Molecular Determinants of Site-specific Inhibition of Human DNA Topoisomerase I by Fagaronine and Ethoxidine

RELATION TO DNA BINDING*

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DNA topoisomerase (top I) inhibition activity of the natural alkaloid fagaronine (NSC157995) and its new synthetic derivative ethoxidine (12-ethoxy-benz[c]phenanthidine) has been correlated with their molecular interactions and sequence specificity within the DNA complexes. Flow linear dichroism shows that ethoxidine exhibits the same inhibition of DNA relaxation as fagaronine at the 10-fold lower concentration. The patterns of DNA cleavage by top I show linear enhancement of CPT-dependent sites at the 0.016–50 μM concentrations of fagaronine, whereas ethoxidine suppress both top I-specific and CPT-dependent sites. Suppression of top I-mediated cleavage by ethoxidine is found to be specific for the sites, including strand cut between A and T. Fagaronine and ethoxidine are DNA major groove intercalators. Ethoxidine intercalates DNA in A-T sequences and its 12-ethoxy-moiety (absent in fagaronine) extends into the DNA minor groove. These findings may explain specificity of suppression by ethoxidine of the strong top I cleavage sites with the A(+1), T(–1) immediately adjacent to the strand cut. Fagaronine does not show any sequence specificity of DNA intercalation, but its highly electronegative oxygen of hydroxy group (absent in ethoxidine) is shown to be an acceptor of the hydrogen bond with the NH₂ group of G base of DNA. Ability of fagaronine to stabilize top I-mediated ternary complex is proposed to be determined by interaction of its hydroxy group with the guanine at position (+1) of the DNA cleavage site and of quaternary nitrogen interaction with top I. The model proposed provides a guidance for screening new top I-targeted drugs in terms of identification of molecular determinants responsible for their top I inhibition effects.

The benzo[c]phenanthridine alkaloid fagaronine (Fig. 1), isolated from the roots of Fagara zanthoxyloides Lam. (Rutaceae) (1), exhibits antitumor activity against P388 and L1210 murine leukemias in vivo and toward colon 26 (1, 2). It has been shown to induce differentiation in murine erythroid Friend cells, human K562 erythroleukemia and promyelocytic HL60 cells (3–5). Fagaronine was proved to be a DNA intercalator (6), it inhibits DNA and RNA polymerase activities and protein synthesis (7, 8). Fagaronine also inhibits reverse transcriptases from different sources (5, 9–10) and was proposed to act through at least two different mechanisms: inhibition of nucleic acid synthesis due to interaction with DNA and inhibition of the elongation step of protein synthesis (7). Further studies revealed that fagaronine is able to stabilize top I ternary cleavable complexes at low concentrations and to inhibit both top I and top II ternary complexes at higher concentrations (12, 13). The most potent fagaronine derivative nitidine (Fig. 1), isolated from extract of a climbing shrub Zanthoxylum niti- dum (14), was observed to trap both, top I- and II-cleavable complexes. Nearly 100 naturally occurring alkaloids in this class have been isolated from plants, and many more have been synthesized, but they are generally not markedly better than nitidine and fagaronine (15) and do not exhibit any significant activity against solid tumors. It is worth noting that the only benzo[c]phenanthridines alkaldoids found thus far to stabilize the top I-cleavable complexes are those that have previously been shown to have antitumor activity in experimental animal models (12). So, new structural analogues of benzophenanthridines, top I inhibitors with an enlarged spectrum of activity, are highly desirable.

In terms of structure-activity relationship, two main points can be emphasized: (i) all the compounds synthesized and studied so far carry the iminium charge on the benzo[c]phenanthridine ring (Fig. 1), which seems to be necessary for their biological action, and (ii) the reactivity of the iminium toward nucleophilic attack has been put forward to explain the antileukemia activity of these series (15). Recently, the iminium bond electrophilicity within the benzo[c]phenanthridines was shown to be a factor which requires consideration in ternary complex formation with reverse transcriptase (16). The other molecular determinants playing the key role in the benzo[c]phenanthridines anticancer or enzymes-inhibition activity are not known yet and need to be identified.

