A Promising Camptothecin Derivative: Semisynthesis, Antitumor Activity and Intestinal Permeability.

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Abbreviations: CPT, 20-(S)-Camptothecin; CDiox, 9, 10-[1, 3]-dioxinocamptothecin; SAz, sodium azide; Pgp, P-glycoprotein 1; Annexin V-FITC/PI, annexin V–fluorescein isothiocyanate / propidium iodide

Abstract

Oral administration of camptothecin (CPT) derivatives and other antitumoral agents is being actively developed in order to improve the quality of life of patients with cancer. Though several lipophilic derivatives of CPT have shown interesting oral bioavailability in preclinical and clinical studies, only Topotecan has been approved for this route of administration. Semisynthesis, antitumor activity, biological inhibition

mechanism, and *in situ* intestinal permeability of 9, 10-[1, 3]-Dioxinocamptothecin (CDiox), an unexplored CPT derivative, have been studied in this paper. The hexacyclic analog was as effective as Topotecan and CPT in different tumor cell lines, showing an expected similar apoptosis cell mechanism and high ability to inhibit DNA synthesis in HeLa, Caco-2, A375 and MDA-MB-231 cell lines. Furthermore, *in vitro* and *in situ* pharmacokinetics transport values obtained for CDiox displayed more favourable absorption profile than CPT and Topotecan.

Keywords: dioxinocamptothecin, antitumor, camptothecin, oral, permeability, transport

Highlights:

a) Synthesis, citotoxicity and intestinal permeability of DioxinoCPT analog is presented

b) Permeability value of hexacyclic derivative was 5-fold higher than Topotecan

c) Permeability value of the hexacyclic derivative was slightly higher than CPT

d) DioxinoCPT is a potentially effective candidate for cancer treatment by oral administration

1. Introduction

Camptothecin (CPT), a natural alkaloid isolated from *Camptotheca acuminata*, was first reported by Wall et al. to have a potent antitumor activity against a broad spectrum of tumors [1, 2]. As soon as the identification of the enzyme Topoisomerase I (TopoI) as the major cellular target of CPT was confirmed (with the discovery of overexpressed levels of TopoI in tumor cells relative to normal cells), the elucidation of the structure-activity relationship of the alkaloid and the molecular mechanism of inhibition were priority in many investigations in the area of medicinal chemistry [3].

It is well established that successful inhibition of TopoI requires an unmodified lactone E ring moiety in the structure, however, the pH-dependent reversible equilibrium of the α-hydroxy-δ-lactone ring is shifted toward the carboxilate open-ring form at physiological pH or above [4]. CPTs are S-phase-specific drugs and the stabilization of the covalent TopoI-DNA complex by CPT is a required step in its antitumor activity. Because of that, prolonged or repetitive exposure of this kind of drugs is necessary to increase cell killing, since the S phase is a short phase of the cell cycle [5].

Several CPT derivatives have been developed to improve the lactone stability and to limit the uncontrolled toxic effects. Among them, Topotecan (Hycamtin®) [6] and Irinotecan (Camptosar®) [7] are the only two analogs approved for clinical practice. While Irinotecan is used exclusively by i.v. infusion, Topotecan is also administered by oral route for the treatment of relapsed small cell lung cancer (SCLC) in patients with a prior complete or partial response who are ≥45 days from the end of first-line chemotherapy [8].

