Modulation in Kinetics of Lactone Ring Hydrolysis of Camptothecins upon Interaction with Topoisomerase I Cleavage Sites on DNA†

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ABSTRACT: The kinetics of hydrolysis of the α-hydroxylactone ring of anticancer agents belonging to the camptothecin (CPT) series has been followed using their fluorescence emission. Data obtained for CPT, CPT-11, and SN-38, either in their free form or in the presence of DNA and/or topoisomerase I (top1), have been compared. DNA was modeled using three types of double-strand oligonucleotides corresponding to top1 cleavage site enhanced in the presence of the drug (olg1), top1 site independent of CPT (olg2), and nonspecific synthetic oligonucleotide containing only AT and no GC base pairs (olg3). Cleavage assays indicated the absence of top1-mediated cleavage on olg3, both in the presence and in the absence of CPT. The kinetics data also showed ratio-dependent stabilization of the lactone forms of CPTs when in the presence of an excess of olg1 or olg2, but not of olg3. These observations correlate with the previously reported preferential binding of CPTs to guanines. Although lactone hydrolysis was not perturbed by top1 alone, this enzyme hindered lactone stabilization by specific oligonucleotides. After addition of top1 to CPT−olg1 or CPT−olg2 complexes, the lactone ring of the drug was destabilized. No lactone stabilization was observed when olg1 was added to CPT−top1 complexes or when olg1−top1 complexes were added to CPT.

Mammalian DNA topoisomerases (including topoisomerase I, top1)[1] are ubiquitous enzymes involved in multiple processes, including DNA replication, transcription, and illegitimate recombinations (for a review, see ref *1*). Top1 is a monomeric enzyme that binds to duplex DNA and creates a transient single-strand break via the formation of a covalent link between the 3′-phosphate of the cleaved strand and a tyrosyl residue of the enzyme. These intermediates are commonly referred to as cleavable complexes (*1*). Under physiological conditions, top1 catalyzes the religation of the 5′-hydroxyl group of the broken DNA after passing of the intact strand through the transient break. Recently, top1 was also identified as one of the specific protein-kinases that phosphorylates SR protein splicing factors, and a binding site for ATP was evidenced (*2*). Therefore, top1 is able to catalyze either breakage and religation of one DNA strand or transfer of phosphate residue on serines of the arginine/serine-rich domain of SR proteins.

Camptothecin (CPT; Figure 1) is a plant alkaloid first isolated in the late 1960s (*3*). There has been a renewed interest in this agent and its derivatives due to their remarkable activity against a broad range of human tumors studied in preclinical models and the discovery of its mechanism of action (for review, see refs *4* and *5*). Numerous semisynthetic analogues were developed to achieve better water solubility and fewer side effects. The water-soluble analogue, CPT-11 [7-ethyl-10-(4-[1-piperidino]-1-piperidino)carbonyloxycamptothecin], exhibited excellent antitumor efficacy against a wide spectrum of tumor types, including colon cancer (*6*, *7*), and had fewer unpredictable side effects than CPT (*7*, *8*). However, marginal in vitro activity of CPT-11 led to the discovery of its active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin) produced by hepatic carboxylesterases (*9*−*11*).

CPT inhibits the religation step of top1 that leads to an accumulation of stable cleavable complexes. CPT was also found to inhibit the phosphorylation of SR protein in vitro, but only in the presence of added DNA, and to modulate the state of hyperphosphorylation of SR proteins in treated cells (*2*). Although the molecular mechanism of interaction of CPT with top1 is not completely solved, there is now good evidence that cleavable complex stabilization is the major event that precedes cell death (*12*, *13*). There are presently no data supporting a function of SR protein phosphorylation in the cell death mechanisms induced by camptothecin. Since cleavable complex stabilization by CPT appears to be a readily reversible process, additional events occurring during replication and transcription must convert it into irreversible lesions (*12*−*15*).

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[1] Abbreviations: CPT, camptothecin; CPT-11, 7-ethyl-10-[[[4-(1-piperidino)-1-piperidino]carbonyl]oxy]camptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; top1, topoisomerase I; DMSO, dimethyl sulfoxide.

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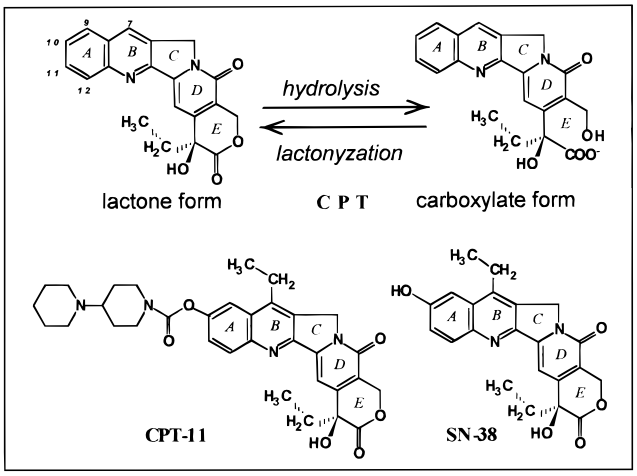
Drugs:



Oligonucleotides:

olg1: **5'-CAA-AGT-CAG-GTT-GAT-GAG-CAT-ATT-TTA-CTC-3'**
3'-GTT-TCA-GTC-CAA-CTA-CTC-GTA-TAA-AAT-GAG-5'

olg2: **5'-CAA-AGT-CAG-GTT-GAT-TAG-CAT-ATT-TTA-CTC-3'**
3'-GTT-TCA-GTC-CAA-CTA-ATC-GTA-TAA-AAT-GAG-5'

olg3: **TAA-AAT-TAA-ATT-AAT-AAA-TAT-ATT-TTA-TTT**
ATT-TTA-ATT-TAA-TTA- TTT-ATA-TAA-AAT-AAA

FIGURE 1: Structural formulas of camptothecins and oligonucleotides. The underlined bases are those differing from olg1 to olg2.