Recently, a new fagaronine derivative ethoxidine (Fig. 1), has been synthesized by one of us (17), and its activity against human immunodeficiency virus, type 1 reverse transcriptase

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1 The abbreviations used are: top I, DNA topoisomerase I; FLD, flow linear dichroism; SERS, surface-enhanced Raman scattering; CT, calf thymus; CPT, camptothecin; PBS, phosphate-buffered saline; LD, linear dichroism; bp, base pair(s).
Escherichia coli PBS. Prepared by mixing the drug stock solutions with the DNA solution in dissolved in PBS to 5 mg/ml stock solution. Drug-DNA complexes were diluted by buffer to desired concentration. DNA and polymers were from insect cells using a two-step procedure as described (21, 22).

Biochemicals, respectively. Stratagene. Bovine pancreatic DNase I and Klenow fragment of ethoxidine were prepared as 1 mM stock solutions in methanol and were synthesis of ethoxidine was described previously (17). Fagaronine and was supplied by National Cancer Institute (Bethesda, MD). The synthesis of the drug chromophores responsible for their DNA binding and top I poisoning or catalytic inhibition.

EXPERIMENTAL PROCEDURES

Materials—CT DNA and the double-stranded poly(dA·dT)·poly(dA·dT) and poly(dG·dC)·poly(dG·dC) polymers were purchased from Sigma. Their concentrations in the DNA base pairs were determined by using molar extinction coefficients of 13,200, 13,900, and 13,200 M⁻¹ cm⁻¹, respectively (20). CPT was purchased from Sigma and fagaronine was supplied by National Cancer Institute (Bethesda, MD). The synthesis of ethoxidine was described previously (17). Fagaronine and ethoxidine were prepared as 1 mM stock solutions in methanol and were diluted by buffer to desired concentration. DNA and polymers were dissolved in PBS to 5 mg/ml stock solution. Drug-DNA complexes were prepared by mixing the drug stock solutions with the DNA solution in PBS.

Plasmid pGEM7Z(+) and restriction endonucleases were purchased from Promega and Escherichia coli strain “Sure” was purchased from Stratagene. Bovine pancreatic DNase I and Klenow fragment of E. coli DNA polymerase I were purchased from Sigma and Roche Molecular Biochemicals, respectively.

Recombinant 68-kDa human DNA top I was purified to homogeneity from insect cells using a two-step procedure as described (21, 22). Specific activity of top I used in our assays was found to be 1.8 × 10⁶ units/mg, where one unit of activity is an amount of enzyme yielding 100% of relaxation of 300 ng of supercoiled pGEM7Z(+) plasmid DNA in 30 min at 37 °C.

DNA Plasmid Constructs—Preparation of the top I DNA substrates in the form of plasmid constructs containing top I-specific and CPT-dependent cleavage sites was described (23). The constructs were purified from the cells and analyzed by DNA sequencing method of Sanger (24).

For 5′-end labeling, plasmid DNA constructs were cleaved with HindIII and Apal and labeled with [α-³²P]dATP in the presence of the Klenow fragment of DNA polymerase I according to (24). The 3′-labeled DNA fragments were purified by electrophoresis on a nondenaturing 5% (w/v) polyacrylamide gel and isolated by electrophoresion followed by ethanol precipitation.

Topoisomerase Cleavage Assays—Cleavage was carried out by incubating 50 units of top I with a 5 μl of the solution of the radiolabeled DNA fragment (3,000–10,000 cpm) in 10 mM Tris-HCl (pH 7.8), 5% glycerol, 0.5 mM EDTA, 0.3 mM 2-mercaptoethanol (final volume 20 μl). For the analysis of DNA cleavage by top I in the presence of the drugs, reaction mixtures were incubated at 25 °C for 20 min, then SDS and proteinase K were adjusted to 0.5% (w/v) and 1 mg/ml, respectively. After incubation for a further 45 min at 37 °C, DNA was purified by phenol extraction, precipitated with ethanol, washed with 70% ethanol, and dried.

Gel Electrophoresis—The samples of DNA were dissolved in 1.5 μl of the formamide-dye mixture (90% formamide containing 15 mM EDTA (pH 8)), heated 1 min at 90 °C, and applied to 8% denaturing polyacrylamide gel. Electrophoresis was proceeded for 65 min at 65 watts (2,500 V). The gels were fixed with 10% acetic acid and dried on glass pre-treated with Bind-silane (Amersham Pharmacia Biotech). Cleavage products were identified by comparison with “A + G” Maxam-Gilbert sequencing ladder.

UV-visible and Circular Dichroism Spectroscopy—UV-visible spectra were recorded with a JASCO V-530 UV-visible scanning spectrophotometer. CD spectra were recorded in the region 200–500 nm with a Jobin Yvon Mark III dichrograph. CD and UV-visible measurements were performed using quartz cells of 1 and 0.5 cm, respectively.