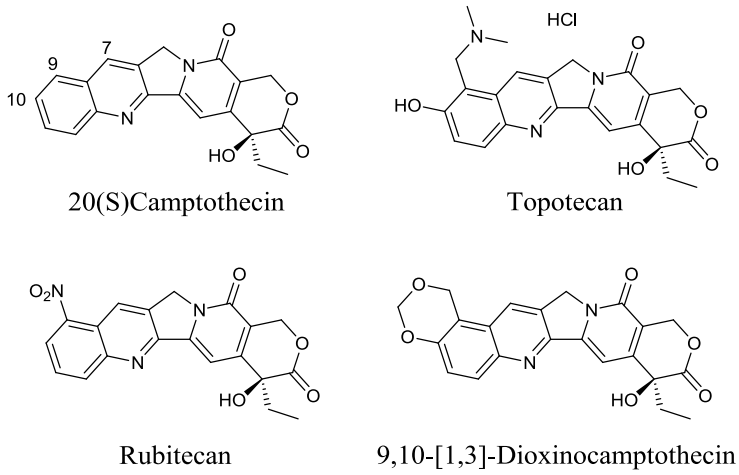

Figure 1. Camptothecin, Dioxinocamptothecin (CDiox) and representative analogues

Further studies focused on the nature of the CPT derivatives, argue that highly lipophilic analogues provide several pharmaceutical advantages relative to water-soluble such as lactone stability, lack of metabolic conversion, broad antitumor activity, oral bioavailability and optimized therapeutic efficiency [9, 10].

According to previous modifications of CPT, additional ring combination in positions 10-11, 7-9 and 9-10 showed potent antitumor activity, probably due to the extended planarity exerted by an additional hexacyclic-fussed ring [11]. Oxazines, amidines, furan and dihydrofuran rings fussed on CPT structure have been extensively studied with exceptional *in vitro* results [12, 13, 14]. 9,10-[1,3]-Dioxocamptothecin (CDiox), which can be regarded as a close structural variant of 9,10-(ethylendioxy) CPT, has not been considered for any prior study in spite of being as active as CPT in enhancing topo I-mediated cleavage of the DNA duplex [15, 16]. In addition, no violations of Lipinski rules, adequate polar surface area and a lipophilic cLogP value, complete a favorable theoretical calculation behavior for developing a more consistent study for this hexacyclic derivative [17].

On the basis of the lipophilic behavior of CDiox and the promising results published for other water-insoluble CPT derivatives [18], a preliminary study to establish the oral availability of this analogue is presented in this work. To elucidate permeability and transport mechanism, experiments were carried out using Caco-2 cells and *in situ* perfusion studies in rats.

2. Results and Discussion

2.1 Chemistry

CDiox was prepared by straightforward condensation of 10-hydroxycamptothecin with an excess of formaldehyde in neat triflic acid. Although the reaction is effective in solvents such as nitromethane, acetic acid and acetonitrile with catalytic amounts sulfuric acid or triflic acid at 50ºC, optimal conditions were obtained by performing the synthesis in neat triflic acid from 0ºC to room temperature overnight. The derivative was characterized by means of ^{1}H-NMR, ^{13}C-NMR, elemental analysis and exact mass. In the best of our knowledge no synthesis has been previously reported for this drug

2.2 Biological studies.

2.2.1 Cytotoxicity

The *in vitro* antitumor activity of CDiox was assayed by MTT method against five human cancer cell lines, including cervical cancer (HeLa), colon adenocarcinoma (Caco-2), malignant melanoma (A375), T cell lymphoblast (Jurkat), and breast cancer hormone sensitive cell line (MDA-MB-231). CPT as parent compound and Rubitecan and Topotecan as well known effective oral CPT derivatives were used as reference drugs. IC50 (μM) values are expressed in Table 1. The results indicate than CDiox is as potent as standard compounds across all the cell lines, revealing that the 1,

3-dioxine ring fussed on the CPT structure retains the antiproliferative activity. Results indicate that CDiox and Rubitecan are slightly more active against HeLa and Caco-2 cells than CPT and Topotecan, while, unmodified CPT is more effective against MDA-MB-231.

