Biochemical Pharmacology, Vol. 55, pp. 1163–1174, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00508-X

Spectroscopic and Biochemical Characterisation of Self-Aggregates Formed by Antitumor Drugs of the Camptothecin Family

THEIR POSSIBLE ROLE IN THE UNIQUE MODE OF DRUG ACTION

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ABSTRACT. We describe the effect strongly influencing the biological activity of some camptothecin (CPT) drugs, the inhibitors of DNA topoisomerase I (topo I), namely, the formation of J-type aggregates in an aqueous buffer solution. These aggregates were built up under certain dilution conditions of the stock DMSO solutions of 20-*S*-camptothecin (20(S)CPT), 10,11-methylenedioxy-CPT (10,11-CPT) and 7-ethyl-10-hydroxy-CPT (SN38). The aggregates were found to be stereospecific, not being detectable for the 20(*R*)-stereoisomer of CPT. They were formed by the stacking interaction between quinoline rings of CPT chromophores with the inverse position of the nitrogen atoms. The aggregates were stable at acidic and neutral pHs, but dissociated at basic pHs. Self-aggregation prevented hydrolysis of the lactone ring at neutral pHs, thus preserving the drugs in a biologically active form. Addition of BSA did not induce either disaggregation or hydrolysis of the lactone ring, whereas the monomeric form of the drugs was shown to undergo rapid conversion to an inactive carboxylate form in the presence of human serum albumin [5]. The drugs did not form the aggregates in the presence of topo I. Moreover, rapid dissociation of the aggregates was observed if a self-aggregated drug solution was added to topo I alone or to the DNA-topo I cleavage assay. Neither DNA alone nor oligonucleotides derived from the sequences of the CPT-enhanced or topo I-induced cleavage sites in SV40 plasmid DNA induces changes in the aggregation state of the drugs. These observations are indicative of interaction between the aggregates and topo I. The aggregates were found to penetrate within the cells with much higher efficiency than a monomeric form of the drugs. Cellular uptake of aggregated and nonaggregated species correlated well with cytotoxic effects produced by the drug. In this manner, CPT's self-aggregation should be regarded as a favourable phenomenon producing species with a more stable biologically active structure of the lactone ring and exhibiting enhanced cellular uptake levels relative to the monomeric forms of medications. BIOCHEM PHARMACOL **55**;8:1163–1174, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. camptothecin; topoisomerase I; J-aggregates; circular dichroism; fluorescence; antitumour effects

The anticancer agent CPT** controls proliferation of cancer cells as do several related derivatives of the CPT family of drugs (Fig. 1), by stabilisation of the reversible topoisomerase (topo) I–drug–DNA (cleavable) ternary complex [1]. The data suggest important structural requirements both for passive diffusion of the drug into cancer cells and for successful interaction with the topo I target. A wide range of CPT derivatives with substitutions in the A and E rings of the drug has recently been analysed in terms of their ability to stabilise the cleavable ternary complexes, the kinetics of lactone ring hydrolysis, and the level of cellular uptake of the medications [1, 2]. These studies demonstrated the possibility of CPT–topo–DNA interactions through the A and E rings of the drug molecule.

The antitumor activity of CPT drugs decreases following dissolution of the compounds in aqueous media due to the

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** *Abbreviations:* CD, circular dichroism; CPT11, 7-ethyl-10[4(1-piperidino)-1-piperidino]carbonyl-oxy-camptothecin; HSA, human serum albumin; $9NH_2$-CPT, 9-amino-camptothecin; PBS, potassium-buffered saline; SN38, 7-ethyl-10-hydroxy-camptothecin; 10,11-CPT, 10,11-methylenedioxycamptothecin; topo I, DNA topoisomerase I; 20(*S*)CPT, 20-*S*-camptothecin; 20(*R*)CPT, 20-*R*-camptothecin.

Received 23 September 1996; accepted 5 September 1997.

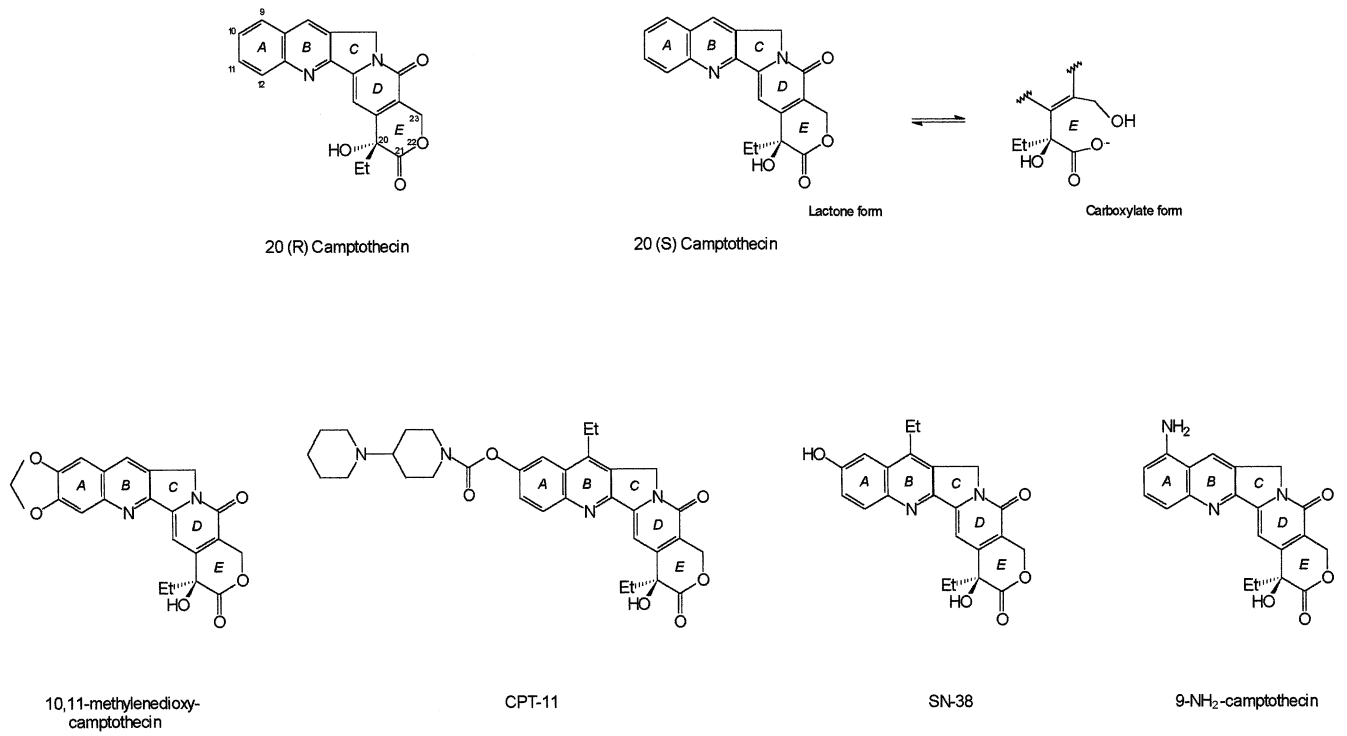

FIG. 1. Chemical structures of CPT drugs.

presence of the hydrolyzable α-hydroxy-δ-lactone ring moiety [2]. The closed lactone (E) ring is widely regarded as being an essential pharmacophore for activity against cancer cells, and the ring opening is thought to result in a loss of potency of medication. Cell-free experiments which directly examined the structural basis of drug potency against the topo I target indicate that ring opening results in a significantly attenuated activity [3]. In addition, the ring opening results in charged drug species exhibiting limited diffusibility through the lipid bilayer of a low dielectric constant, thereby altering diffusibility characteristics of the molecules [4]. For that reason, factors influencing a lactone-carboxylate equilibrium within the CPT molecule are clearly important determinants of the agent's function. Human serum albumin (HSA) was shown to bind preferentially with the carboxylate form, resulting in the lactone ring's opening more rapidly [5]. On the other hand, a lipid bilayer maintains the stability of the lactone moiety of CPT drugs [4].