Flow Linear Dichroism Spectroscopy—FLD spectra were recorded from the region 220–450 nm with a Jobin Yvon Mark III dichrograph equipped with a self-made achromatic λ/4 device. The self-made flow cell described in Ref. 21, with optical length 0.5 mm and volume 200 μl was used for orientation of DNA in the flow. Inhibition of top I-induced DNA relaxation reaction was monitored using a measurement of the FLD signal at 260 nm. 3 μg of pGEM7Z(+) plasmid in 200 μl of a reaction buffer (10 mM Tris-HCl (pH 7.8), 100 mM NaCl, 0.05 mg/ml bovine serum albumin, 0.5 mM EDTA, 5% glycerol) was placed into the flow cell. Then about 3 units of top I were added directly into the cell. Time kinetics was observed as an increase of the FLD signal.

Inhibition of DNA relaxation was performed by addition of fagaronine or ethoxidine stock solutions into reaction mixture to obtain the final concentrations of 4.3 or 0.86 μM fagaronine and 0.45 or 0.22 μM ethoxidine.

Linear dichroism (ΔA) is the difference between the absorbance for light polarized parallel (A∥) and perpendicular (A⊥) to the flow. The reduced linear dichroism (LD) is defined by LD = ΔA∥ − ΔA⊥/Ao, where A is the isotropic absorbance of the sample.

Measurements of the linear dichroism in the region of absorption of the drug was used to determine the drugs chromosome orientation relative to the DNA axis. The angle β between the transition moment of the dye chromophore and the orientation axis of the DNA was calculated from the measured ratios of LD, for the DNA bases and for the drugs,

\[
\Delta A/\Delta A_{h0} = (3\cos^2\beta - 1)/(3\cos^2\alpha - 1)
\]

where α = 86° is the angle between transition moment of the bases and the orientation axis of the DNA molecule (25).

Surface-enhanced Raman Scattering Spectroscopy—SERS spectra were recorded with a spectrometer Coderg, model PHO, with double monochromator in the frequency range 300–1800 cm⁻¹. Ar ion laser (Coherent Radiation, model Innova 2020) operating at 475.9 nm (for fagaronine) or 488 nm (for ethoxidine) was used for spectra excitation. SERS spectra were recorded for 1 scan with a 1 s time constant. Silver hydrosol was prepared according to the protocol published before (26).

RESULTS

Modulation of Topoisomerase I-mediated DNA Cleavage by Fagaronine and Ethoxidine—The model DNA plasmids with inserted oligonucleotides corresponding to the top I cleavage sites were constructed by modification of the DNA pGEM7Z (f+) plasmid and contain the random combination of the top I recognition sites within the 100–200-bp region (23). Restricted DNA fragment of the selected model DNA plasmid 1454 with the strong top I-specific and CPT-dependent cleavage sites
Suppression of DNA cleavage sites by ethoxidine was found to be sequence-specific. To establish dependence of ethoxidine-induced suppression of DNA cleavage by top I on the local base sequence immediately adjacent to the cleavage site, we have analyzed the site-by-site intensities of DNA cleavage by top I in the presence of ethoxidine. The sites B, C, E, and H include 3'-AT-5'-sequence in position of the strand cut (Group 1), whereas the sites A, D, and G include 3'-GT-5'-sequence in position of the strand cut (Group 2). Densitometric analysis of dependence of DNA cleavage intensities on the drug concentration shows that the sites B, C, E, and, partially, H, are strongly suppressed even at 10 μM ethoxidine concentration (9.1, 15.0, 18.0, and 22.8% of initial cleavage intensity, respectively), whereas the intensities of DNA cleavage in the sites A, D, and G were found to be 33.3, 40.0, and 23.5% from the initial level of DNA cleavage, respectively. An increase of ethoxidine concentration up to 50 μM induces additional preferential suppression of DNA cleavage in the sites B, C, E, H (5.41, 5.0, 16.4, and 16.3% of initial level, respectively), whereas the cleavage in the sites A, D, and G remains significant (27.8, 37.3, and 22.3%, respectively). Obviously, ethoxidine suppresses the sites of the first group more effectively than the sites of the second group.

