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Commentary

Relevance of extracellular and intracellular interactions of camptothecins as determinants of antitumor activity

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ABSTRACT

Camptothecins are potent antitumor agents that stabilize the covalent binding of topoisomerase I to DNA forming a reversible ternary complex which, following collision with the replication forks, converts the single-strand breaks into lethal double-strand breaks. This cytotoxic mechanism has been originally ascribed to the closed lactone form, because opening of the lactone ring resulted in loss of antitumor activity. Since the lipophilic lactone favours passive diffusion into the cancer cells, the stability of the closed form is expected to be predictive for activity. Thus, the *in vivo* pharmacological behavior of camptothecins, which is dependent on the pH-dependent dynamics, is likely a critical determinant of their antitumor efficacy and therapeutic index. The physicochemical properties could influence a number of cellular and *in vivo* interactions, including stability of the ternary DNA–enzyme–drug complex, binding to serum proteins, recognition by transport systems. These interactions are also implicated in the processes responsible of toxic side effects and drug resistance which are major limitations of the efficacy of camptothecin-based therapy. A number of strategies have been developed to overcome the limitations associated with the peculiar *in vivo* reactivity and the reversibility of drug–target interaction. Modifications with hydrophilic or lipophilic substituents at specific positions may have a variable (and somewhat opposite) influence on interaction with the intracellular target and plasma proteins and on recognition by membrane transporters. Here, we highlight the interactions of camptothecins which could be exploited to optimize therapeutic efficacy.

1. Introduction

The cytotoxic and antitumor activity of camptothecins (CPT) is ascribed to their ability to stabilize the covalent binding of topoisomerase I (Top1) to DNA (the cleavable complex) forming a reversible ternary complex. These stabilized single-strand DNA breaks are fully reversible and non-lethal. However, when a DNA replication fork collides with the cleavable complex, single-strand breaks are converted into irreversible double-strand breaks (Fig. 1). On the basis of S-phase-specific activity of camptothecins and of the reversibility of cleavable complex, prolonged drug exposure is a critical requisite for therapeutic efficacy.

CPT has a peculiar pentacyclic structure containing an hydrolyzable ring E (Fig. 2) [1]. The molecule in solution is almost planar with an asymmetric carbon in position 20 of ring

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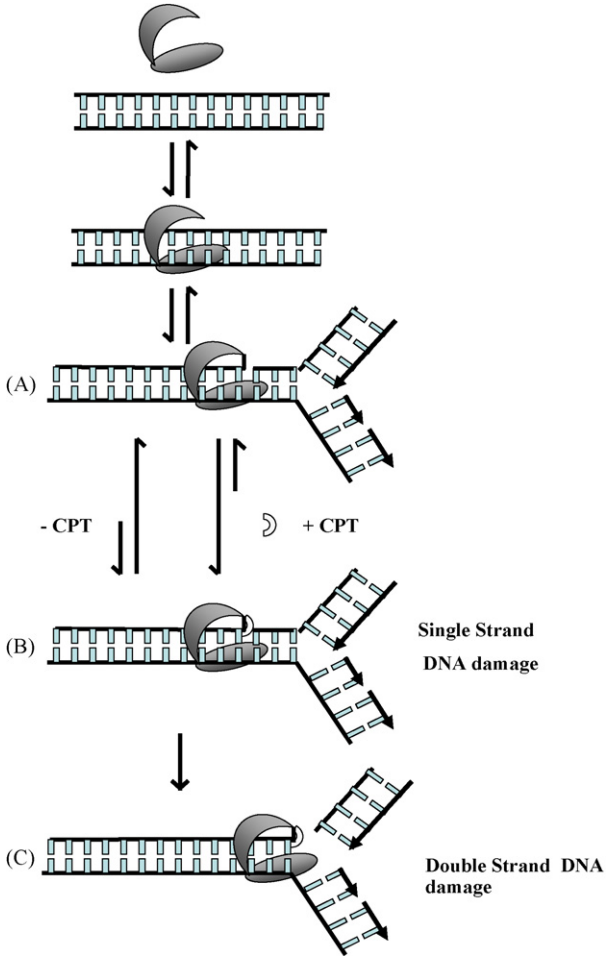

Fig. 1 – Mechanism of DNA damage mediated by topoisomerase I. (A) Cleavage reaction of topoisomerase I. (B) Drug-stabilized clavable complex. (C) Collision with the replication fork.