	HeLa	Caco-2	A375	Jurkat	MDA-MB231
CPT	0.420±0.025	0.131±0.011	0.187±0.005	0.110±0.007	0.342±0.015
Topotecan	0.380±0.011	0.119±0.008	0.162±0.012	0.127±0.009	0.473±0.023
Rubitecan	0.343±0.012	0.112±0.005	0.138±0.014	0.128±0.008	0.479±0.013
CDiox	0.345±0.013	0.104±0.004	0.186±0.007	0.124±0.010	0.476±0.018

Table 1. *In vitro* antitumor activity of CDiox analog and reference compounds against five human tumor cell lines, were measured by the MTT assay after 24h of incubation and expressed as IC50 (μM)

2.2.2 Flow cytometry assay. Apoptosis induction

Cell death mechanism was investigated using Annexin V-FITC/PI flow cytometry assay. Experiments allow analyzing whether CDiox, CPT, Rubitecan and Topotecan were able to induce apoptosis in Caco-2 cells at concentrations of 0.05μM and 0.5 μM after 24h of incubation. In order to determine if the apoptosis induced by the compounds is time dependent, assays were also carried out after 48 h of incubation.

As shown in Table 2, all tested compounds induced cell death mainly through apoptosis. Interestingly, the exposition of cell cultures to increased concentrations of the compounds significantly increased the number of apoptotic cells (early+ late apoptosis), while no important changes in the number of necrotic cells are observed (especially after 48h of incubation). CDiox activity is similar or even better than the compounds used as reference suggesting a promising anticancer candidate. The ability of

compounds to induce cellular apoptosis was in agreement with the results of the MTT assay.

Figure 2 shows an example of flow cytometry scheme for untreated cells and cells exposed to 0.05µM or 0.5 µM of CDiox for 48 h

		Vital cells An-/PI-	Early apoptosis An+/PI-	Late apoptosis An+/PI+	Necrosis An-/PI+
	Control cells	96.67±1.02	0.72±0.03	2.20±0.77	0,41±0.02
24h	CDiox 0.05µM	61.56±1.32	11.8±1.11	24.72±3.56	1.92±0.02
	CDiox 0.5 µM	47.78±1.47	8.16±0.67	42.88±3.43	1.18±0.03
	CPT 0.05µM	83.26±4.21	15.22±1.83	1.40±0.03	0.12±0.01
	CPT 0.5 µM	81.62±2.33	1.64±0.04	13.70±1.95	3.04±0.01
	Topotecan 0.05µM	71.40±3.84	5.42±0.03	19.54±2.35	3.64±0.02
	Topotecan 0.5 µM	53.08±1.53	4.40±0.02	33.98±1.72	8.54±0.04
	Rubitecan 0.05µM	65.04±1.78	11.08±0.02	21.03±1.16	3.85±0.05
	Rubitecan 0.5 µM	45.05±1.38	8.24±0.77	39.82±1.85	6.89±0.03
48h	CDiox 0.05µM	57.00±2.29	3.56±0.55	33.54±1.44	5.90±0.12
	CDiox 0.5 µM	37.04±1.39	3.68±0.03	56.08±2.56	3.20±0.05
	CPT 0.05µM	51.12±1.29	3.18±0.06	41.38±1.92	4.32±0.02
	CPT 0.5 µM	36.98±4.56	4.10±0.06	56.16±2.81	2.76±0.02
	Topotecan 0.05µM	45.20±1.25	2.36±0.01	48.06±1.73	2.36±0.01
	Topotecan 0.5 µM	38.26±2.92	5.74±0.02	53.12±1.88	2.88±0.01
	Rubitecan 0.05µM	49.24±1.15	2.90±0.02	42.9±1.88	4.96±0.01
	Rubitecan 0.5 µM	38.74±2.67	3.76±0.02	53.44±1.94	4.06±0.03

Table 2. Percentage of Caco-2 cells in each state after treatment with CDiox, CPT, Topotecan or Rubitecan for 24 or 48 h of incubation.