Inhibition of the Topoisomerase I-mediated Plasmid DNA Relaxation by Fagaronine and Ethoxidine—Addition of top I into the flow cell with the plasmid DNA induces enhanced FLD signal saturating within approximately 5 min of DNA/top I incubation (Fig. 3A). In the presence of fagaronine or ethoxidine, kinetics of the DNA relaxation was found to be much slower than in the absence of the drugs. Moreover, at the low drug concentrations 0.22 μM ethoxidine exhibited the same top I inhibitory effect as 0.86 μM fagaronine, whereas at the higher drug concentrations 0.45 μM ethoxidine exhibited the same effect as 4.3 μM fagaronine (Fig. 3A). Finally, the same level of inhibition of plasmid DNA relaxation by top I was observed at the up to 10-fold lower ethoxidine concentration compared with fagaronine. So, ethoxidine appears to be much more potent top I inhibitor than fagaronine.

Orientation of Fagaronine and Ethoxidine Chromophores within the DNA Complexes—Fig. 3B shows reduced linear dichroism as a function of [DNA]/[drug] ratios for fagaronine and ethoxidine DNA complexes. Both curves have the maxima with the highest value of the reduced linear dichroism of fagaronine at 1/2 DNA bp ratio, and of ethoxidine at 1/4 DNA bp ratio. The maxima of the reduced linear dichroism curves indicate the points of DNA “saturation” with the drugs. Thus, fagaronine and ethoxidine interact with the DNA in binding stoichiometry of 1 drug molecule per ~2.0 bp of DNA and 1 drug molecule per 4.0 bp of DNA, respectively.

The FLD technique enables 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 the minor groove binders induce the positive signal (28). FLD signals from fagaronine and ethoxidine bound to DNA at the saturation ratios were found to be negative in the regions of all electronic transitions (Fig. 4B). The angles between the short axis electronic transition of the fagaronine and ethoxidine chromophores and the axis of the DNA molecule calculated with Equation 1 are −73° and 79°, respectively. So,
the plane of the drug chromophores appears nearly normal to the DNA orientation axis, that is almost parallel to the plane of the DNA bases.

**UV-visible Spectra of Fagaronine and Ethoxidine**—UV-visible spectra of fagaronine and ethoxidine show two groups of bands corresponding to $\pi \rightarrow \pi^*$ electronic transition (Figs. 4 and 5): $1L_a$ (380–420 nm) and $1L_b$ (270–350 nm). The $L_a$ electronic transition is directed along the long ($z$ axis) of chromophore, whereas $L_b$ lies along its $y$ axis (29).

Deprotonation of fagaronine OH group results in disappearance of bands corresponding to $1L_b$ electronic transition of the chromophore and an increase with a bathochromic shift of the bands of the $1L_a$ transition (Fig. 4A). The pH dependence of the fagaronine’s UV-visible spectrum is determined by its OH group with $pK = 8.0$ (Fig. 4, inset). Therefore, at the physiological pH the solution contains both protonated and deprotonated forms of fagaronine.

UV-visible spectra of ethoxidine in Me$_2$SO, ethanol, methanol, and PBS are found to be practically identical (spectra not shown). On the other hand, the profile and relative intensities of the fagaronine spectra are modified upon Me$_2$SO-ethanol-methanol-PBS transitions (Fig. 4C). The molecules of polar solvents are presumed to form the hydrogen bonds with the oxygen of OH group of fagaronine, and this effect leads to a decrease of the influence of the strong negative charge of the oxygen on the conjugated chromophore system. So, the distribution of electronic density in fagaronine chromophore in polar solutions becomes more similar to this in ethoxidine, and the UV-visible spectra of fagaronine and ethoxidine in PBS are found to be closer than their spectra in Me$_2$SO. The trace 6 in Fig. 5A shows the differential spectra of fagaronine in PBS minus Me$_2$SO. The profile of this difference spectrum may be used as a reference for the effects induced in the case of formation of hydrogen bond between the oxygen of fagaronine’s OH group and a less electronegative moiety.