Some indirect evidence indicated that CPT probably forms a ternary complex with the enzyme and DNA. CPT was shown to be selectively active at top1 cleavage sites bearing a guanine residue at the 5′ terminus of the top1-mediated DNA break (*16*−*18*). Under photoactivation conditions, CPT presented some specific interaction with guanine residues of DNA (*19*). However, no detectable modification of the DNA structure by CPT was demonstrated under physiological conditions (*20*, *21*). Furthermore, biochemical data support the notion that CPT cannot interact with top1 alone (*21*), but only in the presence of DNA (*17*, *18*).

Structure−activity studies indicated some important structural requirements for the biological activity of the members of the CPT series. Substitutions in the quinoline part (positions 7, 10, and 11 of rings A and B, Figure 1) allow increased activity against top1 (*22*). In contrast, the activity is dramatically decreased with modifications of the α-hydroxylactone ring (ring E, Figure 1). The (*S*) stereochemistry and the hydroxyl group at position 20 are essential for activity of CPTs (*11*). The opening of the lactone upon hydrolysis at neutral and basic pH values results in the formation of the carboxylate form that is void of biochemical and biological activities, as nonhydrolyzable derivatives (*22*). Therefore, the lactone−carboxylate interconversion of CPTs is of great importance with regard to the formation of the cleavable complex. The aim of the present study is to analyze under physiological conditions the kinetics of hydrolysis of the lactone ring in the presence of the different partners involved in the cleavable complex. Analysis of the fluorescence emission spectra of CPT derivatives, based on spectral identification of their respective closed and open forms, as well as the effects of substitution, pH, and polarity of the molecular environment, allows a nonperturbing "real time" quantification of the lactone hydrolysis reaction (*23*). Although the results we have obtained for free CPTs were comparable to those by high-performance liquid chromatography, the spectroscopic approach allowed us to overcome the major limitations of HPLC, in particular, nonphysiological conditions, and to study the drug−target complexes.

This methodology was used here to compare the kinetics of lactone hydrolysis in free camptothecins and in the presence of specific and nonspecific oligonucleotides and/or top1. The specific 30-mer oligonucleotides correspond to strong camptothecin-inducible (olg1, Figure 1) or camptothecin-independent (olg2) cleavage sites in SV40 DNA (*16*). These sequences bear guanine residues. The model nonspecific 30-mer oligonucleotide (olg3) is a sequence completely void of GC base pairs.

MATERIALS AND METHODS

Drugs. 20(*S*)-Camptothecin was purchased from Sigma; CPT-11 and SN-38 were provided by Rhône-Poulenc Rorer (France). The samples were used without further purification. The drugs were dissolved in dimethyl sulfoxide (DMSO) at 10^{-4} M, aliquoted, and stored at −20 °C. For the reaction mixtures, the drugs from DMSO were diluted 100 times in an aqueous buffer (20 mM Tris-HCl, 0.5 mM dithiotreitol, 10 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 4 μg/mL aprotinine; pH adjusted to the values cited in the figure legends by addition of either H_2SO_4 or KOH) to obtain the final drug concentration of 2×10^{-7} M.

Pure lactone or pure carboxylate forms of CPTs were prepared by dilution of the stock solutions in a buffer at pH 5.0 or 9.9 (±0.05), respectively. For SN-38, the pH of the solutions of the lactone or carboxylate forms was readjusted to a value of 7.0 just before the measurements. This was intended to preserve the same degree of deprotonation of the 10-hydroxyl group (described below) for both the open-ring and closed-ring forms of SN-38 (*23*). The spectra were recorded immediately after the dilution in buffer (within 20−30 s).

It is important to note that lactone hydrolysis in CPTs strongly depends on the buffer pH. A relatively slight ($\Delta = 0.2$) increase of pH results in a dramatic decrease of the equilibrium portion of the intact lactone form and the hydrolysis half-life (*23*, *24*). For this reason, the pH of the control stock solution of the buffer was measured before and after each kinetic measurement. Within 8 h, the stability of the pH of the buffer stocked in the closed tubes was checked and was found satisfactory (pH decrease was <0.07). For the samples exposed during the kinetic runs, it was difficult to measure the pH because of the small volumes used (100 μL). Even if some slight pH decrease of these samples can be supposed during the measurements (up to 120 min), this should not significantly affect the comparative analysis of the kinetic runs (all held at the same conditions). Anyhow, we discuss our results in terms of the relative effects on the hydrolysis and not of the absolute hydrolysis parameters. For the long-time measurements (up to 16 h), the samples were stocked in the closed tubes and small aliquots were taken for the fluorescence measurements.