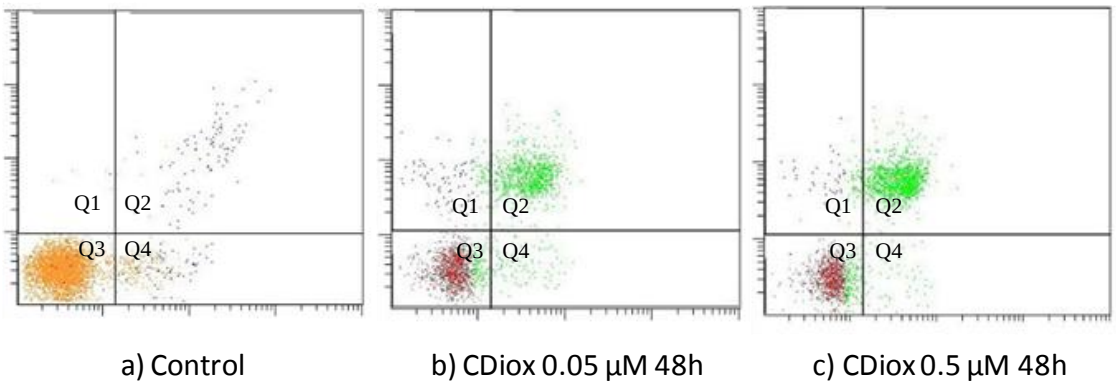

Figure 2. Representative flow cytometry scheme of apoptotic Caco-2 cells stained with annexin V–FITC and PI. *a)* Control untreated cells; *b)* cells treated with 0.05µM of CDiox for 48 h; *c)* cells treated with 0.5 µM of CDiox for 48 h. Q1 quadrant (necrotic cells). Q2 quadrant (late apoptotic cells). Q3 quadrant (normal cells). Q4 quadrant (early apoptotic cells).

2.2.3 Measurement of DNA synthesis

Since TopoI inhibition has been shown to be a feasible strategy to block cancer cell proliferation, the effect of the compounds on DNA synthesis was evaluated by measuring the incorporation of BrdU into the DNA of HeLa, A375, Caco-2 and MDA-MB-231 cancer cell lines. Results, summarized in Figure 3, showed that all CPT derivatives, including CDiox, exhibited high ability to inhibit DNA synthesis in HeLa, A375, Caco-2 and MDA-MB-231 cell lines. Reduction values of BrdU incorporation can be considered similar for CDiox and the reference compounds when compared the effect at 0.8 µM. At higher concentration (1.6 µM) the activity of reference drugs is higher, showing a concentration-dependent effect. These results indicate that CDiox is a potent analogue with similar activity to CPT, Topotecan and Rubitecan. Moreover, it is remarkable the strong inhibition of DNA synthesis observed for 1.6 µM of CDiox in HeLa and Caco-2 cell lines.