E, and with a single out-of-plane nitrogen atom of ring C connected with the slightly out-of-plane carbonyl group of ring D [2]. The CPTs can undergo a pH-dependent reversible interconversion between the lactone form and the ring-opened carboxylate. At neutral or physiologic pH, the equilibrium between the two species favours the carboxylate form (Fig. 2). A closed lactone ring strengthens the covalent structure of the CPT chromophore and its hydrolysis leads to relaxation of the system and to inversion of the position of the nitrogen atom in ring C, from up to down, relative to the plane of the chromophore molecule, similar to that induced by 20(*S*)-20(*R*)-CPT isomerisation [2]. The extracellular stability of the lactone form of CPT is critical for its anticancer activity. Indeed, the carboxylate form shows a poorer diffusibility through the lipid bilayer than the lactone form and binds preferentially to human serum albumin (HSA) [3]. Factors influencing the lactone-carboxylate equilibrium are clearly important determinants of CPT activity and the hydrolysis of lactone ring represents one of the major drawbacks of this class of agents. Although the only recognized target of CPTs is Top1, their complex polycyclic structure and the presence of various structural functionalities may confer ability to interact with other proteins which influence critically their pharmacological behavior. In the present commentary, we examine the multiple drug interactions which may influence the pharmacological properties of CPT and could be exploited to optimize therapeutic efficacy.

2. Interaction of camptothecins with plasma proteins

CPTs exist in an equilibrium between closed lactone and open carboxylate forms, which is dependent on the pH of the medium, interaction with blood components and likely other factors. The different lactone/carboxylate ratios in plasma of mice versus humans is the most convincing explanation of the interspecies variability of CPT efficacy. The pharmacological basis of this difference is the increased affinity of CPTs for HSA, which is the most important interaction with plasma proteins affecting the toxicity profile and efficacy of these drugs.

HSA is the most abundant plasma protein (5 g/100 ml) which has a high affinity for a wide range of drugs and metabolites. HSA consists of three homologous domains (I–III) probably derived through gene multiplication. A hydrophobic pocket is formed in domain II and is thought to be a binding site for many drugs. Although crystallographic analysis of CPT/HSA complex is not available, spectroscopy studies indicate that carboxylate CPT binds within the domain II [4]. In particular, interactions of CPT with HSA are supposed to include both hydrophobic contacts and an interaction of the CPT carbonyl group of ring D and the carboxylate function of hydrolysed ring E with charged amino acid residues of HSA [4].

The preferential binding of HSA with the carboxylate form of CPTs results in the more rapid opening of lactone ring (Fig. 3). The role of HSA in influencing the lactone/carboxylate equilibrium of CPT has been confirmed in many *in vitro* studies. Cell-free system experiments have shown that at

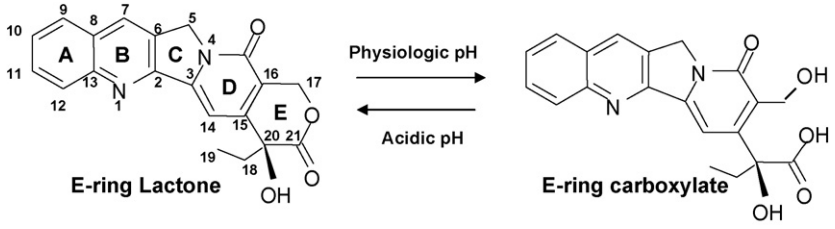

Fig. 2 – Chemical structure of the natural camptothecin and interconversion of the lactone E ring into to the carboxylate form.