Oligonucleotides and Topoisomerase. Complementary strands of the specific 30-mer oligonucleotides corresponding

to strong camptothecin-inducible (olg1, Figure 1) or camptothecin-independent (olg2) cleavage sites in SV40 DNA (*16*) were purchased from Eurogentek. The primers of the model nonspecific 30-mer oligonucleotides void of GC base pairs (olg3, Figure 1) were synthesized by J.-F.R. (Rhône-Poulenc Rorer, France). The primers were dissolved (2×10^{-3} M final concentration in base pairs) in the buffer at pH 7.0, the same as that used for preparation of the drug solutions and complemented by 10 mM $MgCl_2$ and 150 mM NaCl. Annealing was performed by mixing equal amounts of each complementary strand. These reaction mixtures were heated to 95 °C for 5 min and left at room temperature overnight before the measurements. The oligonucleotide stock was diluted to the desired concentration with the buffer to assemble the final reaction mixture that contains drugs and/or top1.

Calf thymus top1 (10 units/μL) was purchased from Gibco BRL.

Instrumentation. Fluorescence emission spectra of CPTs were recorded with a computer-controlled M51 scanning microspectrofluorometer (Dilor, France). The UV laser excitation (2 mW of a 365 nm line of an argon laser, Spectra-Physics, model 2020) was focused through the objective ($\times$2.5) of the microscope within a quartz cell (100 μL). Line-scanning mode of excitation has been used to minimize irradiation effects on the samples during spectrum accumulation (10 s). Between the measurements, laser excitation was closed by a computer-controlled shutter. Experimental parameters such as laser power, focalization conditions, and irradiation time have been strictly controlled to maintain similar conditions of UV irradiation for all experiments.

Experimental conditions are described in more detail in the figure legends. All spectra and kinetic runs were reproduced at least three times for different preparations of solutions.

Kinetic Measurements with Free CPTs and CPTs in the Presence of Oligonucleotides. Prior to the kinetic measurements, the spectra characteristic for the pure lactone (L) and pure carboxylate (C) forms of each compound (generated at the same molar concentrations as described above) were recorded. These spectra (Figure 2) were used thereafter as the model spectra for the decomposition of the time-dependent spectra of the kinetic measurements.

The dilution of the stock solution (in DMSO) of the drug in aqueous buffer (reaction volume 100 μL) is considered as a starting point ($t = 0$) for kinetic runs. For kinetic studies of the complexes, the measurements were always started with the free drug solution. Thus, the initial percentage of the lactone form in the sample has been controlled. In the time interval between two measured points (the corresponding moments are shown by dashed lines in figures with kinetic runs), small volumes of oligonucleotides (2−4% of the volume of the reaction mixture) have been added to the drug solution, which was carefully mixed just after.

The recording of the fluorescence spectra at intervals of $\geq$4 min (see the figures and their legends) was computer-controlled using Labspec software developed by Serguei Sharonov (Laboratoire de Spectroscopie Biomoléculaire, Reims). In this way, a large set of fluorescence spectra was recorded for a given kinetic run.

It is worth noting that this work benefited from several important instrumental advantages. The first one was the use of a microspectrofluorometer equipped with a confocal entrance scheme to deal with very small sample volumes. This not only allows the study of CPT−target complexes in vitro but offers a possibility to investigate intracellular interactions and distribution of CPTs within living cancer cells (*25*−*27*). The second advantage was the computer-controlled recording of the fluorescence spectra. This significantly improved the facility of the kinetic measurements and the precision of the mathematical treatment of the spectra (filtering, baseline correction, etc.). Particularly advantageous was the possibility to perform the decomposition (described below) of each spectrum of a kinetic run into the model spectrum of the respective lactone and carboxylate forms. Although we have previously demonstrated that satisfactory quantitative data can be obtained using two-wavelength fluorescence measurements (*23*), spectral decomposition is intended to provide more rapid and precise calculation of the residual lactone percentage in the sample at any time.

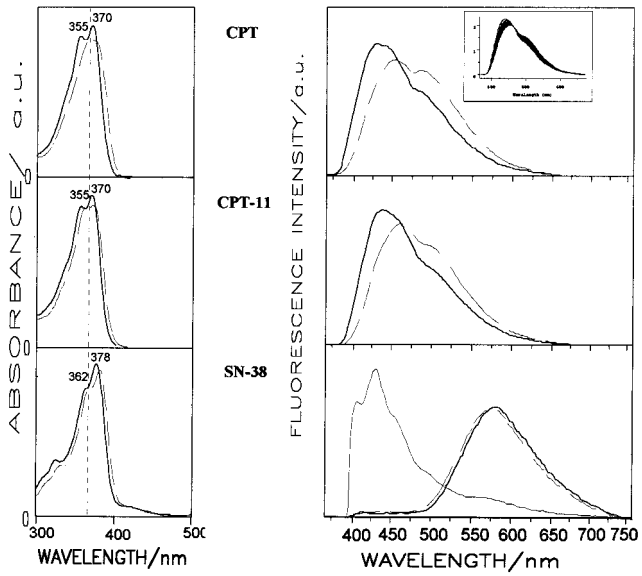


FIGURE 2: Spectral changes in the absorption (left) and fluorescence emission (right) spectra of camptothecins upon going from the lactone (solid lines) to the carboxylate (dashed lines) forms. The short-dash line in the absorption spectra indicates the position of the excitation (365 nm line of the argon laser). The present fluorescence spectra were used as models for decomposition of the spectra of kinetic runs. For SN-38, the third model (thin line) is the blue emission of the drug in DMSO. The box inserted in the right top of the box for CPT shows an evolution of the fluorescence emission upon lactone hydrolysis.

