Molecular and Cellular Interactions between Intoplicine, DNA, and Topoisomerase II Studied by Surface-enhanced Raman Scattering Spectroscopy

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ABSTRACT

The surface-enhanced Raman scattering spectra of the new antitumoral agent, intoplicine (RP 60475, NSC 645008), and those of its complexes with DNA and topoisomerase II in vitro and in K562 cancer cells were obtained. Intoplicine was found to unwind DNA and to inhibit purified calf thymus topoisomerase II via a stabilization of the ternary cleavable complex. The intensity of the surface-enhanced Raman scattering spectrum of intoplicine was not modified by the addition of plasmid pBR322 or calf thymus DNA. In the complex of this antitumoral agent with topoisomerase II, the signal of intoplicine was completely abolished, indicating that at least some portion of intoplicine binds to an internal part of the enzyme. During the formation of the ternary complex, intoplicine was released from the interior of the protein and formed hydrogen bonds via its hydroxyl and/or amino groups.

INTRODUCTION

The molecules adsorbed on metal surfaces turned out to have an unusually large Raman cross-section. This phenomenon is called SERS. The magnitude of Raman scattering cross-section enhancement depends on the chemical nature of the adsorbed molecules, the type of metal surface, and its structure. Chromophore fluorescence is totally quenched and the normal Raman scattering is magnified by several orders of magnitude, yielding structural information not always obtainable with other methods. The greatest enhancement occurs on silver, gold, and copper; the metal surface must be rough and have specific adsorption properties (for review, see Ref. 1).

Recently, the SERS technique has been effectively used to study free anticancer drugs belonging to the anthracycline series, as well as the complexes they form with DNA or with their pharmacological targets within living cells (2–8). The strong fluorescence of these drugs poses a problem for recording highly detailed normal resonance Raman spectra. SERS spectra of these drugs have been recorded at concentrations as low as 10⁻¹⁰ m and are completely free of the fluorescence contribution. Moreover, the structures of the complexes of these drugs with DNA inferred from SERS are consistent with those obtained by high-resolution X-ray measurements and likewise indicate that the drugs intercalate with DNA (2, 6, 8). In fact, SERS spectroscopy has proved to be the most sensitive and least destructive technique for analyzing drug-target complexes both in vitro and in living cells. The unique structural and functional information that this method provides can be applied to the design of new drugs (5–7).

One of the most recent and important discoveries in the field of cancer research has been the identification of DNA topoisomerases as targets for several classes of antitumor drugs (9, 10). DNA topoisomerases are nuclear enzymes that interconvert topological isomers of DNA by breaking and resealing phosphodiester bonds (10). They are thus involved in transcription, replication, chromosome segregation, and DNA repair (10). Two types of topoisomerases, Topo I and Topo II, have been identified in mammalian cells. Their activities are most readily detected with highly transcribed genes (11, 12), and it has been suggested that these enzymes are involved in modulating the torsional waves generated by transcription (13).

A number of anticancer drugs specifically inhibit Topo II (14). By stabilizing an intermediary complex of the Topo II reaction, these drugs block the religation of breaks in the DNA strand. Stabilization of the Topo II-DNA complex is thought to interfere with replication and transcription in rapidly growing cancer cells and ultimately to lead to cell death (9, 14).

Two classes of drugs having this effect have been described: intercalating agents, such as the acridines, anthracyclines and ellipticines; and nonintercalating drugs, such as the epipodophyllotoxins (9, 14). These drugs act via the same biochemical mechanism but have different experimental and clinical activities, perhaps because they interact with DNA at different critical sites (15, 16).

The synthesis of new potential topoisomerase inhibitors is an objective actively being pursued by many groups. Intoplicine (RP 60475, NSC 645008) (Fig. 1) is a representative of a new series of topoisomerases inhibitors (17). This compound has been selected for clinical trials (17, 18) because it displays potent activities in various cellular and animal models, and it inhibits topoisomerases I and II (19–21).

The aim of the present study was to characterize the molecular interaction of intoplicine with purified Topo II and DNA by several methodologies: measurement of DNA unwinding; detection of Topo II-associated DNA breaks; and SERS spectroscopy. In addition, we recorded selective micro-SERS spectra of intoplicine located in the nucleus and cytoplasm of treated K562 cancer cells, which are sensitive to this drug. Finally, the structural features of drug-target complexes in vitro and in living cells are compared.