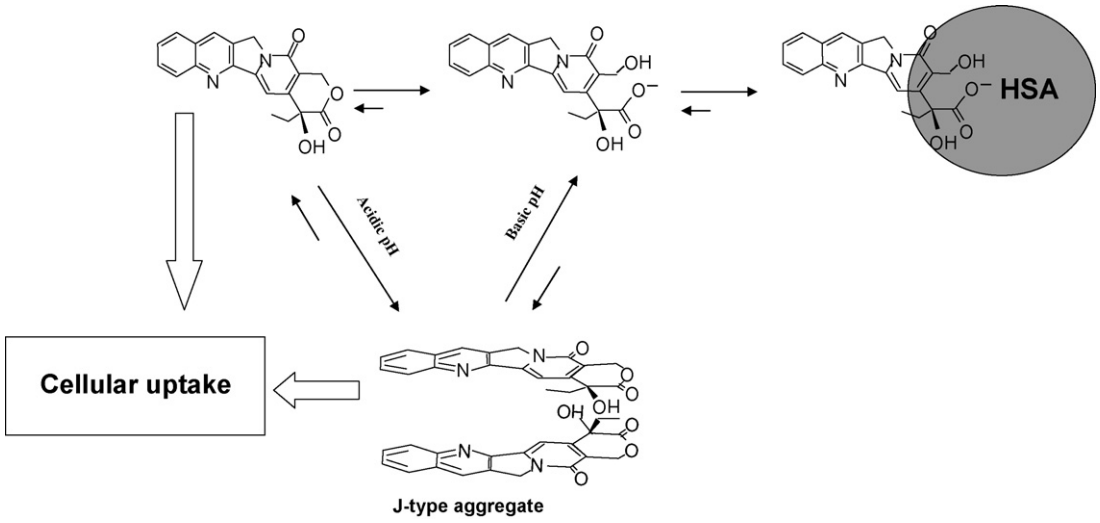

Fig. 3 – Influence of camptothecin structure on binding to human serum albumin (HSA) and on cellular pharmacokinetics. A schematic representation of camptothecin aggregates is shown.

37 °C in phosphate buffered saline, equal amounts of the various CPTs analogues are present as the pharmacologically active lactone form, with values of 17%, 19%, 15%, 13% and 15% for CPT, 9-aminoCPT (9-ACPT), topotecan (TPT), irinotecan (CPT-11), and SN-38, respectively [5]. Addition of 40 mg/ml HSA shifts the equilibrium for CPT and 9-ACPT towards the carboxylate form, with approximately 1% present in the lactone form at equilibrium [5,6]. In contrast to CPT and 9-ACPT, HSA actually stabilizes the lactone moiety of CPT-11 and SN-38, with 30% and 39% of the concentration, respectively, present as the lactone form at equilibrium, while almost no effect was seen for TPT [5–7]. These findings suggest that the differences in the percentages of the lactone form at equilibrium are related to steric features of the various substituents at the C7, C9, and C10 positions. These substituents cause sterical hindrance and prevent binding of the carboxylate forms to HSA, thereby driving the equilibrium towards the lactone species.

In addition to chemical modifications with specific substituents, physicochemical properties of CPTs play a role in modulating lactone reactivity. The introduction of lipophilic substituents at the 7-position have been reported to result in CPT stability by favouring lactone partitioning into the lipid bilayers of red blood cell membranes [8–10]. The incorporation of the 7-*t*-butyldimethylsilyl function increases membrane permeability and the dual 7-alkylsilyl and the 10-hydroxy substitutions of DB-67 enhance substantially blood stability, by altering interaction with HSA. Plasma stability and persistence has been reported for the lipophilic 7-oxyiminomethyl analog, gimatecan [11,12].