**UV-visible Spectra of Drugs in the Complexes with DNA and with Alternating Double-stranded Polynucleotides**—Addition of CT DNA to fagaronine or ethoxidine solution results in a hypsochromic shift in their absorption spectra and an increase in the band at $\sim400$ nm accompanied by relative changes in the bands in the 270–340 nm region (Fig. 5). Characteristic difference spectra of fagaronine and ethoxidine within the DNA complexes are shown in Fig. 5, trace 3. These pronounced spectral modifications induced by DNA binding were used to evaluate the binding constants. For that purpose, DNA was titrated by the drugs (not all the spectral curves are presented in the Fig. 5 for clarity), and the drug/DNA binding constants were determined (Table I).
Molecular Determinants of Fagaronine and Ethoxidine Activity

Fig. 5. UV-visible spectra of fagaronine (A) and ethoxidine (B) PBS solutions and their DNA complexes. Traces 1, fagaronine and ethoxidine solutions at the concentration $5 \times 10^{-5}$ M and $7.7 \times 10^{-4}$ M, respectively. Traces 2, fagaronine and ethoxidine DNA complexes. DNA concentrations were $7 \times 10^{-5}$ M for fagaronine and $4 \times 10^{-5}$ M for ethoxidine. Traces 3–5, difference spectra of drugs in complex with CT DNA, poly(dA-dT), and poly(dG-dC) poly(dG-dC), respectively. Drug/DNA$_{hp}$ ratios ($r$) were 0.3. Trace 6, difference spectrum of fagaronine solutions in PBS (pH 7.4) and in Me$_2$SO.

Table I

| DNA/ligand                        | Fagaronine | Ethoxidine |
|-----------------------------------|------------|------------|
| Calf thymus DNA                   | $10^6$     | $2.5 \times 10^5$ |
| Poly(dG-dC)poly(dG-dC)            | $1.1 \times 10^6$ | $10^5$ |
| Poly(dA-dT)poly(dA-dT)            | $10^6$     | $2.5 \times 10^6$ |

its binding constants, fagaronine does not show base preference of intercalation (Table I). The binding constants on the level of $10^6$ M$^{-1}$ were found for its interaction with all used sequences as well as with CT DNA. Contrary, ethoxidine shows strong preference of intercalation within the AT sequences. Specificity of ethoxidine binding with the poly(dG-dC)poly(dG-dC) and with CT DNA are very similar, and they are drastically different from those of fagaronine-poly(dA-dT)poly(dA-dT) complexes (compare trace 4 with the traces 3 and 5, Fig. 5A). Otherwise, we did not observe any differences in the spectral profiles of difference spectra of ethoxidine complexed with CT DNA and with a number of alternating polynucleotides (Fig. 5B, traces 3–5).

Trace 6 of Fig. 5A shows the difference spectrum of fagaronine solutions in PBS and in Me$_2$SO. At the same time, the spectra of ethoxidine in Me$_2$SO and PBS solutions were found to be identical (no difference spectrum). Moreover, the profile of the fagaronine difference (PBS minus Me$_2$SO) spectrum in the 1La spectral region corresponds exactly to effect of fagaronine binding with poly(dG-dC)poly(dG-dC) and with CT DNA (compare trace 6 with traces 3 and 5 in Fig. 5A). So, participation of the oxygen of the OH group of fagaronine in the hydrogen bond with the molecules of the polar solvent induces the same spectral effect as its molecular interactions upon DNA and poly(dG-dC)poly(dG-dC) binding. It is reasonable to suggest that the oxygen of the fagaronine OH group is an acceptor of proton coming from the guanine NH$_2$ group (see “Discussion”).

**Induced Circular Dichroism Spectra of Drug-DNA Complexes**—Since the fagaronine and ethoxidine are the planar and achiral chromophores, only those molecules complexed to the asymmetric DNA matrices are able to display induced CD (Fig. 6). These induced CD signals, which are indicative of interactions between the drugs and host DNA duplex, can be used to detect and to monitor any CD-active DNA binding mode(s). The CD spectra of fagaronine and ethoxidine contain all bands corresponding to 1La (400–420 nm) and 1L$_d$ (270–340 nm) electronic transitions. As is known, the 1L$_a$ (oriented along chromophore’s long axis) and 1L$_b$ electronic transitions lie in the plane of the chromophore’s aromatic system and are normal to each other (29, 30). The fact that all the bands of the both electronic transitions become optically active indicate that both electric dipole moments of the transition participate in DNA interaction which may be possible only in the case of drug-DNA intercalation.

Titration of the DNA with the varied amounts of ligands may give a hint about the character of ligand-DNA interactions, the number of the binding centers, and relative orientation of the plane of the DNA bases with respect to the drug’s chromophore. An increase of the fagaronine and ethoxidine content does not induce modification of the profile of their CD spectra clearly indicating the only one binding center for each molecule (Fig. 6).