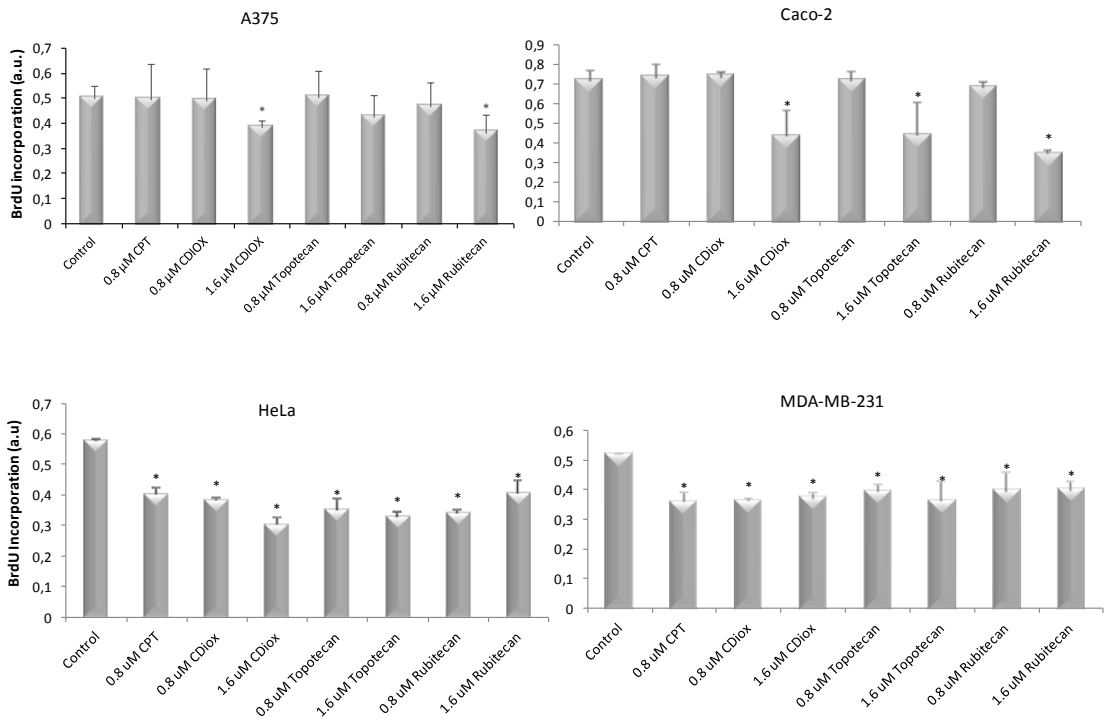

Figure 3. DNA synthesis measured by the BrdU incorporation assay (Roche) after incubation of cancer cells with different amount of compounds CPT, CDiox, Topotecan, and Rubitecan. a) in A375 melanoma cells. b) in Caco-2 cells. c) in HeLa cells. d) in MDA-MB-231 cells. BrdU incorporation values are the mean from three separate experiments with triplicate samples. * $p<0.05$, statistically significant difference from control value

2.2.4 Apoptosis pathway: evaluation of the role of p53 Pho-p53

In order to obtain more insights about the apoptosis pathway, the expression of the pro-apoptotic p53 and its activated form pho-p53 (Ser15) proteins were analyzed in the tumor panel cells lines treated with CDiox, CPT, Topotecan and Rubitecan for 24 h. Figure 4 reflects the results obtained from the Western blot assay. The results were referred to a negative control (without treatment). Coomassie blue stained gel was used to show that equivalent amounts of proteins were charged in all SDS–PAGE lines.

Results indicate that the four compounds were able to induce phosphorylation of p53 (at Ser15) in MDA-MB-231 cells (Figure 4). In A375 cell line, p53 was phosphorylated by Rubitecan, CDiox and Topotecan and only slightly by CPT (figure 4). Data showed that the apoptosis induce by CDiox compound associated with increased pho-p53 levels is similar in magnitude to Rubitecan and Topotecan in MDA-MB-231 and A375 cancer cells. This pathway mediates the activation of the apoptosis in MDA-MB-231 and A375 cells but not in Caco-2 and HeLa cells.

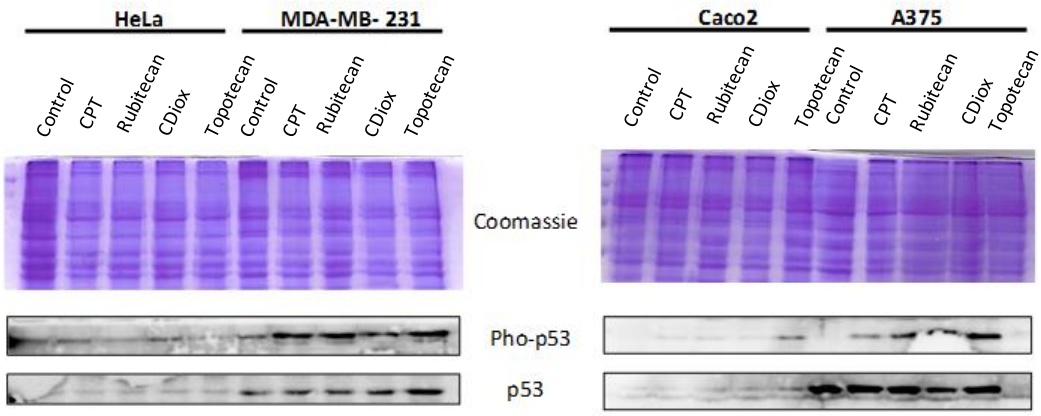

Figure 4. The p53 tumor suppressor protein activation in HeLa, MDA-MB-231, Caco-2 and A375 after different compound treatments. Activation of p53 was monitored by measuring phosphorylated (Ser15). P53 is phosphorylated by ATM, ATR and DNA-PK after signaling of DNA damage responses in order to block cell cycle and activate apoptosis.