MATERIALS AND METHODS

DNA, Enzymes, and Chemicals. Intoplicine, 11-(3-dimethylaminopropylamino)-3-hydroxy-8-methyl-7H-benzo[e]pyrido[4,3-b]indole dimethane sulfonate, was prepared as described previously (17).

A stock solution of intoplicine (10⁻⁷ m) was diluted with PBS just before the measurements. Calf thymus DNA (Sigma Chemical Co.) was dissolved in PBS. The concentration of DNA (phosphate) was estimated on the basis of a molar absorption coefficient of 6600 M⁻¹ cm⁻¹ at 260 nm. Drug/DNA complex was prepared by mixing the components in PBS at the ratio of 1 drug molecule/1000 base pairs of DNA, i.e., 10⁻⁶ m intoplicine·10⁻³ m DNA. pBR322 DNA, EcoRI and HindIII restriction endonucleases, Klenow polymerase, and proteinase K were purchased from Boehringer Mannheim (Meylan, France). Drug/pBR322 DNA complex was prepared by mixing the components at the ratio of 1 drug molecule/20 base pairs of DNA, i.e., 10⁻⁶ m intoplicine·2.08 x 10⁻⁸ m DNA. [α-³²P]dATP (3000 Ci/mmol) was purchased from Amersham. Calf thymus Topo I (10 units/μl) was purchased from GIBCO BRL. Chloroquine and ethidium bromide were purchased from Sigma.
Preparation of Topoisomerase II. Calf thymus DNA Topo II was purified from frozen glands (100 g) by adapting the procedures described previously (22, 23). Briefly, nuclei were isolated and lysed with 0.35 M NaCl, and nucleic acids were precipitated by the addition of 0.1% polyamine P. The supernatant was successively chromatographed through phosphocellulose, hydroxyapatite, and DNA cellulose columns. Topo II active fractions were assembled at each step. Topo II was purified by glyceral gradient separation and stored at -70°C in 50% glycerol-10 mM EDTA. The final nucleotide concentration of the solution was stopped by 5 μl of loading buffer containing 0.1% bromophenol blue, 50 mM EDTA, and 50% (w/v) sucrose.

Unwinding Assay. Supercoiled pBR322 DNA (0.5 μg) was incubated for 30 min at 37°C with Topo I (10 units), with or without intoplicine, in 20 μl of a reaction mixture containing 20 mM Tris-HCl (pH 7.5), 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 30 μg/ml bovine serum albumin. The reaction was stopped by 5 μl of loading buffer containing 0.1% bromophenol blue, 50 mM EDTA, and 50% (w/v) sucrose. The final nucleotide concentration of the reaction is 41.6 μM. Samples were electrophoresed in 0.8% agarose gels containing 1 X Tris borate EDTA buffer and 5 μM chloroquine. Electrophoresis was carried out at 2 V/cm overnight. After extensive washing out of chloroquine by distilled water, the gels were stained with ethidium bromide (10 μg/ml), washed, and photographed under UV light.

Topoisomerase II Cleavage Reaction. Two × 10⁴ dpm of 3’ end-labeled pBR322 DNA (8.3 μM) was equilibrated with or without drug in 20 μl Tris-HCl (pH 7.5), 10 mM MgCl₂, 60 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 30 μg/ml bovine serum albumin in ice. Topo II (20 units in 20 μl final reaction volume) was then added. Reactions were performed at 37°C for 10 min. They were stopped by adding SDS to a final concentration of 0.25% and proteinase K to 250 μg/ml, followed by incubation for 30 min at 50°C. Five μl of loading buffer (see above) were added to each sample. The samples were electrophoresed on 1% agarose gels in 1 X Tris borate EDTA buffer and 0.1% SDS at 2 V/cm overnight. The gel was dried between two sheets of 3 MM paper (Whatman) and autoradiographed with hyperfilms MP (Amersham) for 1 day.

Definition of Topoisomerase Unit. One Topo II unit decatenates 50% of 0.25 μg kinetoplast DNA incubated at 37°C for 30 min under the condition of the assay (23).