An additional effect influencing the biological activity of CPTs and their interaction with HSA is the formation of J-type aggregates (Fig. 3). Stable J-type aggregates are formed by the stacking interaction between the quinoline rings of the CPT chromophores with the inverse position of the nitrogen atoms and are thought to be dependent on an intermolecular bond implicating the hydroxyl group at position 20 [2]. Since aggregation is totally abolished with the 20(*R*) stereoisomer of CPTs, the *R*-orientation of the 20 ethyl/hydroxyl groups is thought to produce a steric hindrance which hinder the formation of aggregates. J-type aggregates are stable at acidic and neutral pHs and partially prevent hydrolysis of the lactone ring at neutral pH value. An increase in pH produces a parallel increase of monomeric species (Fig. 3). This feature is a general characteristic for all CPTs exhibiting a tendency to form aggregates. J-type aggregates penetrate within the cells with much higher efficiency than the monomers of the drugs (up to 10-fold) and dissociate within the cells possibly as a result of localization in subcellular compartments and interaction with Top1 [2]. Hence, the stabilization of the lactone ring at acidic/neutral pH by self-aggregation of the CPTs results in reduced HSA interaction and in enhanced cellular uptake of the drug.

A peculiar protein interaction is that produced by CPT-11 with esterase enzymes. A unique characteristic of CPT-11 is its bulky dipiperidino side-chain linked to the CPT molecule via a carboxyl ester bond. This hydrophilic side-chain, although providing water solubility, leads to a substantial reduction in cytotoxic activity as a consequence of reduced intracellular accumulation. Cleavage of the side-chain forms the metabolite SN-38 which is as much as 1000-fold more potent in inhibition of Top1 than CPT-11. The esterase enzymes implicated in CPT-11 activation are carboxylesterases and cholinesterases [13,14]. Previous reports have indicated that carboxylesterases are responsible for the cleavage of CPT-11 and increased levels of cellular carboxylesterase correlate with increased sensitivity of tumor cells to CPT-11 [15,16]. Among the cholinesterease enzymes, acetylcholinesterase and butyrylcholinesterase are known to interact with CPT-11. While CPT-11 inhibits acetylcholinesterase, butyrylcholinestarease, which is present in a large amount in plasma, acts by hydrolyzing the prodrug to SN-38 [13,14]. During intravenous administration of CPT-11 a cholinergic syndrome is frequently observed [17,18]. This includes rhinitis, increased salivation, miosis, lacrimation, diaphoresis, flushing, and intestinal hyperperistalsis. These symptoms can be rapidly alleviated by atropine, suggesting that the side effects result from interaction of the drug with acetylcholinesterase. The mechanism of cholinergic syndrome

has been ascribed to ganglionic stimulation [17,19]. This finding is based on the structural similarity between the bipiperidino side chain of CPT-11 and dimethylphenylpiperazinium iodide, a known stimulant of nicotinic receptors of autonomic ganglia.

3. Interaction of camptothecins with cellular proteins

3.1. *Cellular accumulation and subcellular distribution*

The mechanisms of cellular accumulation of CPTs are poorly defined. Only a few inconclusive studies have addressed the mechanisms of cellular influx of CPTs, suggesting that drug influx is mediated by both active and passive mechanisms. Carrier-mediated transport and passive diffusion are implicated in intestinal absorption of CPTs [20]. Active transport of TPT and SN-38 has been described in ovarian carcinoma cells [21].