Treatment of the Spectra and Calculation of the Lactone Percentage from the Spectra. After the end of the kinetic measurements, the recorded set of spectra was treated using the Labspec software. The treatment of the recorded data comprised the following procedures: (1) filtering of the spectra to eliminate instrumental noise; (2) subtraction of background, that is, the very low-intensity signal of the buffer solution (no spectra of oligonucleotides or of top1 have been observed with the experimental conditions used); (3) decomposition of each spectrum into two model spectra corresponding to the pure lactone (L) and carboxylate (C) forms of a given compound. The fluorescence emission $F(\lambda)$ of a sample containing [L] and [C] concentrations of the L and C forms can be written as

$$F(\lambda) = [\mathrm{L}]F_{\mathrm{L}}(\lambda) + [\mathrm{C}]F_{\mathrm{C}}(\lambda) \quad (1)$$

where $F_{\mathrm{L}}(\lambda)$ and $F_{\mathrm{C}}(\lambda)$ are the fluorescent spectra of the L and C forms referred to as unit concentration.

These spectra are then normalized to the integrated intensity of the analyzed spectrum F and become $F'_{\mathrm{L}}(\lambda)$ and $F'_{\mathrm{C}}(\lambda)$:

$$F'_{\mathrm{L}}(\lambda) = x_{\mathrm{L}}F_{\mathrm{L}}(\lambda);\ F'_{\mathrm{C}}(\lambda) = x_{\mathrm{C}}F'_{\mathrm{C}}(\lambda) \quad (2)$$

Equation 1 then becomes

$$F(\lambda) = [\mathrm{L}]F'_{\mathrm{L}}(\lambda)/x_{\mathrm{L}} + [\mathrm{C}]F'_{\mathrm{C}}(\lambda)/x_{\mathrm{C}} \quad (3)$$

$F(\lambda)$ can be expressed as a sum of two spectral components

$$F(\lambda) = a_{\mathrm{L}}F'_{\mathrm{L}}(\lambda) + a_{\mathrm{C}}F'_{\mathrm{C}}(\lambda) \quad (4)$$

where a_{L} and a_{C} are the surface fractions of L and C normalized spectra. These fractions are determined by a linear least-squares regression.

From eqs 3 and 4, the percentage of lactone form [L, %] can be calculated as

$$[\mathrm{L},\%] = 100 \times [\mathrm{L}]/([\mathrm{L}] + [\mathrm{C}]) = 100 \times a_{\mathrm{L}}/(a_{\mathrm{L}} + (1 - a_{\mathrm{C}})k) \quad (5)$$

where $k = s_{\mathrm{L}}/s_{\mathrm{C}}$ is the integrated intensity ratio of the L and C forms, taken at the same molar concentration.

RESULTS

Hydrolysis-Related Changes in the Fluorescence Emission Spectra of CPTs. We have previously described the fluorescence emission spectra of CPT and six other congeners, in their respective closed- and open-ring forms, recorded with a macrofluorometer (*23*). It has been demonstrated that opening of the α-hydroxylactone ring of hydrolyzable CPTs was accompanied by dramatic changes in their fluorescence emission spectra. Similar effects have been observed in the fluorescence emission spectra recorded with a confocal microspectrofluorometer (Figure 2). For CPT and the majority of its derivatives, a pronounced red shift of the emission was observed on going from the closed-ring to the open-ring forms (Figure 2 and ref *23*). These changes in the fluorescence emission spectra of the closed and open forms of CPTs were used for quantification of the lactone/carboxylate fraction in the samples.

The 10-hydroxyl-substituted CPTs (here SN-38) are peculiar with regard to their emission spectra, which are very different from those of the other congeners (Figure 2 and ref *23*). Depending on the polarity of the environment, the 10-hydroxyl-substituted CPTs exhibit a large red emission with a maximum around 500−550 nm (in aqueous solution) or a blue emission with a maxima under 450 nm (in the less polar solvents, for instance DMSO). Spectral changes upon hydrolysis of the lactone ring in SN-38 are also particular. The red fluorescence maximum (Figure 2) is blue-shifted (*23*). The blue emission of SN-38 slightly increases in relative intensity upon lactone hydrolysis, but it is more influenced by pH and polarity of molecular environment (*23*). In view of this, we decomposed the spectra of the kinetic runs of SN-38 into three models (Figure 2): unshifted fluorescence emission band (characteristic of the lactone form), shifted emission band (characteristic of the carboxylate form), and blue fluorescence emission (characteristic of SN-38 in DMSO). The decomposition was then corrected by the contribution of the blue emission to calculate the percentage of the lactone/(lactone + carboxylate). The large deviations in the kinetic data for SN-38 (Figure 5B) are related to the fact that the shift of the emission of hydrolyzed SN-38 is less pronounced than the shifts of the emission of CPT or CPT-11 noted above.

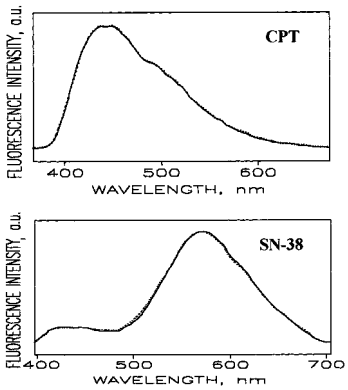


FIGURE 3: Fluorescence emission spectra of CPTs just before (solid lines) and immediately after (pointed lines) addition of olg1 (1/1300 total drug/base pairs molar ratio): (A, top) CPT; (B, bottom) SN-38.