Both ethoxidine and fagaronine, when complexed with the DNA, reveal similar induced CD effects: an increase of the 1La group of bands with a concomitant decrease of the bands corresponding to the 1L$_a$ electronic transition (Fig. 6). Nevertheless, one important difference is obvious: CD signal for the all 1L$_a$ bands of ethoxidine is positive, whereas the 304 nm band of 1L$_a$ electronic transition of fagaronine is negative. This fact implies the difference in geometry of fagaronine and ethoxidine DNA complexes.

Comparison of the symmetry groups of numerous intercalators as well as the results of molecular modeling (16, 29–31) show that the fagaronine long axis is located between the 2-OH- and 8-methoxy group with some shift in the side of OH-substituent (Fig. 1). This is the direction of 1L$_a$ electronic transition. 1L$_a$ electronic is perpendicular to 1L$_b$ and is directed along the short axis of the chromophore. Orientation of the ethoxidine’s long axis should be different due to equivalence of its 2- and 3-methoxy-substituents, which are less electronegative than the 2-hydroxy group of fagaronine. The theory of nondegenerate and degenerate coupled oscillator CD and its practical applications for intercalators (31) shows that the sign of cosine of angle between the orientation of the chromophore long axis and direction of the base pair electronic transition is determined by polarization of electronic transition of interca-
The UV-visible data of fagaronine clearly indicates typical DNA intercalation with the parallel orientation of the chromophore relative to the plane of the DNA bases. The band at 304 nm of its 1L\textsubscript{a} electronic transition is negative, as expected for the classical DNA major groove intercalator (Fig. 6). The SERS spectra of fagaronine-DNA complexes are similar to those for UV-visible analysis described above. The SERS spectral changes observed for fagaronine-DNA complexes with the poly(dA-dT)\text{poly}(dA-dT) or with the poly(dG-dC)\text{poly}(dG-dC) polymers were found to be completely different (data not shown). The spectral profiles of induced CD spectra imply the difference of molecular interactions of fagaronine’s chromophore within these complexes. Moreover, the spectral profile of fagaronine complex with the poly(dG-dC)\text{poly}(dG-dC) appears very similar as in case of CT DNA, whereas its complexation with the poly(dA-dT)\text{poly}(dA-dT) induces completely different spectral features. On the other hand, the profiles of CD spectra induced by ethoxidine were measured to be the same for its complexes with the DNA, poly(dA-dT)\text{poly}(dA-dT) or poly(dG-dC)\text{poly}(dG-dC). These effects were found to be very similar to those for UV-visible analysis described above.

**SERS Spectra of Fagaronine and Ethoxidine DNA Complexes**—The SERS spectra of fagaronine and ethoxidine in solution have been analyzed by us, the assignments of the spectral bands have been made, and the bands sensitive to environment of quaternary nitrogen, O-CH\textsubscript{3}, or OH group of fagaronine have been identified (18).

Fig. 7 demonstrates the most informative regions of fagaronine and ethoxidine SERS spectra. The spectra of free drugs were compared with those of their DNA complexes at a 1/200 bp ratio, where all the drug molecules are ensured to be DNA-bound, and no contribution of the free drugs is present.

As was shown in Ref. 18, the bands in the region 1360–1400 cm\textsuperscript{−1} are sensitive to modification of the N\textsuperscript{−} environment. So, the spectral modifications of ethoxidine spectrum within this region upon DNA binding indicate possible involvement of its quaternary nitrogen in the drug-DNA interaction. Moreover, the band at 1113 cm\textsuperscript{−1} (assigned to the (C-O) vibration) indicates that the exterior O-CH\textsubscript{3} moiety is involved in interaction with the DNA.

The SERS spectral changes observed for fagaronine-DNA complexes (Fig. 7) are more significant than in case of ethoxidine. If modifications of the quaternary nitrogen are less pronounced than that in ethoxidine, the bands attributed to vibrations involving OH group motions will be strongly modified. So, the band at 1273 cm\textsuperscript{−1} corresponds to υ(C-O) vibration, and it was shown to be sensitive to the formation of hydrogen bond with participation of OH groups of various chromophores (18).