In addition, all compounds induced p53-independent apoptosis in HeLa and Caco-2 cells. These data are in good agreement with the results obtained by cell cytometry. In this regard, Caco-2 cells have been shown to contain a G→T transition at codon 204, resulting in a truncated protein form in the central domain. Thus, we did not found p53 signal in Caco-2 cells [19].

This is an interesting finding that suggests two different apoptotic-induced mechanisms by these compounds depending on the cell type studied.

2.2.5 In vitro permeability

CPT, as parent compound, and Topotecan, as the only camptothecin derivative approved for oral administration, were selected to be compared with CDiox to measure *in vitro* permeability values using Caco-2 monolayer in both apical to basal (Pab) and in basal to apical (Pba) directions at different concentrations.

Results are presented in Figure 5. Pab represents the permeability value in the physiological sense (from intestinal lumen to plasma) and Pba represents the hypothetical value of the permeability from the plasma to the intestinal lumen. Pba and Pba/Pab ratio values have not physiological application but they are very useful parameters in order to elucidate the mechanistic aspects of transport of the compounds.

Permeation assays indicate that there are two different patterns of transport across the Caco-2 monolayer: one for Topotecan and another for CPT and CDiox

Regarding to Topotecan, permeability value (Pab) is extremely low despite its effectiveness in oral administration. Moreover, Pba/Pab ratio is clearly higher than one, which is in the common pattern of efflux substrates and it is in accordance with the results described in literature for this compound [20, 21]. Pab is higher when Topotecan concentration increases due to the saturation of the secretion transporter, while Pba values decrease with increasing concentrations.

Nevertheless, CPT and CDiox showed a different behavior. Permeability values of CPT and CDiox decreased with increasing concentrations but in both directions (Pab and Pba). This fact could indicate the contribution of transporters in both directions [22]. From Figure 5 it is possible to observe that at higher concentration (10, 25 and 50 μM) permeability values of

CDiox are higher than CPT indicating a more effective absorption behavior for the hexacyclic derivative.

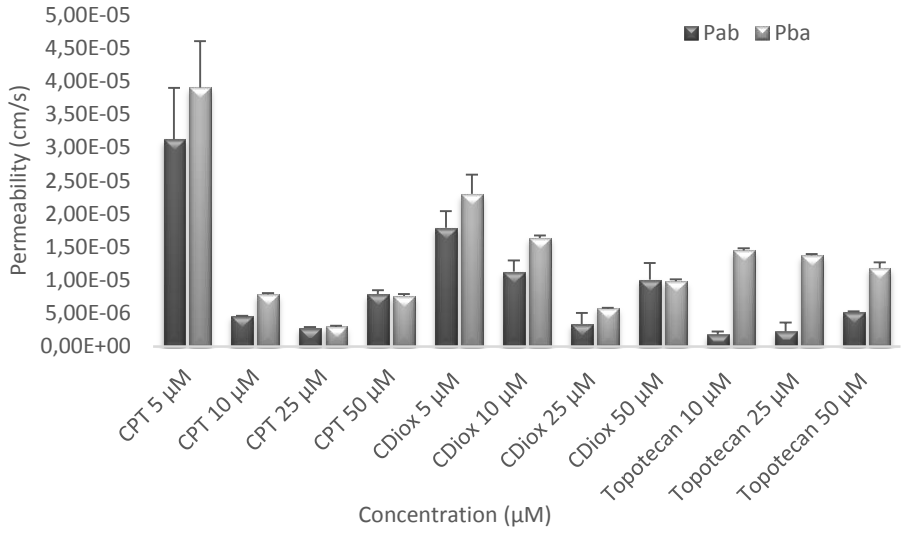

Figure 5. Permeability values obtained from apical to basal (Pab) and from basal to apical (Pba) at different concentrations in Caco-2 cell line. Data correspond to values of three independent experiments

It is well-known that the main limitation of Topotecan is that it is substrate of efflux transporters such as Pgp and BCRP [23, 24]. As consequence, the oral fraction absorbed of orally administered Topotecan is less than 45% and it has been observed high interindividual variability in plasma levels after oral administration.