Effect of the Oligonucleotides on the Kinetics of Lactone Hydrolysis in CPTs. First of all, it should be noted that addition of the oligonucleotides to the solutions of CPTs has not induced any detectable immediate perturbation of the fluorescence spectra of the drugs. Neither intensity nor position of the maximum of the fluorescence emission of CPTs has been affected by the presence of the nucleic bases (Figure 3). Complexes of SN-38 with oligonucleotides merit particular attention. The 10-hydroxyl-substituted derivative exhibits, at pH 7.0, fluorescence emission spectra consisting of two bands (Figure 3). As we have described above, the small band slightly increased in relative intensity upon opening of the lactone ring of the drug. In addition, the short-wavelength emission of the 10-hydroxyl-substituted CPTs has been shown to increase rapidly and to become dominant upon decrease of the polarity of the solvent, for instance, on going from water to DMSO (ref *23* and Figure 2). On the other hand, deprotonation of the 10-hydroxyl group leads to the disappearance of this shorter wavelength fluorescence band (*23*). It is interesting to note here that, after addition of oligonucleotides to SN-38, no detectable modifications of the relative intensities of the fluorescence bands of SN-38 were observed (Figure 3).

In contrast, as shown below, specific oligonucleotides modulate the kinetics of the lactone ring hydrolysis. The hydrolysis reactions are presented in Figures 4−8 as a time-dependent degradation of the percentage of the lactone form. The lactone percentage was calculated from the spectral data as described under Materials and Methods. For a given kinetic run, the error of the decomposition was within 1−2%. The points represented in these figures are the average results of at least three runs. These experimental data appeared to be well described by a one-phase exponential decay function, fitted using the least-squares method.

Before addition of oligonucleotides, all of the kinetic curves were very close. However, within a few minutes of

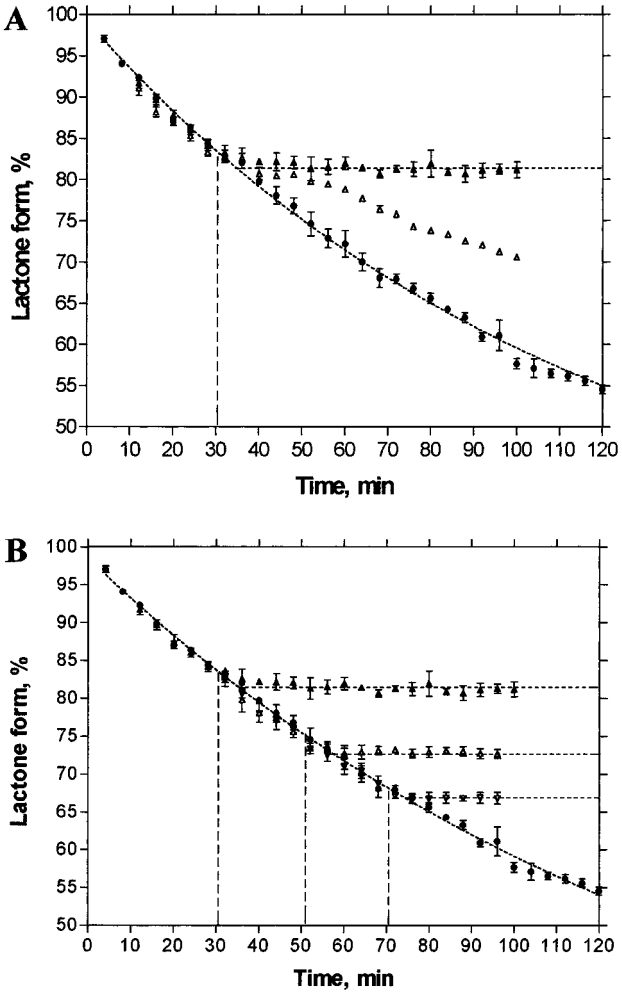


FIGURE 4: Kinetics of the lactone hydrolysis for CPT in the aqueous buffer at pH 7.0: (A) (●) free drug; and when olg1 was added (within 32nd min, corresponding time is shown by a dashed line) to obtain 1/650 (△) or 1/1300 (▲) total drug/base pairs molar ratio (these correspond to molar ratios of lactone form/base pairs of ca. 1/765 or 1/1530, respectively); (B) (●) free drug; and when olg1 (1/1300 total drug/base pairs molar ratio) was added within 32nd (▲), 52nd (△), or 72nd (▽) min of hydrolysis (as indicated by dashed lines).

the addition of the specific oligonucleotides olg1 and olg2, the hydrolysis of the lactone ring of CPTs was slowed (at 1/650 drug per base pairs molecular ratio, Figure 4A) or stopped (at 1/1300 drug per base pairs molecular ratio, Figures 4 and 5). This was observed at pH 7.0 (Figure 4) and pH 7.1 (Figure 5). At pH 7.1 (Figure 5), when the hydrolysis is faster, the stabilization takes a longer time than at pH 7.0 (Figure 4). Nevertheless, when added to nearly the same residual percentage of the lactone form of CPT or CPT-11 (Figures 4A and 5A, respectively), the olg1 stabilized nearly the same portion of the lactone for both congeners. Very similar data were obtained for SN-38, too (Figure 5B). Figure 4B shows the stabilization of lactone hydrolysis by olg1 added at different times, that is, to different residual concentrations of the lactone form of CPT. One can note some faster stabilization of the lactone for later additions of the oligonucleotides.