Addition of DNA induces splitting of this band in two peaks...
DNA construct (involving G(1) and T(2) bases immediately). Fagaronine showed the typical CPT pattern of modulation of tributed top I-specific and CPT-dependent cleavage sites (Fig. the model DNA substrate constructs, containing randomly dis-tribution of function in DNA relaxation activity as fagaronine (Fig. 3A). Our preliminary data show also 3–8-fold lower IC₅₀ values of ethoxidine in the human K562 and A549 cancer cell lines, compared with fagaronine (19). In this work we applied a variety of biochem-ical and biological techniques to the comparative study of mechanisms of effect on the top I specific activity, top I sup-inpressors and top I poisons (7). Both groups of drugs inhibit plasmid DNA relaxation by top I. The suppressors mechanism of activity is determined by the possibility to prevent top I-DNA recognition and binding of the enzyme to its DNA substrate, whereas the poisons stabilize DNA-top I ternary cleavable complex.

Recently synthesized (17) new fagaronine derivative etho-xidine (Fig. 1) is found to be ~10-fold more potent inhibitor of top I DNA relaxation activity as fagaronine (Fig. 3A). Our preliminary data show also 3–8-fold lower IC₅₀ values of ethoxidine in the human K562 and A549 cancer cell lines, compared with fagaronine (19). In this work we applied a variety of biochemical and biological techniques to the comparative study of mechanisms of effect on the top I specific activity, top I suppressors and top I poisons (7). Both groups of drugs inhibit plasmid DNA relaxation by top I. The suppressors mechanism of activity is determined by the possibility to prevent top I-DNA recognition and binding of the enzyme to its DNA substrate, whereas the poisons stabilize DNA-top I ternary cleavable complex.

It is known that fagaronine belongs to the group of top I poisons (12, 13). In the top I cleavage assays, we have employed the model DNA substrate constructs, containing randomly distributed top I-specific and CPT-dependent cleavage sites (Fig. 2). Fagaronine showed the typical CPT pattern of modulation of top I-mediated DNA cleavage, all CPT-dependent sites of the DNA construct (involving G(+1) and T(−1) bases immediately adjacent the strand cut) were equivalently enhanced, and no detectable differences of cleavage intensities between the individual sites were found. The mechanism of modulation of top I-mediated DNA cleavage by ethoxidine was found to be completely different from that of fagaronine. The presence of ethoxidine at the same concentrations as fagaronine induced strong suppression of both top I-specific and CPT-dependent cleavage sites in a concentration-dependent manner (Fig. 2). Site-by-site densitometric analysis of intensities of DNA cleavage as a function of ethoxidine concentration demonstrate the sequence specificity of the effect: the sites, including A(+1) and T(−1) bases immediately adjacent to the strand cut (within the top I-specific sites), were found to be suppressed by ethoxidine much more effectively than the others. The results of these assays show that the 12-ethoxy substitution of fagaronine chromophore changes completely the mechanism of top I inhibition by the drug, from top I poisoning by fagaronine to suppression of top I-DNA recognition by ethoxidine.

Finally, the biochemical data emphasize that ethoxidine is not able to trap top I-cleavage complex, but suppresses the DNA cleavage in the sequence-specific manner. Comparative structural analysis of fagaronine and ethoxidine DNA complexes was further employed to identify molecular determinants of DNA binding and top I poisoning by the drugs.

Two related structural factors are thought to account for the expected biological effects of intercalated drugs. One is geometry of the intercalation complex, allowing an orientation of the drug propitious to interactions with proteins such as DNA topoisomerases, polymerases, or transcriptional factors. The second factor is the presumed sequence-dependent conformational perturbations in DNA induced by the drug, which may lead to disturbance of the DNA-protein recognition (32) or, inversely, to stimulate the recognition of specific topologies by such an enzymes as DNA topoisomerase I (33).

Fagaronine and ethoxidine are shown to be the DNA major groove intercalators (15). FLD analysis of drug-DNA complexes (Fig. 3B) shows that fagaronine and ethoxidine intercalate into DNA with the stoichiometries 1/2 and 1/4 DNA bp, respectively. Lower stoichiometry of ethoxidine DNA intercalation may be explained by the sterical limits due to its spacious 12-alkoxy substituent excluding close approach of the neighboring DNA intercalated chromophores. Induced CD spectra of ethoxidine DNA complexes show that its long axis penetrates within the DNA minor groove. The plane of ethoxidine chromophore is slightly rotated relative to the plane of the DNA bases due to the steric effect of its spacious 12-ethoxy substituent being disposed within the DNA minor groove.

The CT DNA binding constants for ethoxidine and fagaro-nine are very similar (Table I), whereas they reveal different sequence specificity of DNA binding. Ethoxidine shows a 25-fold higher binding constant with the poly(dA-dT)polym(dA-dT) than with poly(dG-dC)poly(dG-dC), whereas fagaronine binds both polymers with nearly the same binding constants (Table I). AT specificity of ethoxidine intercalation may be explained by the lower rigidity of AT than GC base pairs, so, its spacious 12-ethoxy substituent may penetrate more easily within d(AT) versus d(GC) DNA duplexes.