In the present report, we characterise a new effect on the stability of CPT drugs, the efficiency of cellular uptake and inhibition of cell growth exerted by the derivatives 20-*S*-camptothecin (20(*S*)CPT), 7-ethyl-10-hydroxy-CPT (SN38) and 10,11-methylenedioxy-CPT (10,11-CPT). The phenomenon is concerned with the forming of small and very stable stereospecific J-type aggregates in the aqueous buffer solutions. We adapted UV-vis, steady-state fluorescence, and circular dichroism methodologies to characterise in detail the electronic properties and structural basis of self-aggregation of CPT derivatives. The self-aggregated species were found to penetrate easily within the cancer cells with an up to 10-fold enhanced level of uptake relative to monomeric forms of the medications. The self-aggregates dissociated within the cells, possibly as a result of interactions with topo I. No changes in the aggregation were observed when BSA or oligonucleotides with the sequences corresponding to strong topo I-induced cleavage sites were added. Aggregation strongly decreased hydrolysis of the lactone CPT ring at neutral pHs. The higher tendency toward self-aggregation may prevent (at least in part) a lactone form of the drug from hydrolysis at neutral pHs, while a diminished tendency of the species to aspecific interactions allows the rate of drug accumulation within the cells and its availability at the receptor site to be increased.

MATERIALS AND METHODS

Drugs, Enzymes, Oligonucleotides and Chemicals

20(*S*)CPT, 20-*R*-camptothecin (20(*R*)CPT), 10,11-CPT and 9-amino-camptothecin ($9NH_2$-CPT) sodium salts were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. 7-ethyl-10[4(1-piperidino)-1-piperidino]carbonyl-oxy-CPT (CPT11) and SN38 were generously provided by the Rhône-Poulenc Rorer. Drug stock solutions were made in DMSO (A.C.S. spectrophotometric grade) at concentrations of 10, 1 or 0.1 mM, and aliquots were stored in the dark at −70°. Further dilutions were made by mixing aliquots of a drug solution in DMSO with the corresponding amount of PBS immediately before use, and the corresponding amounts of DMSO were adjusted so that the same DMSO concentration was present

in all preparations. Variations in pH in the drug aqueous solutions were accomplished by dropwise addition of 0.1 M of HCl or NaOH. BSA was purchased from Sigma and used as received. All other chemicals were of reagent grade and used without further purification.

Calf thymus topo I (2.6×10^6 units/mg) was purified to homogeneity from calf thymus and prepared for measurements as described [5]. One unit of topo I corresponded to the amount of enzyme necessary to relax (or decatenate) 50% of 0.5 μg of pBR322 DNA (or kinetoplast DNA) when incubated for 30 min at 37° under the assay conditions. Topo I was aliquoted and stored, without detectable loss of activity, at −70° in the conservation buffer containing 10 mM of Tris-HCl, pH 7.5, 20 mM of 2-mercaptoethanol, 0.5 mM of EDTA, 100 μg/mL of BSA, and 50% glycerol. The preparation of topo I and drugs for CPT reactions was carried out as described [6, 7].

The oligonucleotides used directly for CPT reactions were received from The Drug Synthesis and Chemistry Branch, National Cancer Institute. They corresponded to a strong CPT-induced topo I cleavage site in SV40 DNA [8]. Annealing was performed by mixing equal amounts of each oligonucleotide in 80 μL of the annealing buffer (10 mM of Tris-HCl, pH 7.5, 100 mM of Na_2SO_4, 1 mM of EDTA). The reaction mixture was heated to 65° for 5 min and left at room temperature until cooled to 20°. The concentration of DNA (phosphate) was estimated on the basis of the molar absorption coefficient of 6,600 M^{-1} cm^{-1} at 260 nm. The DNA-topo I cleavage assay was performed as described previously [6, 7].

Cells

K562 is a human erythroleukemia cell line established from a patient with chronic myelogenous leukemia in blast transformation [9]. The cells were kept in exponential growth at $5–8 \times 10^5$ in RPMI culture medium (Life Technologies) supplemented with 10% fetal calf serum and 2 mM of L-glutamine. Cell growth and viability were determined after 48 hr of cell cultivation by phase-contrast microscopy.

For the drug treatments of the cells, 4.5×10^6 cells were incubated with 10^{-5} M of the drug for 30 min at 37° in the RPMI culture medium and washed twice with PBS by centrifugation (5 min, 200 *g*, 4°). Under the conditions used, drug incorporation into the cells was found to be completed in the first 30 min of the incubation [10].

For the lysis of drug-treated cells, two procedures were used. The first was a traditional thermal shock. The cells, after being washed twice with PBS, were resuspended in water, deposited in liquid nitrogen for several minutes and then in the incubator at 37°. The procedure was repeated twice. The second procedure of cellular lysis was realised by centrifugation of the cells followed by resuspension of the pellet in DMSO. Separation of the water-soluble and nonsoluble fractions of lysates after both procedures was performed by centrifugation of the lysates (4 min, 200 *g*, 4°). The water-soluble fraction was immediately used for fluorescence analysis, and the nonsoluble fraction was resuspended in water before the spectra were recorded.

UV Absorption, CD, and Fluorescence Spectra

Absorption spectra were recorded with a Philips PU 8720 UV/VIS scanning spectrophotometer, and Jobin Yvon, Mark III and JASCO-500C dichrographs were used to measure CD spectra. Experimental conditions are indicated in the figure legends. Measurements were performed using quartz cells of 1, 0.1, 0.01, and 0.001 cm in path length. Fluorescence spectra were recorded with a Shimadzu RF530 fluorescence detection set at excitations of 370 nm for CPT and CPT11, and 385 nm for 10,11-CPT.

RESULTS

Electronic Properties and Spectroscopic Characterisation of the Monomeric Forms of CPT Drugs

UV-vis spectra of CPT and CPT11 in DMSO solution were found to be very similar (Fig. 2) in the 275–450 nm region. The differences in the absorption profiles for SN38 and 10,11-CPT as compared with those of CPT and CPT11 were manifested in a slight bathochromic shift (4–8 nm) and changes in relative intensities of the bands at *ca.* 372 and 391 nm. The amino group at position 9 of the A ring of CPT ($9NH_2$-CPT derivative) induced dramatic changes in the UV-vis spectra of the drug. Figure 2F shows the linear dependence of the absorbance of CPT solution in DMSO on the concentration of the drug. This behaviour was found to be typical for all CPT drugs and demonstrates that no aggregation in DMSO solutions occurred in this range of concentrations.

CD spectra of the drugs in DMSO solutions were measured at concentrations up to 10^{-2} M. CD signals were found to be quite weak, and their positions and relative intensities corresponded well to the electronic transitions of the drugs in the UV-vis spectra (Fig. 3). All CD bands for 20(*S*)-CPT, CPT11, SN38, $9NH_2$-CPT, and 10,11-CPT had the same sign, whereas the CD spectrum of 20(*R*)CPT proved to be mirror-symmetrical as compared with the spectra of all other drugs (Fig. 4).

A transfer of the drugs from the diluted (10^{-5}–10^{-4} M) stock solutions in DMSO to the aqueous buffer led to a blue shift of both the absorption profiles (Fig. 2) and the corresponding CD bands (Fig. 4). These similarities confirm that the molecules were in the form of monomers (see Discussion).