Topoisomerase Reaction for SERS Experiment. The assay was performed with various concentrations of calf thymus Topo II (20 to 0.1 decatenation units) in a 20-μl final reaction volume containing 0.25 μg of supercoiled pBR322 DNA, 20 μM Tris HCl (pH 7.5), 60 mM KCl, 10 mM MgCl₂, 30 μg/ml bovine serum albumin, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 1 μM intoplicine or water. The final nucleotide concentration is 20.8 μM. The reaction was assembled in ice and the reaction mixture was then incubated at 37°C for 5 min. Then sample was 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, intoplicine alone (1 μM), DNA alone, Topo II + intoplicine, and DNA + intoplicine were performed under the same conditions, except that distilled water was used to adjust the reaction volume to 20 μl.

Cell Culture and Drug Treatment. The K562 human erythroleukemia cell line was established from a patient with chronic myelogenous leukemia (24).

Cells were in the exponential growth phase at 5–8 × 10⁵ in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (Seromed) and 2 mM L-glutamine. Cell growth and viability were determined by phase contrast microscopy and by using the trypan blue test. Cells (2 × 10⁶) were incubated with 1 μM intoplicine for 1 h at 37°C, washed twice with PBS by centrifugation (200 × g at 4°C) and resuspended in 200 μl PBS.

SERS Spectroscopy. Aqueous silver hydrosol was prepared by reducing silver nitrate with trisodium citrate as described by Nabiev et al. (1), preaggregated by the addition of sodium perchlorate up to the final concentration of 0.06 M, and used for recording SERS spectra of intoplicine and its complexes as described (7). D₂O colloids were made in exactly same fashion, substituting D₂O (100%; Sigma) for water.

For the analysis of intracellular interactions of intoplicine with living K562 cancer cells, the following procedure has been used. One hundred μl of the treated cell suspension (10⁶ cells) were introduced into 400 μl of preaggregated silver hydrosol, centrifuged (10 min; 200 × g at 4°C) and incubated at room temperature for 40 min. Then the cells with hydrosol were resuspended in PBS, washed to remove hydrosol as well as hydrosol-intoplicine complexes outside the cells as described (7), and used for micro-SERS measurements. The viability of treated cells incubated with silver hydrosol before and after micro-SERS measurements was controlled by microscopy with 0.1% trypan blue. The percentage of surviving cells was always higher than 95%. Images of the cells incubated with a hydrosol were recorded on an image analyzer (Samba 2005; Alcatel TTTN, France) as described (7) and revealed an endocytotic penetration of the hydrosol micelles inside the cells.

Drop of suspension of cells treated by drug and by hydrosol was deposited on the glass slide under the Raman microscope. SERS spectra were measured by a computer-controlled Dilor Omars-99 confocal Raman spectrometer connected to an Olympus BH-2 microscope equipped with a 100× water immersion objective (Leitz fluorat), using the 514.5-nm line of an argon laser (Spectra-Physics; Model 2020-03). Spatial resolution has been found to be ~1 μm. The cytoplasm and nucleus regions of the cells have been chosen from the optical image and the SERS spectra have been recorded from the regions of maximum incorporation of the hydrosol. Experimental conditions are described in the figure legends.

All spectra were reproduced at least 3 times for different cells and for different focusing points inside the same cell. The spectral differences between the nucleus and cytoplasm were reproducible from one cell to the next and over multiple experiments.

RESULTS

DNA Unwinding Properties of Intoplicine. The DNA-unwinding activity of an intercalating agent can be assessed by (a) relaxing a DNA molecule with Topo I in the presence of the agent and (b) electrophoresing the DNA through a gel containing a known unwinding agent such as chloroquine (25). In our experiments, the topoisomerase control supercoiled pBR322 DNA migrated through gels containing 5 μM chloroquine as a series of bands between the nicked and supercoiled forms, indicating various degrees of relaxation (Fig. 2).