For CPTs with olg1 or olg2, the lactone percentage was stable for >1 h (Figures 4 and 5). Further observation of these samples during 16 h revealed gradual loss of the lactone

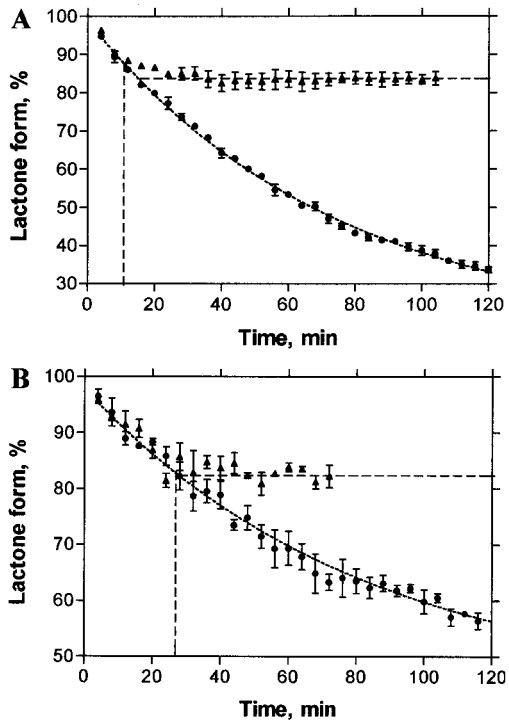


FIGURE 5: Kinetics of lactone hydrolysis for CPTs in the aqueous buffer at pH 7.1: (A) CPT-11; (B) SN-38; (●) free drug; (▲) when olg1 (1/1300 total drug/base pairs or 1/1530 lactone form/base pairs molar ratio) was added at time as indicated by dashed lines.

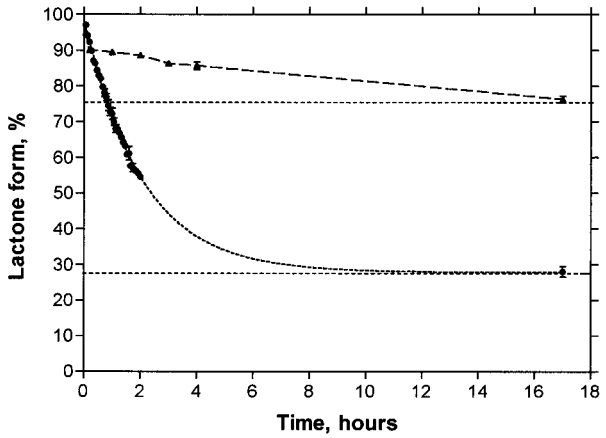


FIGURE 6: Long-time observation of the kinetics of lactone hydrolysis for CPT in aqueous buffer at pH 7.0: (●) free drug; and when the olg1 was added (▲), within 32nd min, to obtain 1/1300 total drug/base pairs molar ratio.

percentage (Figure 6). Thus, already hydrolyzed CPTs were not "lactonized"; that is, the lactone percentage did not increase in the described environment.

In contrast to olg1 and olg2, no stabilization of the lactone ring has been observed after the addition of olg3 to CPTs (Figure 7).

To determine whether AT oligonucleotide could be a substrate for top1, the double-stranded olg3 5′-end labeled on both strands was submitted to top1-induced DNA cleavage reaction in the presence or absence of 30 μM CPT and analyzed by sequencing gel electrophoresis. This protocol (*16*) allows us to detect even weak DNA cleavage sites located either on one or on both strands of the duplex DNA. The described method demonstrated that no DNA cleavage could be detected for olg3 in the presence of top1 alone or

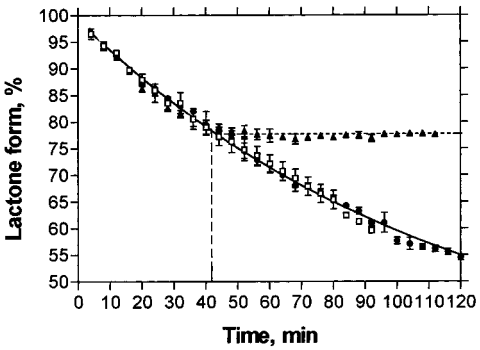


FIGURE 7: Kinetics of lactone hydrolysis for CPT in aqueous buffer at pH 7.0: (●) free drug; and when oligonucleotides were added (within 32nd min, as indicated by dashed line): (▲) olg2, (□) olg3. Total drug/base pairs molar ratio was 1/1300.