The hydrolysis of the lactone ring is believed to be strongly pH-coupled, being sluggish at acidic pH, increased at neutral pH and accelerated dramatically at pH > 7 [11]. We analysed the spectral effects of lactone hydrolysis at pH 7.4 (under conditions avoiding ionisation of other groups in the CPT chromophores, e.g. OH-substitution in the A ring for SN38) as well as at basic pHs. In the aqueous media (pH 7.4), the hydrolysis scarcely affected the absorption spectra

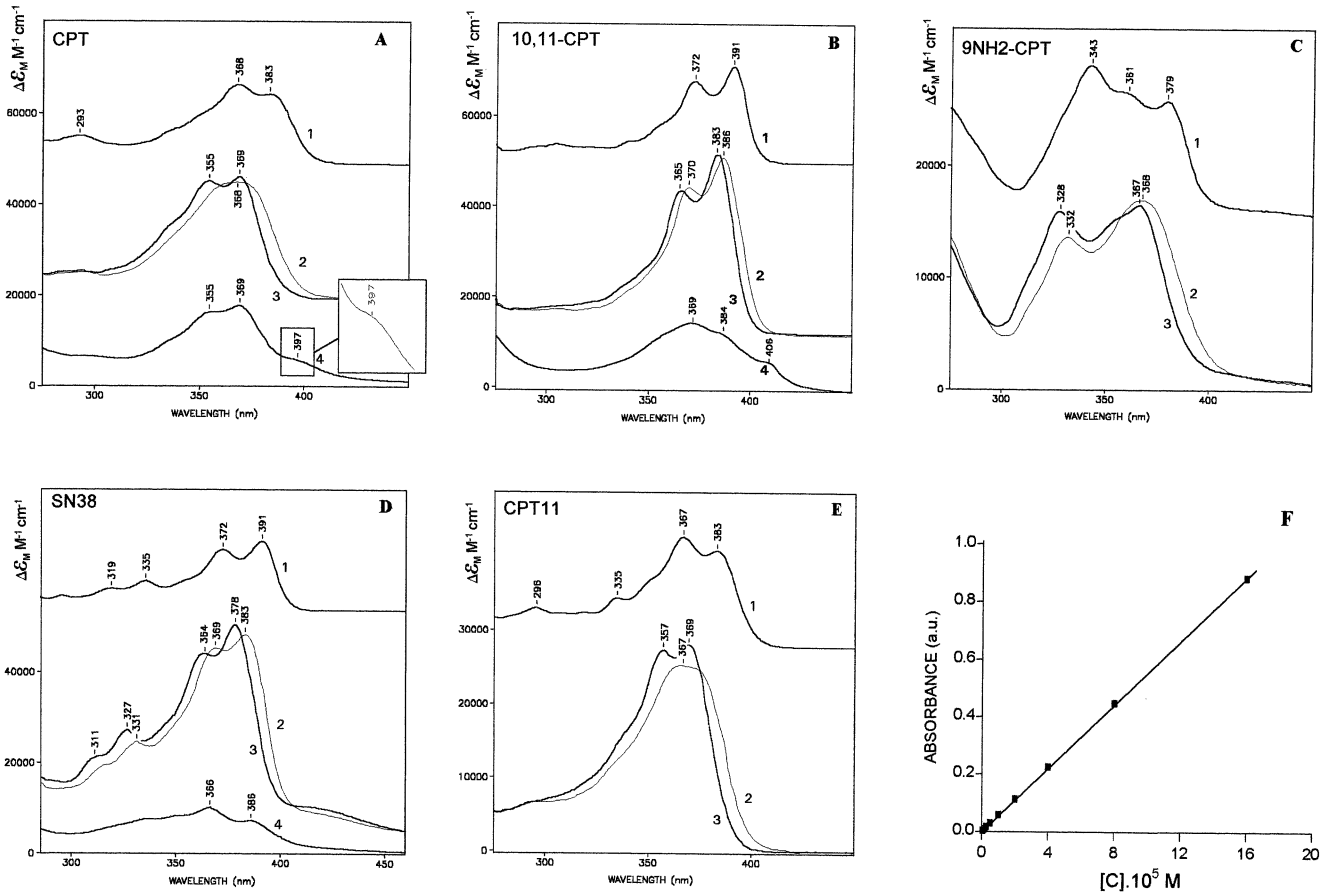

FIG. 2. UV-vis spectra of the monomeric (1–3) and self-aggregated (4) forms of CPT (A); 10,11-CPT (B); 9NH_2-CPT (C); SN38 (D); and CPT11 (E). 1) Solution in DMSO, concentration 2×10^{-5} M; 2) Solutions in the aqueous buffer (pH 7.4); concentrations: 2×10^{-5} M for CPT, CPT11 and 9NH_2-CPT and 4×10^{-6} M for 10,11-CPT and SN38. The samples were prepared by dilution of 10^{-3} M (for CPT, 9NH_2-CPT and CPT11) or 10^{-4} M (for 10,11-CPT and SN38) stock solutions in DMSO. The spectra were recorded after 6 hr of incubation of samples to achieve total hydrolysis of the drug lactone rings; 3) Solutions of the drugs in the aqueous buffer under the same conditions as for (2), but with spectra recorded immediately after dilution; 4) Solutions in the aqueous buffer under the same conditions as for (2) and (3). The samples of CPT and SN38 and the sample of 10,11-CPT were prepared by dilution of 10^{-2} M and 10^{-3} M stock solutions in DMSO, respectively. The spectra were recorded immediately after dilution of the drugs in the aqueous buffer and were found to be the same after incubation of the drugs in the aqueous buffer for 4–6 hr. (F) Dependence of the absorbance at 368 nm on the concentration of CPT in DMSO.

of all the drugs (Fig. 2). On the other hand, the CD spectra varied dramatically with time, so that the sign of the CD bands in the 280–450 nm region was inversed, proceeding the lactone hydrolysis (Fig. 4). The rapid hydrolysis of CPT, CPT11, 9NH_2-CPT, and 10,11-CPT at basic pHs induced spectral changes identical to those found during the hydrolysis at neutral pHs (*ca.* 7.4). On the other hand, a pH increase dramatically affected both CD and absorption spectra of SN38 (Fig. 5C). The bands at 330, 368 and 383 nm actually disappeared, whereas new electronic transitions at 339 and 414 nm became dominant. These variations were found to be completely reversible: decreasing pH to *ca.* 7 restored the absorption profile of SN38 on the minute-scale. It is well-known that the lactonisation of the carboxylate form of the drugs at pH 7 can be accomplished only within several hours [11]. Hence, appearance of new electronic transitions in an SN38 molecule correlates to deprotonation of the OH-substituent of the A ring rather than to hydrolysis of the lactone ring. Moreover, the pK values of pH-dependent transitions in the UV-vis spectra correlate well to the pK of OH-group of the A ring of SN38 [12].

Decreasing the pH to *ca.* 1 in the water solutions of the carboxylate or lactone forms of all drugs resulted in similar peculiarities in their absorption spectra (Fig. 5A; SN38 used as an example). The bands at 325, 364 and 378 nm greatly decreased in intensity at acidic pHs, whereas the electronic transitions at *ca.* 390 and 413 nm became dominant in the absorption spectrum, with the pK value of this process corresponding well to the pK of protonation of the nitrogen of the B ring [12]. These changes were also found to be reversible when the pH was increased from *ca.* 1 to 7.