When pBR322 DNA was submitted to the action of Topo I, relaxation occurred and the DNA appeared in the chloroquine gel in a highly positive supercoiled state (Fig. 2, Lane 2). When DNA was relaxed in the presence of low concentrations of intoplicine (0.03, 0.1, and 0.3 μM corresponding to Lanes 3, 4, and 5, respectively), no change in the distribution of the topoisomeres was observed. In the presence of 1 μM intoplicine (Lane 6), however, chloroquine induced less supercoiling than it had in the absence of this compound. When 3 μM intoplicine were added (Lane 7), the DNA remained in a relaxed state equivalent to that of the control DNA (Lane 1). As the concen-

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Fig. 1. Chemical structure of intoplicine.

Fig. 2. Unwinding activity of intoplicine. Lane 1, pBR322 DNA alone (0.5 μg); Lane 2, pBR322 DNA + Topo I; Lanes 3–5, (pBR322 DNA-Topo I) + intoplicine: 0.03; 0.1; 0.3; 1; 3; 10; and 30 μM. N, nicked; S, supercoiled; R, relaxed.
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Fig. 3. Stimulation of Topo II cleavage reaction by intoplicine. Lane 1, pBR322 DNA alone (0.1 µg); Lane 2, pBR322 + Topo II (20 units); Lanes 3-9, pBR322 + Topo II (20 units) + intoplicine, 0.01; 0.03; 0.1; 0.3; 1; 3; and 10 µM. Arrows, the major cleavage sites stimulated by intoplicine.

Stimulation of Topoisomerase II Cleavage Reaction. The effect of intoplicine on the cleavage reaction catalyzed in vitro by Topo II was examined. Purified calf thymus Topo II yielded a single Mr 165,000–170,000 band on a silver-stained SDS polyacrylamide gel. The formation of the complex between Topo II and pBR322 DNA was revealed indirectly by the appearance of double-stranded breaks in the DNA when a protein denaturing treatment (SDS and proteinase K) was applied at the end of the reaction. DNA breaks were then analyzed by agarose gel electrophoresis. A typical experiment is presented in Fig. 3. Control DNA migrated as a single band. DNA exposed to Topo II appeared as discrete fragments, indicating the formation of the cleavable complex (Lane 2). Intoplicine, added to the reaction mixture at concentrations between 0.01 and 1 µM, stimulated the cleavage reaction dose dependently (Lanes 3–7). Maximal stimulation was obtained with 1 µM intoplicine (Lane 7).

The enhancement of the cleavage reaction by intoplicine was site specific; cleavage at two sites was markedly stimulated (Fig. 3, arrows). Further increases in the intoplicine concentration progressively decreased the cleavage products, except those derived from cleavage at these two predominant sites (Lanes 8–9, arrows). Thus, intoplicine inhibited the Topo II reaction, apparently by stabilizing the Topo II-DNA complex. Under the conditions of our experiments, this inhibition was maximal at a concentration of intoplicine of 1 µM. Similar results were obtained when P388 murine Topo II was used in the reaction or when unlabeled pBR322 DNA, at concentrations up to 41 nM, was added to the reaction mixture.

In Vitro SERS Data. We compared the conventional Raman spectrum of intoplicine obtained with a 10⁻² M water solution with its SERS spectrum obtained at the 10⁻⁷ M concentration of the drug in the aqueous hydrosol and in the D₂O colloid (Fig. 4). The SERS spectrum of intoplicine is well resolved and does not appear to be greatly changed either in band frequencies or relative intensities with respect to the normal Raman spectrum, apart from the band ~1625 cm⁻¹, which had slightly increased in intensity, and the bands ~1428 cm⁻¹ and 1061 cm⁻¹, which had decreased in intensity. Identifying all the bands for this type of chromophore (Fig. 1) is quite difficult and could be made on the basis of normal mode calculations, but some tentative assignments could be made on the basis of the chromophore structure which includes coupled indole, phenyl, and phenol groups and an analysis of the spectra recorded in deuterium oxide (see "Discussion").

Fig. 5 shows the SERS spectra of intoplicine (10⁻⁶ M) and its complex with calf thymus DNA (10⁻³ M), at a ratio of 1 drug molecule/1000 base pairs of DNA and corresponding to 99% drug bound to DNA, recorded at the same spectroscopic experimental conditions. The absolute intensities of the spectra were completely identical and there were only slight changes in the relative intensities of a few of the bands (1359, 1409 cm⁻¹). Moreover, no changes in the relative intensities were observed when these SERS spectra were compared to those of intoplicine and its complex with pBR322 plasmid DNA, at a ratio of 1 drug molecule/20 base pairs of DNA, and corresponding to 47.6% drug bound to DNA (Fig. 5).