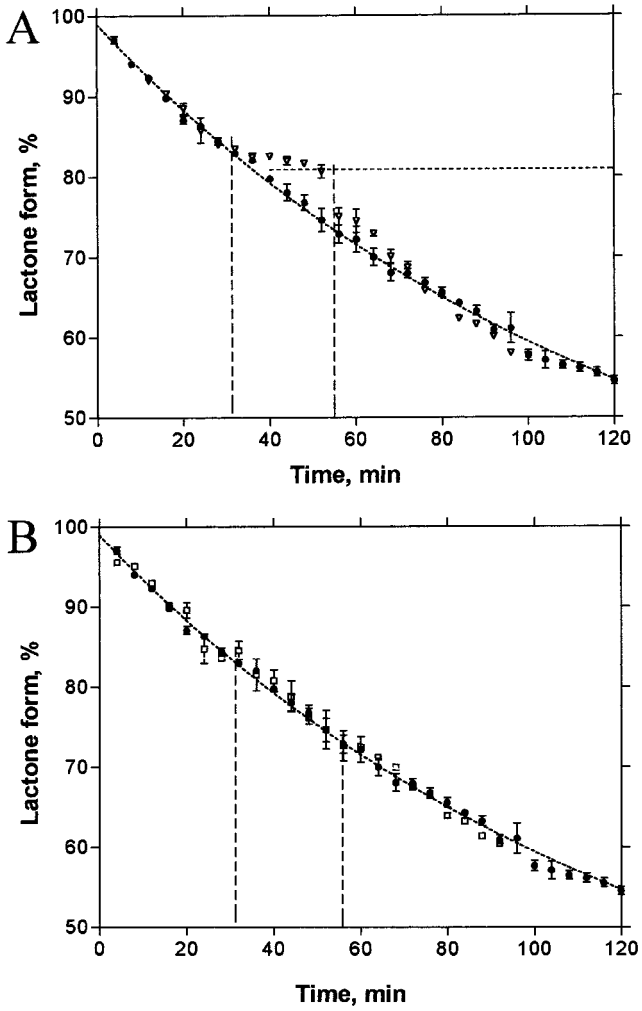


FIGURE 8: Kinetics of lactone hydrolysis for CPT in aqueous buffer at pH 7.0: (●) free drug; and after the addition of top1 (1/12 drug/enzyme molar ratio) and/or olg1 (1/1300 total drug/base pairs molar ratio) in the following sequences: (A) (▽) CPT + olg1 (32nd min) + top1 (54th min); (B) (□) CPT + top1 (32nd min) + olg1 (54th min).

in the presence of top1 with CPT, compared to untreated oligonucleotide (J.-F.R., unpublished data). In contrast, DNA cleavage was found in these conditions using olg1, as has already been described (*16*−*18*).

Effect of Top1 on the Kinetics of Lactone Hydrolysis in CPTs. Addition of top1 (hereafter, 1/12 drug/enzyme molar ratio), either alone or in the presence of the oligonucleotides, did not induce any immediate modification of the fluorescence emission spectra of CPT (data not shown).

Influence of top1 on the kinetics of lactone hydrolysis in CPT has been analyzed with respect to the different sequences of the ternary complexes formation.

(a) Lactone Hydrolysis in CPT + Olg1 + Top1 Ternary Complexes. All kinetic curves presented in Figure 8 started with free CPTs. When olg1 was added (the corresponding moments are shown with dotted lines), the curve was stabilized at a certain percentage of the residual lactone content (Figure 8A). These are represented by the horizontal regions of the kinetic curves. The addition of top1 was followed by a destabilization of the lactone form. The kinetic curves restarted descending.

(b) Lactone Hydrolysis in CPT + Top1 + Olg1 Ternary Complexes. The hydrolysis kinetic curves of free CPT were not affected by the addition of top1 (prepared in a buffer with the same pH as the pH of the reaction mixture), either immediately after or within more than hour after the samples had been mixed. When olg1 was added to this CPT−top1 sample, the lactone hydrolysis was still close to that for the free drug solution (Figure 8B).

(c) Lactone Hydrolysis in CPT + (Top1 + Olg1) Ternary Complexes. In these experiments, the previously prepared top1 + olg1 complexes were added to the CPT solutions. These top1 + olg1 complexes were prepared by incubation of the enzyme/oligonucleotide mixtures during 15 min at 37 °C. The addition of the (top1 + olg1) complexes did not induce any detectable effects on the kinetic runs of CPTs (data not shown).

DISCUSSION

This study aimed to shed light on lactone−carboxylate interconversion at the principal stages of the cleavable complex formation with intermediate CPTs. The obtained results can be discussed in two main parts, according to the effects induced by partners of CPTs in these complexes.

Effect of Oligonucleotides on the Kinetics of Lactone Hydrolysis in CPTs. We have to analyze all possible reasons of the lactone stabilization observed after addition of olg1 and olg2.

(i) pH Decrease. First, addition of the very small volumes (2−4% of the total sample volume, see the Materials and Methods) of oligonucleotide solutions prepared in the same buffer as the drug solutions should not be intended to modify the pH of the reaction mixture. Consistent with this, the pH-sensitive emission of SN-38 did not exhibit any features attributable to pH modification after addition of the oligonucleotides (Figure 3). No lactonization (possible in the case of the decreased pH) has been detected for the partially hydrolyzed CPTs stocked overnight in the presence of olg1 or olg2 (Figure 6). In contrast, some loss of the lactone percentage, probably upon degradation of the complexes, has been noted during a long observation time. The lactone stabilization was observed at pH 7.0 and 7.1 (Figures 4 and 5A, respectively). All of this indicates that lactone stabilization after the addition of the specific oligonucleotides cannot be related to a decrease of pH. Therefore, it seems reasonable to favor the following hypothesis.