It is important to note that the positions and relative intensities of the bands in the CD spectra of all drugs were always in close correlation with those of the UV-vis spectra of the same preparations. The only difference observed (as noted above) was the reversal of the sign of the CD bands

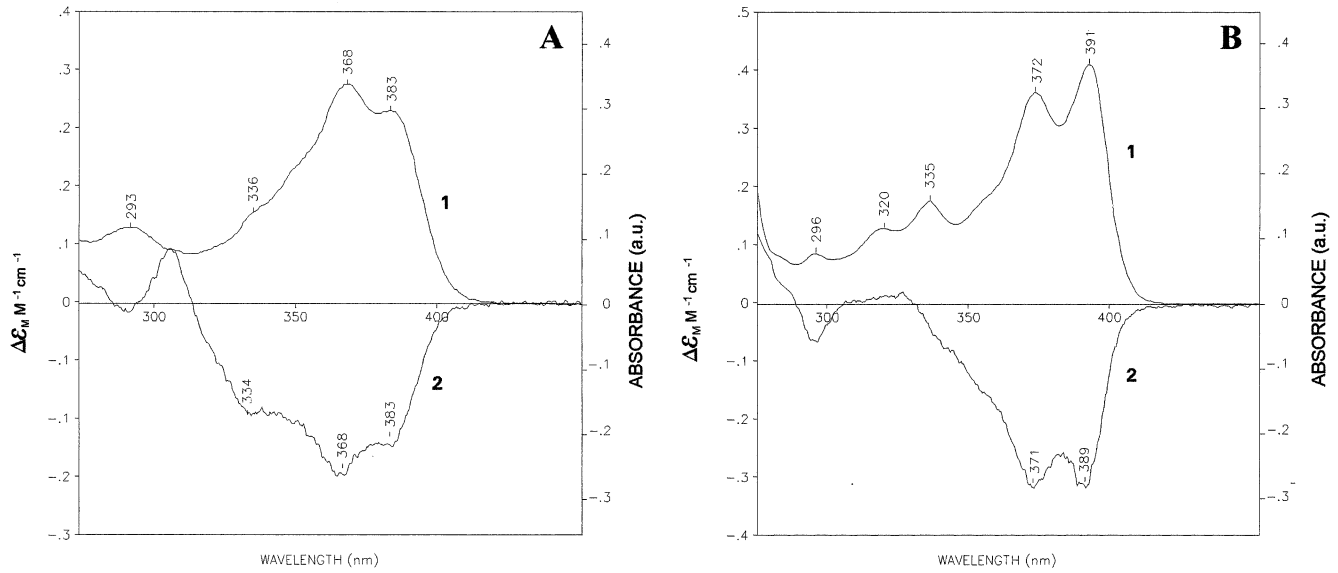

FIG. 3. UV-vis (1) and circular dichroism (2) spectra of DMSO solutions of CPT (A) and SN38 (B). CD spectra were recorded from the 10^{-2} M stock solutions of the drugs and UV-vis spectra from 10^{-5} M solutions. Cuvette path lengths: 1 cm for UV-vis and 0.005 cm for CD.

in the 280–450 nm region, induced by the hydrolysis of the drug lactone ring.

Formation of the Self-Aggregates and the Conditions of Disaggregation of the Drugs

A comparison of the UV-vis spectra of two 20(S)CPT aqueous buffer solutions of the same (2×10^{-5} M) concentration but prepared by dilution of either 10^{-2} or 10^{-3} M stock solutions of the drug in DMSO displayed remarkable differences (Fig. 2). The solutions prepared from the more concentrated stocks were characterised by *ca.* 40–50% lower extinction as well as by a lower ratio of intensities of the bands at 355 and 369 nm. Moreover, an additional absorption band at *ca.* 397 nm appeared for the solutions prepared from the 10^{-2} M stock. Fluorescence emission of the samples prepared from the more concentrated stock solution was also found to be *ca.* 50% less intense as compared with the corresponding preparation from the 10^{-3} M stock solution (Fig. 6C). Even stronger

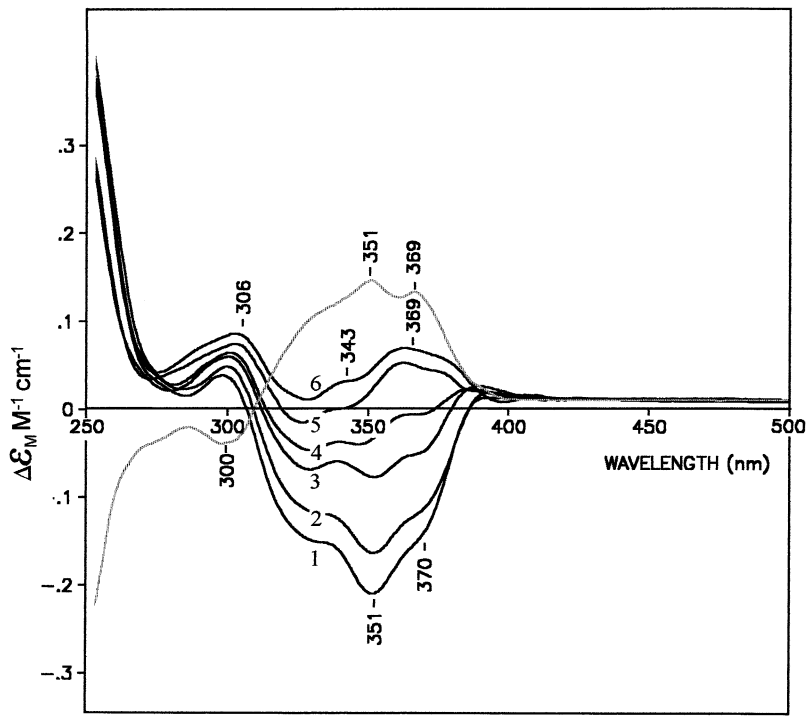

FIG. 4. CD spectra of the monomeric forms of 20(S)CPT (solid line) and 20(R)CPT (dashed line) and hydrolysis kinetics (1–6) for 20(S)CPT in the aqueous buffer at pH 7.4. The spectra 1–6 were recorded at 20-min time intervals. Concentration of the drugs was 2×10^{-5} M. The aqueous buffer solutions were prepared by dilution of the 10^{-3} M stock solution.