The formation of a complex between intoplicine and Topo II dramatically decreased the absolute intensity of drug signal (Fig. 6). The signals from topoisomerase and/or some low-molecular-weight nucleotide-related compounds bound to the enzyme (~729 cm⁻¹ and 2588 cm⁻¹) were either not observed or much decreased. The SERS spectrum of the intoplicine-DNA complex was much lower in intensity relative to that of the drug alone. The SERS spectrum of the intoplicine-DNA complex was much lower in intensity relative to that of the drug alone. The SERS spectrum of the intoplicine-DNA complex was much lower in intensity relative to that of the drug alone. The SERS spectrum of the intoplicine-DNA complex was much lower in intensity relative to that of the drug alone. The SERS spectrum of the intoplicine-DNA complex was much lower in intensity relative to that of the drug alone.
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643 cm\(^{-1}\)) are the only contribution to the SERS spectrum of the intopticine-Tope II complex. In a control experiment in which intopticine and purified bovine serum albumin were used, the intopticine SERS spectrum was not modified (Fig. 6). Another control experiment has been done with the mixture of Topo II and adenine. The SERS spectra of adenine have been found to be of nearly the same intensity both for pure compound and for its mixture with Topo II (data not shown).

To clarify if Topo II can block the surface of silver hydrosol, the mixture of Topo II and dimethylcrocetine was introduced in the hydrosol. The SERS spectra showed superposition of bands corresponding both to the drug and Topo II, suggesting that Topo II does not block the surface of hydrosol.

When pBR322 DNA was added to the intopticine-Tope II complex, the SERS spectrum of the drug reappeared with some changes (Fig. 7) in the 1350–1450 cm\(^{-1}\) region. This difference consisted of a decrease of the relative intensity of the 1363 cm\(^{-1}\) band when the drug was in its bound form and could be attributed to the effect of intopticine binding in the ternary complex.

**Micro-SERS in K562 Cancer Cells.** Micro-SERS analysis was performed on a single living cells. Our results show spectral differences for intopticine between the nucleus and the cytoplasm (Fig. 8, compare curves 2 and 3). These spectral differences probably correspond to that previously observed in vitro in the relative intensities of the 1360–1416 cm\(^{-1}\) band for the drug in free form and in the ternary complex (compare curves 1 and 4 in Fig. 8). This result indicates that in the cytoplasm, intopticine was in free form, while in the nucleus, the drug was complexed with DNA and/or protein.

**DISCUSSION**

In the present study we have shown that intopticine interacts with purified Topo II and with DNA. However, its precise mechanism of action appears to be quite different from that of other known Topo II inhibitors that intercalate with DNA, such as ellipticinium, anthraccline, or 9-aminoacridine derivatives.

The DNA-unwinding assay demonstrated that intopticine, in the presence of chloroquine and excess Topo I, binds DNA and induces torsional constraints in a circular DNA double helix. An unwinding angle of 9.5° ± 2 was found for intopticine. This kind of interaction is well documented for mono-intercalators, such as ellipticinium derivatives, ethidium bromide, anthracclines, and acridine derivatives (25, 27), as well as for bis-intercalators (25). However, nonintercalating drugs, such as distamycin (28), irehdiamine A (29), chloroquine (30), and the quinolones (31), also unwind DNA.

SERS spectra (Fig. 4) were recorded with intopticine at a concentration 5 orders of magnitude lower (10\(^{-7}\) M) than that used to obtain conventional Raman spectra (10\(^{-4}\) M). The bands characteristic of this compound could still be detected by SERS when the concentration was as low as 10\(^{-9}\) M, but at this concentration the contribution from impurities becomes important.