(ii) CPT−Oligonucleotide Interaction. The ability of the photoactivated CPTs to interact with DNA base pairs has

been supposed by Leuteurtre and co-workers (*19*). We will not comment here on the possible photoactivation of CPTs—it is not the subject of this paper. The use of the same experimental conditions for all kinetic measurements allows us to discuss, rather, the relative differences in the hydrolysis parameters for free CPTs and for CPTs in the presence of specific oligonucleotides.

While the lactone forms of CPTs were stabilized in the presence of olg1 and olg2, no data indicated any particular influence of these oligonucleotides on the carboxylate forms of the drugs. The previously reported inactivity of carboxylate forms can be related with the fact that no lactonization has been observed either for totally (data not shown) or for partially hydrolyzed (Figure 6) samples stocked with specific oligonucleotides during 16 h.

It should be emphasized that the described effects are observed as a significant slowing of the lactone ring opening. We have demonstrated previously that substitutions at the quinoline rings of CPTs do not influence significantly the lactone hydrolysis (*23*). Both kinetic and equilibrium parameters of hydrolysis were very close for a series of drugs, consisting of CPT and quinoline-substituted congeners. Addition of the specific oligonucleotides induced lactone stabilization not only for CPT (Figure 4) but also for its quinoline-substituted congeners CPT-11 (Figure 5A) and SN-38 (Figure 5B). When added to nearly the same residual lactone percentage of CPTs, olg1 stabilized nearly the same portion of the respective lactone forms. Therefore, this stabilization seems to be general for CPTs and rather independent of the quinoline substitution.

We have shown that relative intensities of two fluorescence emission bands of the 10-hydroxyl-substituted compounds (SN-38) were particularly sensitive to a state of this group (*23*). The fluorescence spectra of SN-38 were not modified by addition of olg1 or olg2. Therefore, the OH group of the quinoline part of SN-38 is not affected by the presence of these oligonucleotides. This allows the lactone−nucleotide contacts of CPTs with DNA.

As can be expected, the described lactone stabilization by specific oligonucleotides depends on the lactone/base pairs molar ratio: it was somewhat more rapid for the higher excess of oligonucleotides (Figures 4 and 5).

The very important thing was the fact that no lactone stabilization was observed for CPT in the presence of olg3 void of G/C base pairs (Figure 7). DNA cleavage assays demonstrated that AT oligonucleotide is not a substrate for top1−DNA cleavage reaction in the absence or presence of CPT. This indicates that the interaction of CPT with DNA should take place at the GC regions. The latter is in agreement with the previously reported data for the base pairs specificity of the CPTs binding sites (*16*−*18*). In recent studies it was assumed that alkylating CPT derivative can bind at the interface of the top1−DNA complex and interacted with the G^{+1} base of the top1 cleavage site (*17*, *19*).

Effect of Top1 on the Kinetics of Lactone Hydrolysis in CPT. Lactone hydrolysis was not perturbed for CPT in the presence of the enzyme alone. In the presence of both top1 and DNA, that is, in the ternary complexes, different sequences of the cleavable complex preparation have been analyzed. The kinetic curves for CPTs in the presence of top1 and olg1 (Figure 8) or top1 and olg2 (data not shown) indicate that the presence of the enzyme can reverse the lactone stabilization.

The effect of top1 can be induced at the drug level and/or the DNA level. According to these suppositions one can propose the following models.

(*i*) CPT interacts with top1 (drug level), but only in the presence of DNA (*17*, *18*). Such interaction can result in a modified CPT−DNA interaction that does not lead to stabilization of the lactone ring of CPT. In this case, the intact lactone function of CPT should be important as a key factor to the initial step of ternary complex formation, that is, for the drug−DNA complexes. In contrast, since the nonhydrolyzable CPTs were biologically inactive, the next step, that is, drug−top1 interaction, can require the possibility of E ring opening.

(*ii*) CPT does not interact with top1 (*21*) at all (since no evidence for interaction of CPT with top1 alone has been found here or anywhere), or at least CPT does not interact with top1 via the lactone moiety (since top1 does not affect lactone hydrolysis). In this case, the hindrance of lactone stabilization can be related to the structural perturbation induced at specific oligonucleotides by top1 (DNA level) and/or to the competition between lactone function of the drug and the active tyrosine of the enzyme for the interaction with the same sites (*17*) of DNA. Within this hypothesis, our data indicate that interaction between DNA and top1 is stronger than interaction between DNA and CPT and removes free CPT that can continue its lactone hydrolysis.

In conclusion, we have shown here that the lactone ring of CPTs interacts with DNA at GC but not AT base pair regions. This type of binding may provide a reservoir of active (lactone) drug to stimulate top1 cleavage. The next step, that is, formation of the cleavable complexes, can require the possibility of E ring opening.

To understand which of the proposed models should be preferred and if it should be, a more detailed analysis of the molecular interactions of CPTs in the cleavable complexes is required. It would be interesting to understand if the nonhydrolyzable CPTs are able to interact with DNA. This kind of information can be obtained using nondestructive techniques of vibrational spectroscopy (*28*−*30*). The molecular characterization of the lactone and carboxylate forms of a series of CPTs has been performed by Raman and surface-enhanced Raman scattering spectroscopies (*30*), and these techniques are actually used to study CPTs in their complexes with top1 and DNA base pairs in vitro. In vivo investigation of intracellular interaction and distribution of CPTs within living cancer cells by fluorescence (*25*−*27*) and SERS (*31*) microspectroscopies is also in progress in our group.

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