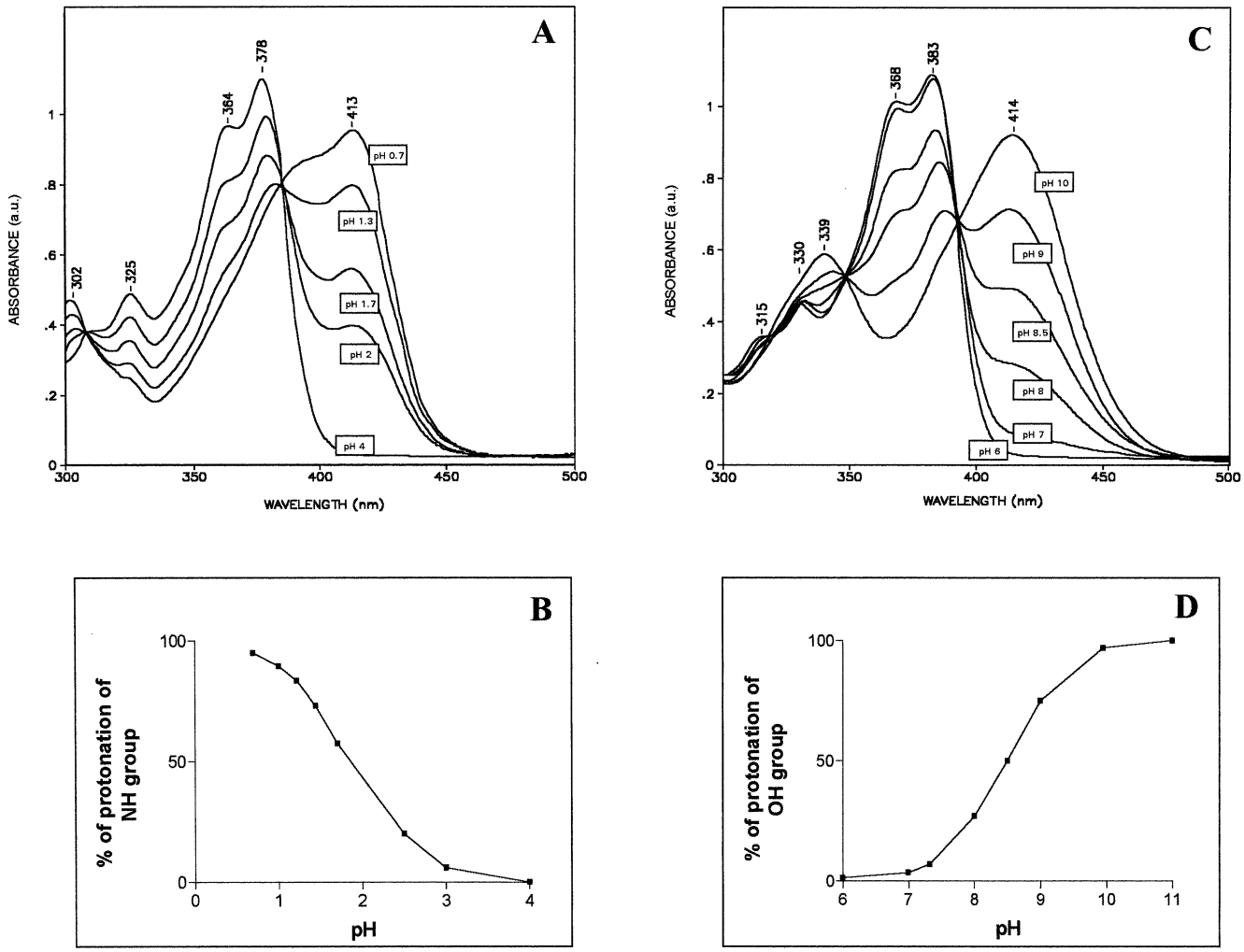

FIG. 5. UV-vis spectra of the monomeric form of SN38 at acidic (A) and basic (C) pHs. Concentration of the drug: 4×10^{-6} M. The samples were prepared by the dilution of the 10^{-4} M stock DMSO solution of SN38. Approximate pK values for the NH group of the B ring (B) and the OH group of the A ring (D) of SN38 determined from the UV-vis data.

effects of the same origin were found for 10,11-CPT and SN38 (Fig. 2). The effects described were found for SN38 and 10,11-CPT when 10^{-3} M or more concentrated stock solutions were used, whereas the dilution of 10^{-2} M stock solutions was required for CPT. UV-vis spectra of $9NH_2$-CPT, CPT11 and 20(*R*)CPT were found to be the same regardless of the stock solutions from 10^{-2} M to 10^{-5} M used to dilute in the aqueous buffer.

Diluting the 10^{-2} M stock solution of 20(*S*)CPT or 10^{-3} M (and more concentrated) stock solutions of 10,11-CPT and SN38 in the aqueous buffers resulted in the appearance of tremendous CD signals (Fig. 6A). These signals did not exist in the DMSO solutions of the drug or when the more diluted stock solutions were chosen to prepare the same molar concentration of the compound in water buffers. These CD signals with molar ellipticity values at the level of 20–40 M^{-1} cm^{-1} were characteristic of the formation of stable self-aggregates (see "Discussion"). It should be noted that the sign of the CD signals of 10,11-CPT aggregates was opposite to those of 20(*S*)CPT and SN38. The formation of aggregates was not influenced by addition of DMSO up to 20% of the volume of the sample. The CD signals of the aggregates did not appear for $9NH_2$-CPT, CPT11 and 20(*R*)CPT.

Incubation of the aggregated species at pH 7.4 for 72 hr did not induce any spectral (i.e. UV-vis, fluorescence, or CD) features characteristic of the hydrolysis of lactone rings of the drugs. Moreover, incubation led to a *ca.* 20% increase in spectral effects typical of the aggregates (both in UV-vis and CD spectra), which in turn were found to be very stable over many days without any changes in the shape of the signal or precipitation of the sample. On the other hand, the CD signals, UV-vis and fluorescence features of aggregates were found to be very sensitive to increases in pH, totally vanishing after 20 min when the pH of the buffer was increased to 10 (Fig. 6). By increasing the pH to 11, the signals of the aggregates disappeared within minutes. An increase in pH led to the rapid hydrolysis of the lactone ring; hence, an increase in pH produced a monomeric species with the hydrolysed lactone ring. These effects seem to be a general characteristic for all drugs exhibiting a tendency to form self-aggregates at neutral pHs (20(*S*)CPT, 10,11-CPT, and SN38).