The recognition of CPTs as substrates for ABC (ATP-binding cassette) transporters may have implications in their intestinal absorption, tissue distribution and pharmacological behaviour. However, membrane proteins belonging to the ABC superfamily have been shown to participate in an energy-dependent efflux of CPTs (Fig. 4) [22]. These transporters are extensively studied because their expression in tumor cells is responsible of a phenomenon, known as multidrug resistance, i.e. resistance to a variety of structurally and functionally distinct agents. Several multidrug transporters of the ABC family have important physiological roles, because they are implicated in protection against a wide range of drugs and xenotoxins by reducing the intestinal uptake and tissue penetration and by mediating active excretion of their substrates [23]. Although these multidrug transporters are not tumor-specific defense factors, their overexpression observed in tumor cells may have relevance as a determinant of chemosensitivity of some tumor types. The first identified transporter, for which a contribution to resistance to CPTs has been documented, is P-glycoprotein (P-gp) encoded by the ABCB1/MDR1 gene, but other members of the family such as multidrug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP) have been shown to confer resistance to CPTs [24–26]. Other transporters among the 49 ABC transporters recently identified in the human genome could also be involved in resistance to CPTs, but no conclusive data have been so far obtained [23]. Based on their sequence similarities, all known efflux transporters can be divided into seven classes (A–G) and members of four of these classes (A, B, C, and G) have been clearly proved to confer drug resistance on cultured cells [23]. Although all share a similar ABC, their domain structures are organized differently, with diverse numbers and locations of trans-membrane domains [26].

P-gp, the best known ABC transporter, is a 170 kDa protein that transports neutral and cationic hydrophobic compounds [23,27–29]. The charge of CPTs has been suggested to affect the interaction with P-gp. In contrast to uncharged CPTs derivatives, such as 9-ACPT, lurtotecan and DX-8951, the positively charged TPT was found to be recognized by P-gp [27,30].

The MRPs have also been recognized as playing a role in resistance to CPTs. Several members of the MRP transporter family are induced by cytotoxic drugs, including CPTs [31,32]. There are currently 10 members of the MRP family and at least seven of them (MRP1-7) confer resistance to one or more antitumor drugs. Like P-gp, MRP1 has been shown to be relevant in clinical drug resistance [33]. MRP1 exhibits substrate specificity similar to P-gp, but drugs recognized as substrates are frequently conjugated with glutathione and other anions, or are cotransported with glutathione [34,35]. Unlike P-gp, MRP1 transports anionic compounds. Recently, MRP4, originally identified as a cyclic nucleotide transporter,