In order to evaluate if CDiox is substrate of energy dependent transporter permeability, assays were carried out in presence of Sodium azide (SAz) as metabolic inhibitor [25]. The presence of SAz leads to inhibition of the active carrier mediated processes by depletion of ATP resources in the cells. Results in presence of the metabolic inhibitor show that permeability values of CDiox (Table 3) did not suffer significant changes, indicating that

transport of CDiox is not mediated by an active transporter in these assay conditions. In conclusion, the permeability behavior of CDiox obtained from *in vitro* assays indicates that this compound is a good candidate to be administered by oral route

	Peff (cm/s) in absence of SAz	Peff (cm/s) in presence of SAz
CDiox (Pab)	$1.00 \cdot 10^{-5} \pm 8.45 \cdot 10^{-7}$	$8.05 \cdot 10^{-6} \pm 5.02 \cdot 10^{-7}$
CDiox (Pba)	$9.84 \cdot 10^{-6} \pm 5.43 \cdot 10^{-7}$	$9.73 \cdot 10^{-6} \pm 4.87 \cdot 10^{-7}$

Table 3: Permeability values of CDiox at 50µM in presence and in absence of Sodium Azide (SAz)

The determination of permeability parameter (Peff) in preclinical studies is a crucial step to determine the suitability of the candidates for oral administration in early stages. Figure 6 and table 4 show the oral fraction absorbed data (Fa) predicted for CDiox and the reference compounds based on the permeability values obtained experimentally and on the previously validated Peff-Fa correlation. This correlation has been obtained with drugs in solution, thus it represents the maximum oral fraction absorbed achievable in absence of solubility or limited solution.

	Peff (cm/s)	Fa
CPT	$7.88 \cdot 10^{-6}$	0.80
Topotecan	$1.77 \cdot 10^{-6}$	0.30
CDiox	$1.00 \cdot 10^{-5}$	0.87
Metropolol	$2.30 \cdot 10^{-5}$	0.99

Table 4. Predicted values of oral fraction absorbed (conc. 50µM)

