effect of these drugs. The next stage of our work will concern the comparative analysis of molecular interactions of FGR and ETX within the ternary cleavable complexes with the DNA and topos. This work should provide us with the information enabling us to correlate the in vitro results with the cytotoxicity effects exhibited by these drugs at the cellular level.

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Glossary

SERS	surface-enhanced Raman scattering
FLD	flow linear dichroism
LD	linear dichroism
CD	circular dichroism
FGR	fagaronine
ETX	ethoxidine (12-alkoxy-benzo[*c*]phénanthridine)
topo (s)	topoisomerase (s)
PBS	potassium-buffered saline
sc	supercoiled

References and Notes

(1) Capranico, G.; Zunino, F. *Curr. Pharm. Des.* **1995**, *1*, 1.

(2) Nabiev, I. R.; Sokolov, K. V.; Manfait, M. *Biomolecular Spectroscopy*; Wiley: London, 1993; Chapter 7, pp 267−338.

(3) Nabiev, I.; Chourpa, I.; Manfait, M. *J. Raman Spectrosc.* **1994**, *25*, 13.

(4) Nabiev, I.; Chourpa, I.; Riou, J.-F.; Nguyen, C. H.; Lavelle, F.; Manfait, M. *Biochemistry* **1994**, *33*, 9013.

(5) Nabiev, I.; Chourpa, I.; Manfait, M. *J. Phys. Chem.* **1994**, *98*, 1344.

(6) Feofanov, A.; Ianoul, A.; Oleinikov, V.; Gromov, S.; Fedorova, O.; Alfimov, M.; Nabiev, I. *J. Phys. Chem. B* **1997**, *101*, 4077.

(7) Smulevich, G.; Feis, A. *J. Phys. Chem.* **1986**, *90*, 6388.

(8) Matsuoka, Y.; Nordén, B. *Biopolymers* **1982**, *21*, 2433.

(9) Bailly, C.; Henichart, J. P.; Colson, P.; Houssier, C. *J. Mol. Recognit.* **1992**, *5*, 155.

(10) Monnot, M.; Mauffret, O.; Simon, V.; Lescot, E.; Psaume, B.; Saucier, J. M.; Charra, M.; Belehradek, J.; Fermandjian, S. *J. Biol. Chem.* **1991**, *266*, 1820.

(11) Wang, J. C. *Annu. Rev. Biochem.* **1996**, *65*, 635.

(12) Fleury, F.; Ianoul, A.; Kryukov, E.; Sukhanova, A.; Kudelina, I.; Wynne-Jones, A.; Bronstein, I. B.; Maizieres, M.; Berjot, M.; Dodson, G. G.; Wilkinson, A. J.; Holden, J. A.; Feofanov, A. V.; Alix, A. J. P.; Jardillier, J. C.; Nabiev, I. *Biochemistry* **1998**, *37*, 14630.

(13) Larsen, A. K.; Grondard, L.; Couprie, J.; Desoize, B.; Comoe, L.; Jardillier, J.-C.; Riou, J.-F. *Biochem. Pharmacol.* **1993**, *46*, 1403.

(14) Wang, L. K.; Johnson, R. K.; Hecht, S. M. *Chem. Res. Toxicol.* **1993**, *6*, 813.

(15) Pezutto, J. M.; Antosiak, S. K.; Messmer, W. M.; Slaytor, M. B.; Honig, G. R. *Chem. Biol. Interact.* **1983**, *43*, 323.

(16) Messmer, W. M.; Tin-Wa, M.; Fong, H. H. S.; Bevelle, C.; Farnsworth, N. R.; Abraham, D. J.; Trojavek, J. *J. Pharm. Sci*. **1972**, *61*, 1858.

(17) Bronstein, I.; Wynne-Jones, A.; Sukhanova, A.; Fleury, F.; Ianoul, A.; Holden, J.; Alix, A. J. P.; Dodson, G.; Jardillier, J. C.; Nabiev, I.; Wilkinson, A. J. *Anticancer Res.*, in press.

(18) Klevens, J.; Platt, M. J. *Chem. Phys.* **1949**, *17*, 470.

(19) Hildebrandt, P.; Stockburger, M. *J. Phys. Chem.* **1984**, *88*, 5935.

(20) Schrader, B. *Raman/Infrared Atlas of Organic Compounds*; VCH: Weinheim, Germany, 1989.

(21) Wilson, E. B. *Phys. Rev.* **1934**, *45*, 706.

(22) Nonaka, Y.; Tsuboi, M.; Nakamoto, K. *J. Raman. Spectrosc.* **1990**, *21*, 133.

(23) Gupta, M.; Fujimori, A.; Pommier, Y. *Biochim. Biophys. Acta* **1995**, *1262*, 1.

(24) Liu, L. F., Ed. *Adv. Pharmacol.* **1994**, *29B*, 305.

(25) Pilch, D. S.; Yu, C.; Makhley, D.; La Voie, E. J.; Srinivasan, A. R.; Olson, W. K.; Sauers, R. R.; Breslauer, K. J.; Geacintov, N. E.; Liu, L. L. *Biochemistry* **1997**, *36*, 12542.

(26) Sukhanova, A.; Fleury, F.; Devy, J.; Ianoul, A.; Duval, O.; Alix, A. J. P.; Jardillier, J. C.; Nabiev, I. *Mol. Pharmacol.*, in press.