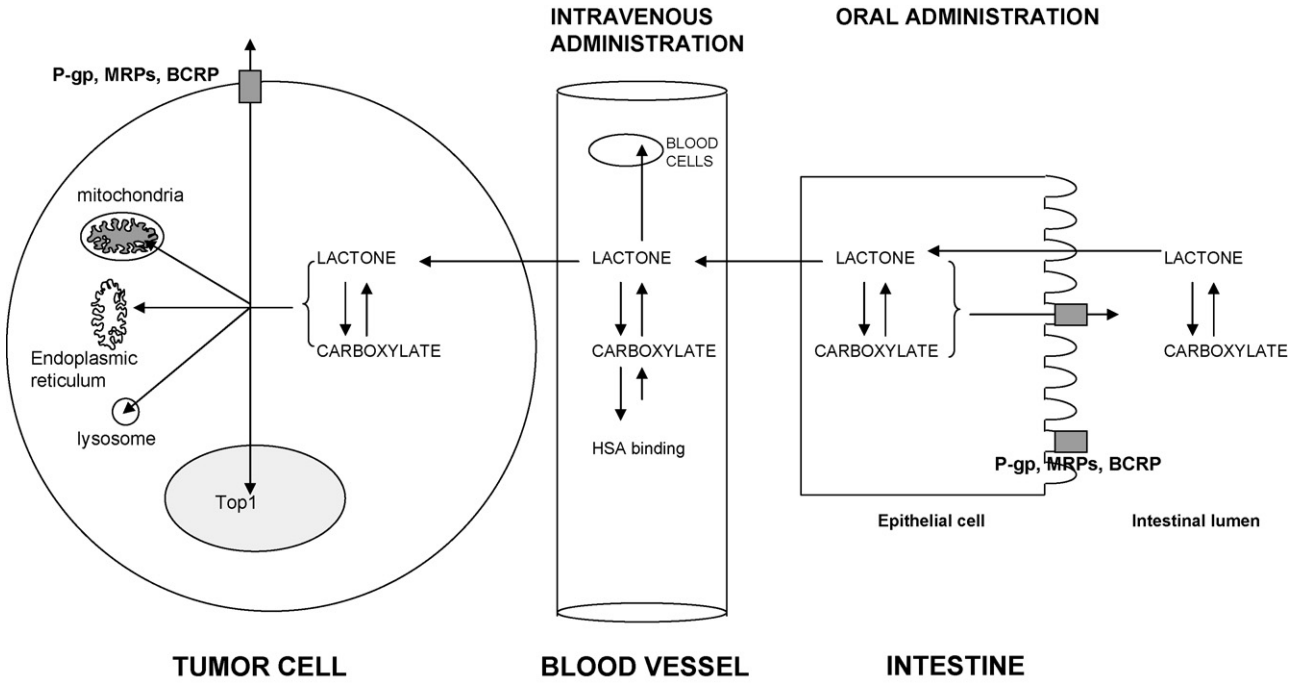

Fig. 4 – Schematic representation of the mechanisms implicated in camptothecins adsorption and distribution.

has been shown to confer resistance to CPTs including CPT-11 and TPT [36,37].

The 72 kDa BCRP is the second member of the subfamily G of the human ABC superfamily and thus also designated ABCG2. BCRP likely affect the oral absorption, tissue distribution and hepatobiliary intestinal elimination of drug substrates. Mutations in amino acid 482 of BCRP may occur in cells selected for resistance to doxorubicin, and this residue has been implicated in substrate interaction [38–41]. Unlike P-gp, which appears to transport unmodified drugs and xenobiotics, BCRP is an organic anion pump able to transport conjugates of sulfates, glutathione, and glucuronic acid and is very efficient in transporting glutathione conjugates [30,42]. Overexpression of BCRP confers resistance to TPT and SN-38, but not to natural CPT or lipophilic CPTs [43–47]. Structure-activity studies have indicated that CPT derivatives with potential for glucuronidation are better BCRP substrates than CPT itself [48]. Because these derivatives bear substitutions in the A and B rings, it has been suggested that BCRP could recognize the A and B ring portions [49]. Recently, homoCPTs have been found to be BCRP substrates, suggesting that the stabilized E ring also contributes to recognition by the transporter [50]. Interestingly, mutations in amino acid 482 (R482G, R482T) of BCRP confer stronger resistance to homoCPT and BN809105 than the wild-type transporter. Recent studies indicated that 9-ACPT and 9-NCPT are not recognized by P-gp, MRP1, and MRP2. In contrast, expression of wild-type or R482T BCRP confers resistance to 9-ACPT, but not to 9-NCPT [51]. In addition to 9-NCPT, other CPT analogues, such as lurtotecan (NX211), exatecan mesylate (DX8951), karenitecin (BNP1350) and gimatecan (ST1481) appear to be poor BCRP substrates [47,52]. However, the affinity of CPTs for BCRP does not appear to be directly related to the lipophilicity of the drugs. Indeed, NX211 and DX8951, which are poor substrates, are hydrophilic while BNP1350 and ST1481 are lipophilic. Studies aimed at defining the specific regions involved in the recognition of the different CPTs by transporters are expected to contribute to the development of novel analogues capable of overcoming specific mechanisms of drug resistance [49].

The cytotoxic effects and antitumor efficacy of CPTs are expected to be strongly dependent on the capability of the drug to reach the nuclear target Top1. The amount of CPT available for poison activity is influenced not only by intracellular accumulation, but also by retention and subcellular localization. A comparison between TPT and gimatecan revealed a different subcellular distribution, likely dependent on the different physicochemical properties of the two CPTs [53]. The hydrophilic TPT localizes in mitochondria and endoplasmic reticulum and this feature is associated to an acidification of these compartments. The lipophilic gimatecan exhibits a lysosomal localization and, since the lactone form is stabilized in the acidic environment of lysosomes, this compartment may represent a store allowing intracellular release of the active drug. Modulation of intracellular [54] and extracellular pH [55] has been reported to influence CPT activity likely as a consequence of changes in cellular pharmacokinetics and subcellular distribution. Indeed, modulation of intracellular acidification by inhibition of vacuolar-H^+-ATPase was found to cause a subcellular redistribution of TPT resulting in an increased drug localization in the nucleus [54]. On the other hand, acidic extracellular conditions could cause down-regulation of the efflux pump BCRP [55]. 7-Halomethyl and 10-amino CPT analogs have been reported to exhibit selective toxicity under acidic conditions [55].