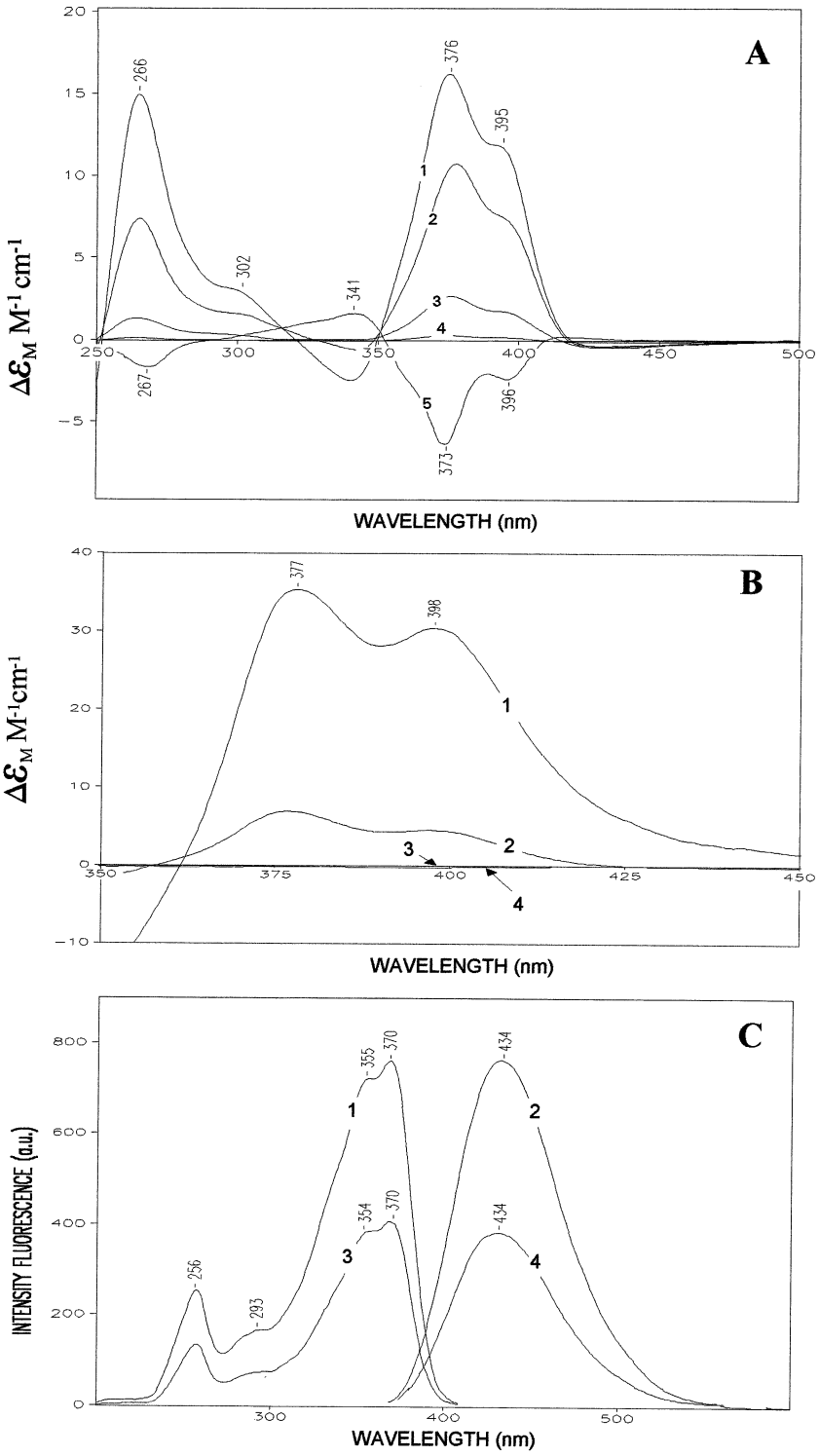

FIG. 6. (A) CD spectra of self-aggregated 20(S)CPT prepared by dilution of the 10^{-2} M stock solution down to 2×10^{-5} M concentration in the aqueous buffer of pH 7.4 (1) or 11 [2–4]. (2) spectrum was recorded immediately after dilution; (3) after 10 min; (4) after 20 min; (5) CD spectrum of 10,11-CPT prepared in the same manner as 20(S)CPT at pH 7.4, but on the basis of the 10^{-3} M stock solution in DMSO. (B) CD spectrum of self-aggregated 20(S)CPT prepared by dilution of the 10^{-2} M stock solution down to 2×10^{-5} M concentration in the aqueous buffer of pH 7.4, with BSA added up to the 5 mg/mL final concentration (1); CD spectra of the same preparation of 20(S)CPT in the presence of 5 mg/mL of topo I (2, 3) and in presence of DNA-topo I cleavage assay (4). Spectra 1, 2 and 4 were recorded within 20 min after mixing of ingredients. Spectrum 3 was recorded within 10 min after mixing of ingredients. (C) The excitation (1, 3) and emission (2, 4) fluorescence spectra recorded from the samples prepared by dilution of the 10^{-3} M (1, 2) or 10^{-2} M (3, 4) stock solutions of CPT down to 2×10^{-5} M concentration in PBS, pH 7.4. The fluorescence detector excitation and emission settings: λ_{exc} = 370 nm, and λ_{em} = 435 nm.

Effect of Topoisomerase I on the Process of Self-Aggregation of the Drugs

Addition of topo I induced disaggregation of 10,11-CPT (Fig. 6B). The effects were found to be identical to those induced by an increase in pH (Fig. 6A). On the other hand, the addition of topo I did not modify the pH (7.4) of a reaction volume. A CD signal of the aggregates was not detectable within 5 min when a 10-μM concentration of the drug and 10 μM of topo I were used in the reaction volume. 20(S)CPT also displayed disaggregation upon topo I addition, although not to the levels achieved in the case of 10,11-CPT (Fig. 6B). This means that the effect of disaggregation was also found to be identical to that observed following the increase in pH (Fig. 6A), with the only difference being the time-scale of the process as compared with 10,11-CPT: disappearance of the CD signal

TABLE 1. Intracellular fluorescence and viability of K562 cancer cells treated with 20(S)CPT

Preparation/ parameters	Fluorescence intensity of cellular lysates*	Viability (% of control cells)
Aggregates: cells treated by 20(S)CPT (10^{-5} M) prepared from the 10^{-2} M stock solution	204 ± 19	16 ± 8
Monomers: cells treated by 20(S)CPT (10^{-5} M) prepared from the 10^{-3} M stock solution	100 ± 2	39 ± 5
Control: untreated cells	30 ± 2	100

*λ_{exc} = 370 nm; λ_{em} = 430 nm. Intensity of fluorescence from the water-soluble lysates of cells treated with 20(S)CPT prepared from the 10^{-3} M stock solution is taken as 100%.

took 10–15 min after the self-aggregated species were mixed with topo I.

The addition of BSA at the same concentration as for the experiment with topo I described above did not induce an effect of disaggregation of the drugs (Fig. 6B). In the case of CT-DNA or oligonucleotides with the sequences corresponding to the topo I-induced cleavage sites, disaggregation effect of the drugs was not found in drug-to-DNA (bp) molar ratios ranging from 1:1 to 1:10. Addition of self-aggregated CPT species to the DNA-topo I cleavage assay [6, 7] induced dissociation of the aggregates in a manner similar to those induced by topo I alone (Fig. 6B).

Effect of the Aggregates on Cellular Uptake of the Drugs and Viability of Cancer Cells

To address the question as to whether self-aggregation of CPT drugs affects drug cellular uptake and growth inhibition of cancer cells, we treated human leukaemia K562 cells with the aqueous buffer solutions of the drugs at equal (10^{-5} or 10^{-6} M) final concentrations, although prepared from 10^{-2} M or 10^{-3} M stock solutions in DMSO. The treated cells were divided into two parts: the first was lysed to measure intracellular drug uptake by fluorescence analysis under conditions of selective excitation of the fluorescence spectra of CPT drugs, whereas the second batch was kept in culture medium without the drug for 48 hr and used to determine cell viability.

The fluorescence level of 20(S)CPT in the cellular lysates obtained by the thermal shock method diluted from the 10^{-2} M stock was twice that of the 10^{-3} M stock solution (Table 1). Fluorescence analysis of the nonsoluble fraction of the cellular lysates demonstrated a negligible level of fluorescence and did not reveal any differences between the two methodologies of cell treatment (data not shown).

The second procedure was used in cellular lysis by treating the cells with DMSO to eliminate the possible effects of aggregation or disaggregation of the intracellular drug on its fluorescence properties. The aggregates do not exist in DMSO solutions. Hence, cellular lysis in the presence of DMSO should destroy aggregates of the CPT drugs and enable the fluorescence signal of the intracellular 20(S)CPT in the monomeric form to be analysed. In fact, this procedure yielded the identical results as the first method (Table 1), suggesting dissociation of the aggregates within the cell.

Fluorescence analysis of the intracellular uptake of 10,11-CPT showed even stronger effects of drug aggregation than did that of 20(S)CPT. The fluorescence of 10,11-CPT for the water-soluble fraction of the cellular lysates of cells treated with the aggregated drug was found to be nearly 10 times that of cellular treatment with the preparation of the monomeric drug (Table 2). Analysing nonsoluble fractions of the cellular lysates again did not show any differences in the level of drug fluorescence. The effects observed for 10,11-CPT were also found to be independent of the lysis procedure: similar results were obtained both for the thermal shock and DMSO destruction of the treated cells.

Viability of the cells treated by the aggregated and nonaggregated preparations of 20(S)CPT and 10,11-CPT was studied and found to be in qualitative correlation with the data on intracellular uptake of the drugs (Tables 1 and 2).

The final control experiment included treatment of the cells with the drug not exhibiting formation of the aggregates in the aqueous buffers. Treatment of the cells with the same concentration of CPT 11 prepared from the 10^{-3} M

TABLE 2. Intracellular fluorescence and viability of K562 cancer cells treated with 10,11-CPT

Preparation/parameters	Fluorescence intensity of cellular lysates*	Viability (% of control cells)
Aggregates: cells treated by 10,11-CPT (10^{-6} M) prepared from the 10^{-2} M stock solution	942 ± 81	17 ± 3
Monomers and aggregates: cells treated by 10,11-CPT (10^{-6} M) prepared from the 10^{-3} M stock solution	209 ± 68	27 ± 5
Monomers: cells treated by 10,11-CPT (10^{-6} M) prepared from the 10^{-4} M stock solution	100 ± 27	32 ± 5
Control: untreated cells	47 ± 9	100

*λ_{exc} = 384 nm; λ_{em} = 420 nm. Intensity of fluorescence from the water-soluble lysates of cells treated with 10,11-CPT prepared from the 10^{-3} M stock solution is taken as 100%.

TABLE 3. Intracellular fluorescence and viability of K562 cancer cells treated with CPT11

Preparation/parameters	Fluorescence intensity of cellular lysates*	Viability (% of control cells)
Cells treated by CPT 11 (10^{-5} M) prepared from the 10^{-2} M stock solution	97 ± 6	68 ± 14
Cells treated by CPT 11 (10^{-5} M) prepared from the 10^{-3} M stock solution	100 ± 5	72 ± 9
Control: untreated cells	4 ± 1	100

*λ_{exc} = 370 nm; λ_{em} = 430 nm. Intensity of fluorescence from the water-soluble lysates of cells treated with CPT 11 prepared from the 10^{-3} M stock solution is taken as 100%.

or 10^{-2} M stock DMSO solutions did not reveal any differences in either the intracellular uptake of CPT11 or viability of the treated cells (Table 3).