UV-visible difference spectral analysis as well as induced CD spectra of drug-DNA complexes show specific molecular inter-actions of fagaronine within the (GC) sequences, whereas no any sequence specificity of ethoxidine interactions was de-tected. UV-visible, CD, and SERS spectroscopy show participa-tion of the oxygen of fagaronine hydroxy group in hydrogen bonding with the less electronegative group (e.g. amino group of the guanine) upon DNA intercalation. SERS spectroscopy shows also involvement of ethoxidine quaternary nitrogen in the DNA interaction, whereas this effect is not so clear for fagaronine DNA complexes.

An attractive explanation for all these results is to propose the following structural model of drug-DNA complex (Fig. 8). While the ethoxidine chromophore interacts with the DNA, its spacious 12-ethoxy chain determines specificity of intercalation within the less stable AT sequences. It is more favorable for the spacious groups to unwind locally the DNA in AT sequences and to penetrate within the double helix than within the GC sequences. The long axis of ethoxidine’s chromophore protrudes within the DNA outside the double helix so that the 12-ethoxy chain is disposed within the DNA minor groove and suppresses recognition of AT sequences by top I. The studies reported here provide experimental evidence that the nature of the side chain in the 12-position is of primary importance in eliciting the observed poisoning of top I. There is no doubt that the substituent at position 12 strongly affects the catalytic activity of the enzyme.

We found it reasonable to suggest three factors determining top I poisoning activity of fagaronine: (i) specific orientation of chromophore within the DNA complex, (ii) molecular interactions of fagaronine hydroxy group with the 2-amino group of DNA G base (probably in a (+1) position adjacent to the strand cut), and (iii) the electrophilic iminium bond of benzo[c]...
phenanthridine can be a subject of a nucleophilic attack from the top I and responsible for formation of a labile covalent bond between the top I and benzo[c]phenanthridine.

The structure and geometry of the fagaronine-DNA complex may determine top I poisoning activity of the drug. Orientation of fagaronine chromophore deduced from the FLD and CD spectroscopic data presumes projection of its OH group from the surface of the minor groove in such a fashion as to promote interaction with the 2-amino group of guanine (the only hydrogen bond donor group exposed in the minor groove). The exact molecular mechanism of the fagaronine’s hydroxy group interaction with G base for the top I poisoning effect is not clear, whereas its role in this process is evident. It is well known that steric and electronic features rather than the chemical nature of different substituents may be critical in determining the positional sequence specificity of the poisons-enhanced DNA cleavage by the topoisomerases (34). The most potent among the benzo[c]phenanthridines so far synthesized and studied, the iminium and nitidine with the DNA G base may induce local cleavage by the topoisomerases (34). The most potent among these benzo[c]phenantridines proposed in Ref. 16.

We propose that exposed DNA minor groove position of fagaronine iminium bond (Fig. 8) enables the nucleophilic attack by some group of top I, formation of labile covalent bond between the enzyme and the quaternary nitrogen of the drug, and stabilization of the top I-fagaronine-DNA ternary cleavable complex. It is not clear if fagaronine is able to interact directly with the top I through its iminium bond. These experiments are in progress now.

The proposed model (Fig. 8) is based on extended biochemical and biophysical data and may be used for screening of new top I-targeted benzo[c]phenanthridine structural analogues, top I inhibitors with an enlarged spectrum of activity.

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FIG. 8. Schematic representation of the proposed ethoxidine-DNA complex (A) and fagaronine-topoisomerase I-DNA ternary complex (B). Ethoxidine chromophore intercalates preferentially within the AT sequences; its spacious 12-alkyloxy group penetrates within the DNA minor groove and blocks access of top I to the DNA top I-specific sites. Fagaronine is an acceptor of hydrogen bond with the 2-amino group of guanine. Fagaronine-topoisomerase I-DNA ternary complex is proposed to be stabilized by the labile covalent bond between the fagaronine and top I formed due to nucleophilic attack of electrophilic iminium bond of the drug by a residue within the enzyme.
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Molecular Determinants of Site-specific Inhibition of Human DNA Topoisomerase I by Fagarone and Ethoxidine: RELATION TO DNA BINDING

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