3.2. *Interaction of camptothecins with topoisomerase I*

The cleavable complex is a transient intermediate of the reaction mediated by Top1, where the enzyme is covalently bound to DNA. Under these conditions, DNA is characterized by unbalanced energetic and structural features that create the binding site for the drug. The capability of CPTs to interact with cleavable complex, termed ''interfacial inhibition'', represents an extreme case of uncompetitive inhibition, in which the drug takes advantage of transient structural conditions that appear only in the presence of the macromolecular complex [56]. Since the S-phase cytotoxicity of CPTs is the consequence of the collision between cleavable complex and the replication forks, the stability of the DNA-Top1-CPT is a critical determinant of antitumor activity (Fig. 1). In this regard, the reversibility of the cleavable complex has stimulated many efforts aimed at improving its stability. The resolution of the crystal structure of the Top1-DNA-TPT ternary complex has elucidated the molecular determinants of CPTs implicated in trapping the cleavable complex [57]. The crystal structure of the ternary complex containing CPT instead of TPT reveals that CPT intercalates at the site of DNA cleavage with a binding mode similar to that of TPT [58]. A slight twist in the orientation of TPT relative to CPT along the vertical axis of the duplex DNA, which positions the C 7, 9 and 10 regions of the TPT into the major groove, could underlie the improved poison activity of TPT over CPT. Taking into consideration drug docking models, 7-substituted lipophilic and hydrophilic CPTs have been synthesized. The lipophilic analogues (e.g., gimatecan) exhibit favorable molecular and pharmacological features resulting in potential therapeutic advantages [11,59–63]. The promising pharmacological profile of analogs of this series likely reflects multiple favorable events including a rapid intracellular accumulation (an event which minimizes drug–plasma protein interaction) and a persistent stabilization of the cleavable complex. However, since recent studies have indicated the participation of water in the drug interaction with the enzyme-mediated cleavable complex providing stabilization, hydrophilic analogs able to form extensive hydrogen bond networks have been prepared [64–66]. The reported hydrophilic CPT analogs form stable cleavable complex with promising *in vivo* activity [64–66].

In particular, the pattern and the persistence of DNA cleavage observed *in vitro* with highly hydrophilic CPT-polyamine conjugates containing free amino groups support a contribution of drug interaction with DNA as a determinant of specific inhibitory effects [66]. However, the incorporation of a free polyamine chain in position 7 of the CPT resulted in a reduction of cytotoxic potency, indicating a limited intracellular accumulation of hydrophilic analogs.

It is generally believed that only the lactone form of CPTs is active. However, there is an interesting observation that the open carboxylate form can bind within the same intercalation pocket [57] or, when the CPT lactone enters the Top1-DNA

active site, E-ring opening occurs [67]. Some chemical modifications of the lactone ring, which shift the carboxylate/lactone equilibrium, support a critical role of the opened form in the mechanism of action of CPTs. Indeed, the sodium salt carboxylate form of CPT induces accumulation of Top1-DNA covalent adducts, whereas CPT ring E modifications which shift the equilibrium in favor of the closed form, such as replacement by an amide group, reduction or removal of the carbonyl oxygen, inactivate the molecule, thus implicating the presence of an intact CPT-type lactone ring as an essential requirement for the cytotoxic activity [68–70]. Relevant to the mechanism of drug interaction in the ternary complex is the observation that water-soluble CPT analogs containing a spermidine moiety linked to the 21-position of the open form through a stable amidic bond retained ability to poison Top1 [71]. In spite of a substantial reduction of the cytotoxic potency *in vitro*, the novel polyamine conjugate retained appreciable antitumor activity. Again, this finding is consistent with the interpretation that the cellular pharmacokinetics is a critical determinant of antitumor potency, because the closed lactone form favours drug uptake, but it is not an essential feature for inhibitory activity at target level. Indeed the closed lactone ring could be replaced by an alternative pharmacophore able to provide specific interactions in the intercalation pocket of the cleavable complex DNA–enzyme.