DISCUSSION

Electronic Properties of Monomeric CPT Chromophores

CPT includes two main chromophores: the A and B rings (quinoline) and the D ring with the hydrolyzable E ring (Fig. 1). The CPT molecule has a single asymmetrical carbon, located at position 20 (Fig. 1); in natural CPT the configuration is S. We set out to investigate which parts of the CPT chromophore provide a main contribution to the electronic (UV-vis, CD, fluorescence) spectra of the drugs. The absorption spectrum of CPT in DMSO (Fig. 2) was very similar to that of quinoline [13], but all main absorption bands exhibited the long wavelength shift ongoing from quinoline to CPT. This shift is typical for conjugation of the AB and DE rings [14]. The comparison of the UV-vis spectra of CPT drugs in the 280–450 nm region in DMSO (Fig. 2) shows that the spectra of CPT, CPT11, SN38, and 10,11-dimethylene-CPT were quite similar. The differences between the UV-vis spectra of these drugs include modifications in relative intensities and band shifts in the 350–390 nm region (Fig. 2).

Dramatic changes in the absorption spectra upon protonation of the nitrogen in the B ring (for all the drugs) or deprotonation of the hydroxyl group in the A ring (for SN38) demonstrate that the most important factors influencing the electronic spectra include the state of the quinoline chromophore (rings A + B, Fig. 1) and, to a lesser extent, the state of the closest substituents through electronic conjugation. On the other hand, the state of the lactone ring could influence the absorption spectrum of the drugs in an indirect manner. Only slight changes in the absorption spectra of all the drugs could be detected upon the hydrolysis of the lactone ring at neutral pH (Fig. 2).

The results show that the CD spectra of CPT in DMSO at both 10^{-2} and 10^{-3} M concentrations corresponded exactly to their absorption spectra, demonstrating that the molecules are in a monomeric form [15, 16]. The same is true for the solution of CPT monomers in the aqueous buffer, for the CD and UV-vis spectra correspond well to each other. Ongoing from 20(S)CPT to 20(R)CPT, the sign of the CD spectrum was inverted, confirming a main role of orientation of the hydroxyl group at position 20 for the CD signal of the drug. Opening the E-cycle induced dramatic changes in the CD spectra: the sign of the CD signal changed from negative to positive, once again correlating well with its absorption spectrum. Opening the cycle by long-term hydrolysis at pH 7.4 or immediate hydrolysis by adjusting pH to 11 gave the same effect for the CD-spectra. Hence, the UV-vis and CD spectra of CPT drugs in DMSO as well as in the aqueous solutions (prepared by dilution of the nonconcentrated stock solutions in DMSO) show a typical behaviour of monomeric chiral chromophores with a high sensitivity to the state and environment of the asymmetrical carbon at position 20.

Electronic Properties and Structure of Self-Aggregated CPT Chromophores

A decrease in the extinction of the bands in the UV-vis spectra, changes in the profile of absorption bands, the appearance of an additional long wavelength absorption band at *ca.* 398 nm (Fig. 2) as well as the appearance of abnormally strong CD signals (Fig. 6A)—the spectral features found to be characteristic of the aqueous buffer solutions of 20(S)CPT, 10,11-CPT and SN38-diluted the more concentrated stock of the drugs down to the same (10^{-5}–10^{-6} M) concentrations in the aqueous buffer solutions. These spectral differences show that CPT, SN38 and 10,11-CPT were able to form aggregates when the more concentrated stock solutions were used for the preparation. This behaviour of the electronic spectra of the drugs could be explained in terms of the molecular exciton theory developed in the works of several researchers, especially those of Davydov [15], and Kasha *et al.* [16]. Three key findings of the molecular exciton theory are as follows: (1) Both "blue" (hypsochromic) and "red" (bathochromic) absorption bands can occur as aggregated species form. These are H-aggregate and J-aggregate absorption, respectively; (2) Fluorescence intensity diminishes upon aggregation of molecules; and (3) an abnormally strong CD signal may appear, reflecting some resonance excitonic energy transfer within the aggregated species.

In the case of CPT drugs, "red" (bathochromic) absorption bands were formed when concentrated stock solutions of 20(S)CPT, 10,11-CPT or SN38 were used to prepare the diluted buffer solutions (Fig. 2). This finding, together with

diminishing absorption (Fig. 2) and fluorescence intensity (Fig. 6C) and the appearance of extremely high characteristic signals in the CD spectra (Fig. 6A) suggests the J-type self-aggregation of the drugs.

We wished to determine how many monomeric molecules participate in the formation of self-aggregates in the case of CPT drugs? Due to $1/N^{1/2}$ dependence (N is the number of monomeric units) of the line width of aggregates [17], only the larger aggregates clearly gave rise to narrow lines, whereas dimers, trimers, and shorter oligomers had rather broad spectral features. The relative amount of oligomers and long chain polymers would be controlled to some extent by concentration and the freezing procedure of the sample. It is worth restating that a sample without the J-band structure may still contain a considerable amount of oligomers [18]. In the so-called monomeric sample, the formation of oligomers cannot be completely avoided. In addition, we could not find considerable narrowing of the J-band; hence, the aggregated species do not include here a significant amount of the monomeric units. We are not able to specify if there were dimers, trimers or tetramers, but there were certainly no aggregates consisting of hundreds of molecules as occurs in the typical cases of cyanine dyes exhibiting a tendency to form J-type aggregates [18].

Incredibly strong signals in CD spectra can obviously be attributed to the stacking interactions of the quinoline rings of CPT molecules in polar solutions [15, 19]. The second possibility, self-association through formation of hydrogen bonds, is less probable. This type of interaction must be enhanced and easily revealed in nonpolar solutions of the drug; here though, the CD spectra of CPT in DMSO were characteristic of the monomeric form of the molecule (Fig. 5), and the concentration dependence of the absorbance (Fig. 2F) of the CPT drug solutions in DMSO was found to be linear. It is most probable that the stacking interaction involves quinoline chromophore (rings A and B) with the inverse position of the nitrogens of the B rings and leads to the formation of internally dissymmetric chromophore. Quinoline groups are known to have a tendency to overlap as follows from the crystallographic data [20]. The cyanine-type stacking interaction between the A-B rings of CPT molecules was confirmed by characteristic changes in the profile of the UV-vis spectra of the drugs (Fig. 2), similar as they are to those found for the aggregation of cyanine molecules [17–19]. The self-aggregation led to a decrease in the long wavelength band in the absorption spectrum (Fig. 2), which was found to be sensitive only to specific substitutions in the A ring or to protonation of the nitrogen of the B ring. On the other hand, this band was found to be nonsensitive to the modifications in the vicinity of the remaining part of the CPT chromophore (Fig. 2).

The stacking interaction between the quinoline rings of the CPT molecules does not appear to be the only reason for the aggregation of the drug. Increasing the pH from 7.4 to 10 led to disappearance of the CD signals of the aggregates and an increase in the extinction of bands in the UV-vis spectra up to the characteristic values for the monomeric forms of all the drugs. It may be assumed that some groups with pK at *ca.* 9 play an additional role in the aggregation of CPT molecules, and deprotonation of these groups induces dissociation of CPT aggregates. The CPT molecule itself does not contain any groups with pK values in this region [12]. Hence, an intermolecular bond could be the only additional factor stabilising the aggregates in the aqueous buffer solutions. As for the groups which could participate in the formation of this intermolecular bond, the role of the hydroxyl at position 20 appears to be the most probable: aggregation was totally abolished when the 20(*R*)-stereoisomer of CPT was used. In other words, the aggregation is stereospecific, and one of the most important factors in the process of CPT aggregation is relative orientation of the ethyl and hydroxyl groups in position 20. Orientation of the ethyl group may also play the role of steric hindrance to form the aggregates by the 20(*R*)-stereoisomers of CPT, whereas the hydroxyl group may participate in intermolecular bonding and stabilising 20(*S*)CPT self-aggregates.