The bands that appear in the conventional Raman spectrum of the whole molecule have not all been attributed. However, some tentative assignments can be made on the basis of the chromophore structure, which is known to consist of a superposition of coupled indole, phenyl, and phenol rings. Thus, the band at ~1360 cm\(^{-1}\) is typical for an NH-bending vibration coupled with a stretching vibration of the indole ring, and the band at ~1409 cm\(^{-1}\) is characteristic of an OH-deformation coupled with a stretching vibration of a phenyl ring (8). Tentative assignments have been confirmed by deuterium-substitution effect for these bands being presented in Fig. 4. Therefore, the slight differences between the conventional Raman and SERS spectra of intopticine, revealed as differences in intensities of the 1360 and 1409 cm\(^{-1}\) bands, can be attributed to the interaction of the drug, in the latter procedure, with the silver surface via its electron system and its hydroxyl and/or amino group.

Complex formation between intopticine and calf thymus DNA, in conditions where almost all the drug is bound to DNA, does not markedly alter the spectrum of the compound (Fig. 5). This result has a great importance for our understanding of the structure of the intopticine-DNA complex. It is not known whether the covalent or electrostatic interactions between chromophore and DNA necessarily perturb the SERS spectra. It may depend on the type of this interaction. One can suppose that the absolute intensity of the SERS spectra would expect to decrease, if the chromophore penetrates partially inside the double helix (8). Such an effect is caused by the short-range mechanism of Raman cross-section enhancement in the silver hydrosol. An alteration of SERS spectra has already been described by J. M. Sequaris et al. (32) for the interaction of the Pt-coordinated complexes with DNA. A dramatic decrease of the absolute intensities of the SERS spectra for intercalators in the anthraccline series in the presence of DNA has also recently described (2, 4, 6, 7). The SERS spectra of ethidium bromide, a typical DNA intercalator, was found to be completely abolished in the presence of DNA, in the same experimental conditions, indicating that the entire molecule penetrated inside the double helix. The SERS spectra obtained with etoposide, a Topo II inhibitor which binds DNA but does not unwind DNA (33), are altered when this compound interacts with DNA, but their absolute intensities are not.\(^5\) Therefore these data suggest that intopticine binding to DNA is different from that of ethidium bromide or etoposide.

\(^5\) H. Morjani, unpublished results.
The intensities of the spectra are only slightly modified if intoplicine is complexed with DNA (see Fig. 5). The absolute intensities of the SERS spectra for free drug and for its complex with DNA were identical, suggesting that the chromophoric part of the molecule is not localized inside the DNA double-stranded helix. However, our observations, particularly those of the changes in relative intensities in the 1359–1409 cm⁻¹ and 1580–1625 cm⁻¹ regions of the spectrum, do not exclude that one part of the intoplicine molecule could fit inside the DNA helix. Furthermore, these drug/DNA interactions may be sufficient to confer the DNA unwinding properties, even though a large part of the intoplicine molecule is outside of the double helix and thus remains accessible to protein interactions. Exact localization of the intoplicine structure in the DNA double helix by direct nuclear magnetic resonance analysis should be of interest to validate this hypothesis.

Intoplicine inhibits the activity of purified calf thymus Topo II in vitro and stabilizes the complex formed between this enzyme and DNA (cleavable complex or ternary complex). SERS analysis showed that the drug binds to an internal region of the enzyme, since only a slight signal of intoplicine was detected. This is not what occurs in the presence of bovine serum albumin (Fig. 6), showing that the decreasing of the drug signal is not the result of simple intervention of protein interactions.
series decreases the antitumoral activity (17) and abolishes its Topo II inhibitory activity (18). We propose that part of the intoplicine chromophore including its -OH or -NH groups associates with the DNA. This interaction may be what causes DNA to unwind. During the formation of the ternary complex, an internal region of the enzyme may interact with the part of the drug that is external to the DNA. On the other hand, the part of the molecule that is hidden in the enzyme may interact with DNA during the cleavage reaction, so as to inhibit the religation step, most likely via removal of the 3’OH end of the DNA from the 5’tyrosyl-DNA linkage, as already has been suggested in the model of interaction between antibacterial quinolones and DNA gyrase (34).

When micro-SERS spectra of intoplicine were recorded from the nucleus or cytoplasm of K562 cells treated with this drug, the changes in relative intensities of the 1360–1416 cm⁻¹ bands corresponded to those detected for intoplicine in the ternary complex and in free form, respectively (Fig. 8). These results suggest that when cells intoplicine interacts with topoisomerase II in the nucleus whereas it remains and is in a free form in the cytoplasm.

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