4. Concluding remarks

Although Top1 is the sole recognized therapeutic target of CPTs, it is now evident that multiple drug–protein interactions play an important role in determining antitumor efficacy and in modulating the therapeutic index. These non-target interactions are particularly relevant in oral delivery of CPTs, because the drug must cross additional biological barriers, i.e. intestinal absorption and hepatic filter, which could limit plasma concentrations (Fig. 4). The identification of CPTs with increased oral bioavailability [60] and the observation that the gastrointestinal apparatus may function after oral intake as a reservoir for the slow release of the drug in circulation emphasize the critical relevance of pre-target interactions which could influence the pharmacokinetic and pharmacodynamic behaviour following oral administration. On the basis of the peculiar mechanism of action (i.e., reversibility of the Top1-mediated single-strand breaks), the pH-dependent dynamics and *in vivo* reactivity, prolonged exposure *in vivo* and stability of CPTs in plasma are considered critical requisites for therapeutic efficacy. Therefore, the pharmacological behaviour (absorption, ability to cross biological barriers, plasma levels, rate of clearance) is expected to play a critical role in optimization of CPT-based therapy. A number of chemical modifications and drug delivery approaches have been reported in an attempt to stabilize the lactone ring and to improve the pharmacological behaviour *in vivo* [72]. In this effort, molecular designs based on structure–activity relationship studies of drug–target interaction, HSA binding and ABC transporter recognition are not obvious because of variable (and somewhat opposite) influence of specific drug features on these interactions. For example, the presence of hydroxyl groups at positions 10 and 11 in the ring A (Fig. 2) is known to prevent high-affinity binding of the carboxylate form to HSA [5–7], but confers recognition by BCRP [49]. New lipophilic analogs characterized by an improved profile in terms of increased stability of the lactone form in human blood and reduction of binding affinity with HSA and efflux pump transporters have been designed in an attempt to favour rapid cellular uptake, and to enhance the intracellular accumulation and the persistence of cleavable complex [72]. These features appear particularly relevant for orally administrated CPTs which have to overcome the intestinal epithelium before reaching the tumor site and the target enzyme in tumor cells [Fig. 4]. An additional advantage of the lipophilic nature of CPTs is the ability of the drugs to cross the blood–brain barrier and thus their potential activity against CNS tumors. On the other hand, CPT analogs containing an hydrophilic side-chain at the 7-position exhibited a remarkable ability to stabilize the cleavable complex [64–66]. However, these hydrophilic CPTs were also characterized by a reduced cytotoxic potency, likely related to a reduction of cellular uptake [65,66]. In addition, the physicochemical properties are expected to modulate several extracellular and intracellular interactions which could influence the pharmacokinetic behaviour (in particular, drug excretion) and subcellular distribution. It remains to be defined if the different drug localization in subcellular compartments (e.g., lysosomes) could confer some selectivity or therapeutic advantages [53,73]. The pH modulation in solid tumors as a consequence of chronic hypoxia and peculiar physiology of tumor cells may influence not only cellular pharmacokinetics but also the expression and function of relevant proteins (e.g., BCRP) which may affect cell sensitivity to CPTs.

In conclusion, the therapeutic efficacy and selectivity of CPTs reflect not only the inhibitory potency at target level but also the pharmacological behavior which is determined by multiple interactions. A better understanding of the determinants of drug–protein interactions may provide a basis for additional progress in improving the therapeutic potential of CPTs.

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