10,11-CPT had a great potential for forming aggregates ongoing from the solution in DMSO to aqueous buffer solution (Fig. 2B). It was capable of aggregating even when 10^{-3} M stock DMSO solution was used for dilution. Moreover, the CD signals of the aggregates were opposite those of CPT (Fig. 4). Hence, 10,11-methylene substitution in the A ring changes the relative orientation of the molecules participating in the aggregates. Potential applications of the aggregation effect produced by 10,11-CPT are currently being investigated in clinical trials (see below).

Biological Roles of Self-Aggregation of CPT Drugs

As was noted above, the antitumor activities of the monomeric form of CPT drugs begin to deteriorate immediately following dissolution of the medications in the aqueous media, due to the presence of the E ring in the structure of each drug. In solution, this functionality readily reacts via acyl cleavage [12], yielding a biologically inactive carboxylate form of the drug. Investigators previously established that the carboxylate of CPT is hardly internalised by cancer cells [21], and the intact lactone moiety is essential for the activity against the topoisomerase I (topo I) target [3, 22]. Furthermore, the α-hydroxylactone ring moiety appeared to be a critical structural feature for activity *in vivo* [23]. Once the molecular mechanisms of the drug interactions are concerned, the structure-activity relationship of some thirty CPT derivatives favor CPT to interact with the topo I-DNA complex via stereospecific binding [1]. The synthetic optical isomer 20(*R*)-CPT has almost no effect on topo I. The binding site probably accommodates the whole molecule of the drug, since the two most distant rings of CPT (A and E) are critical for topo I inhibition [1]. The interaction of the E ring, in the vicinity of position 20, with a receptor is supported by the loss of activity resulting from

either substitution in the 20-hydroxyl group or replacement of 21-lactone by nonhydrolyzable 21-lactam [22]. The 20-hydroxy-21-lactone structure undergoes facile ring opening and closure reactions, whereas ring opening does not occur easily in the lactam or 20-deoxyCPT. This suggests that the opening of the lactone could very well be a crucial point in the pattern of drug action. It was proposed that the ring-opened drug could be reversibly covalently bonded with the enzyme via the ester exchange reaction, likely facilitated by hydrogen bonding of the 20-hydroxyl to the electronegative atom of the enzyme [1]. All these data demonstrate that the factors influencing a lactone-carboxylate equilibrium of CPTs (e.g. self aggregation of some drugs) are the important determinants of their biological activities.

We then wished to know if the self-aggregation of the drug affects the formation of a ternary cleavable ternary complex between the drug, topo I and oligonucleotides. We first observed the stabilisation of the lactone form of the drugs in aggregates. On the other hand, addition of the drugs to topo I alone or to the DNA cleavage assay induced rapid dissociation of the aggregates (Fig. 6B). Addition of the drugs to BSA (Fig. 6B), DNA, or oligonucleotides alone did not exhibit any influence on self-aggregation. Consequently, the aggregates do not interfere with drug interactions with topo-DNA cleavable complexes due to their rapid dissociation, and this disaggregation is evidence of the interaction between the CPT drugs (at least 20(S)CPT, 10,11-CPT and SN38) and topo I. These effects may be evidence of specific interactions between the drug and topo I, which seem to be of greater intensity than those within the aggregates.

Extensive studies of some factors influencing stability of the lactone moiety of CPT drugs and their correlation with the biological activity of medications were performed by Burke *et al.* [4, 5]. They found that lipid bilayer interactions stabilize the lactone moiety of CPT drugs [4]. 10,11-CPT was found to display the highest membrane activities, whereas topotecan, characterised by excellent water solubility, displayed remarkedly reduced binding to lipid bilayers. The lactone moieties of CPT and 10,11-CPT were found to be stable for periods up to 72 hr in the presence of lyposomes. Stock solutions of all drugs were prepared in DMSO at the concentration of 2×10^{-3} M and diluted down to 1–2 μM in the PBS buffer. As was shown above, at these conditions of dilution at least one of the drugs studied (10,11-CPT) exists in a form of the small J-type aggregates. We also found also a significant contribution of the aggregated species for 20(S)-CPT in the PBS buffer prepared from the 2×10^{-3} M DMSO stock solution. The self-aggregation of these medications should be considered as a complement to the interactions of the drugs with the lipid bilayer. Both these effects lead to stabilisation of the lactone moiety of CPTs.

DMSO is a principal solvent used for preparation of stock solutions of CPT drugs. Aqueous buffer solutions of CPTs used for *in vitro* studies of double and ternary CPT/topo I/DNA complexes, for HPLC analysis and studies of CPTs intracellular uptake were prepared in each case from the 10^{-2} or 10^{-3} stock DMSO solutions ([1–6] and references herein). In order to check if the same effects were operative in other solvents, we performed an experiment using PEG400. Dilution of 10^{-2} M CPT in PEG400 down to 10^{-5} M concentrations in PBS led to the appearance of CD signals typical for the aggregates, in the same manner as those described for the DMSO-concentrated stock solutions. Thus, the effects of aggregate formation seem to be of a general character for at least two solvents—DMSO and PEG400.

The data summarised in Tables 1 and 2 indicate that the self-aggregation of 20(S)CPT and 10,11-CPT drugs strongly regulates the cellular uptake and viability of cancer cells. CPT, like the majority of other small drug molecules, are thought to be accumulated in the cells by passive diffusion. The cellular accumulation of 10,11-CPT and $9NH_2$-CPT was compared with that of CPT in CASE, SW-48, and HT-29 human colon cancer cells [24]. It was shown that 10,11-CPT displayed significantly enhanced uptake levels relative to CPT and $9NH_2$-CPT, and this derivative was found to be as a most potent analogue in the series. The potencies of several CPTs in both drug-sensitive and multidrug-resistant KB carcinoma cells were also compared [25]. The order of potency of the medications in both cell lines was found to be 10,11-CPT > CPT > topotecan. According to the data presented in Tables 1–3, the potencies of 10,11-CPT, 20(S)CPT and water-soluble CPT11 to form self-aggregates appear in the same order as their potencies of intracellular penetration and growth inhibition of cancer cells.

All these data taken together show that the self-aggregation of some CPT drugs should be regarded as an additional (and in this case favourable) factor affecting the biological activity of the medications. The tendency to self-aggregation may first provide a diminished tendency to other aspecific interactions and therefore increase the rate of accumulation within the cells and, consequently, the availability at the receptor site. Second, the ring opening results in charged drug species, exhibiting limited diffusibility through lipid bilayer domains of a low dielectric constant [4]. Hence, the stabilisation of the lactone ring at neutral pHs by self-aggregation of the drug should result in enhanced cellular uptake of the medications.

The drugs were found to be disaggregated within the cells. This effect may be caused by the interaction either between the drug and topo I alone or within the ternary cleavable complex. The effect of the direct interaction between CPT and topo I has recently been found by analysing the energy migration between the Trp residue(s) of topo I and CPT [26]. This energy migration induced a dramatic increase in CPT fluorescence in the presence of topo I, whereas the level of fluorescence of the drug was unchanged in the presence of BSA or oligonucleotide substrate. The dissociation constant of the CPT/topo I complex was estimated to be 1.5×10^{-6} M, which is quite

close to those constants calculated from the data on inhibition of the topo I cleavage reaction by the CPT drug [26]. On the other hand, it is not clear whether monomeric or self-aggregated CPT species were used in the experiments described. Disaggregation of the drug in the presence of topo I may induce (at least in part) an increase in the level of CPT fluorescence, but in any event clearly favors specific drug/enzyme interaction not being detectable upon addition of bovine serum albumin.

Molecular interactions of the CPT drugs with topo I alone and in ternary cleavable complexes with topo I and oligonucleotides with the sequences corresponding to the topo I-induced cleavage sites as well as the self-aggregation of the drugs in these interactions and kinetics of the hydrolysis of their lactone moieties are presently under study by means of UV-vis, CD, fluorescence and Raman spectroscopic techniques in our laboratories.

This work was supported in part by Grants 96-04-48763 from the Russian Foundation for Basic Research (Russia) and 1379 from ARC (France).

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