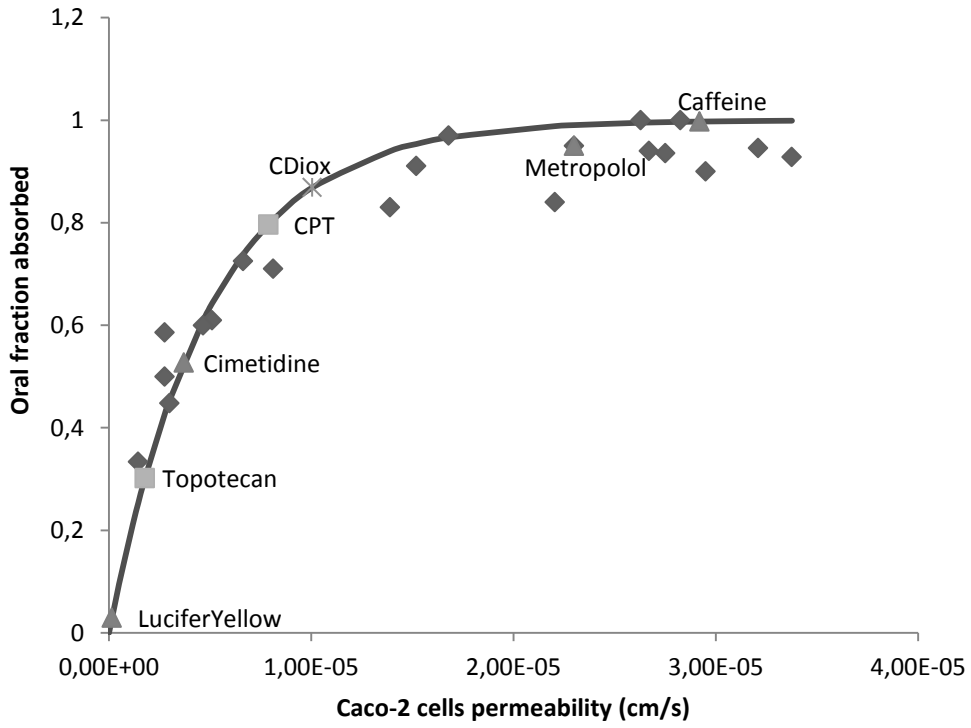

Figure 6. Oral fraction absorbed *vs* Permeability values through Caco-2 monolayers obtained from apical to basal direction (Pab) at 50 µM concentration of the compounds assayed. Grey cross correspond on CDiox values, grey squares correspond on CPT derivative compounds. Diamonds data correspond to the internally validated correlation between Caco-2 permeability and oral fraction absorbed (unpublished data). Grey triangles correspond to reference compounds of high (caffeine and metroprolol), intermediate (cimetidine) and low permeability (lucifer yellow). Data correspond to values of three independent experiments.

A high oral fraction absorbed (higher than 50%) is necessary to be a potential oral drug candidate. Lower values would difficult the development of an oral product and lead to the selection of other extravasal (intramuscular) or intravenous route. Preliminary results indicate that CDiox is a good candidate for oral administration. Its permeability value at 50 µM is 5-fold higher than Topotecan and slightly higher than CPT, showing no (or minimum) affinity for Pgp, which could be a favorable behavior due to this transporter is a common resistance mechanism in tumor tissues.

2.2.6 In situ permeability. Rat perfusion assay

In situ outcomes, carried out using perfusion rat model, are in accordance with obtained from cell culture assays.

Absorption rate constants, Ka, and Peff values, are higher for hexacyclic derivative than for CPT and Topotecan. In addition, CDiox displays much better absorption profile than a well known high oral permeability standard Metropolol. (Table 5 and Figure 7).

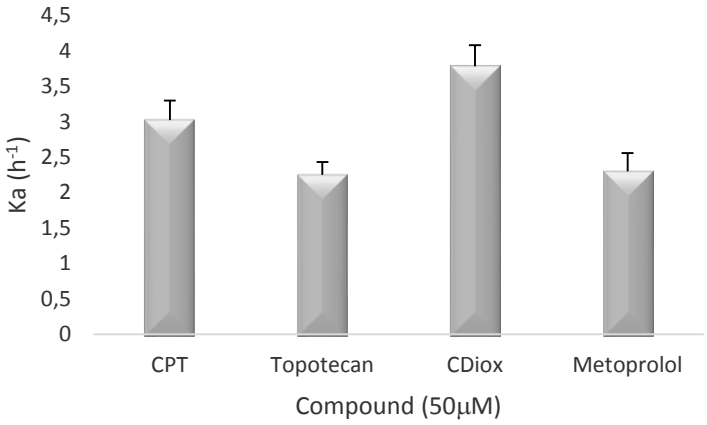

Figure 7. CPT, Topotecan and CDiox absorption rate coefficients in rats.

	Peff (cm/s)	Ka (h^{-1})
CPT	$7{,}48\cdot10^{-5} \pm 7{,}44\cdot10^{-6}$	3,02±0,30
Topotecan	$5{,}58\cdot10^{-5} \pm 4{,}45\cdot10^{-6}$	2,25±0,18
CDiox	$9{,}38\cdot10^{-5} \pm 6{,}83\cdot10^{-6}$	3,78±0,27
Metoprolol	$5{,}53\cdot10^{-5} \pm 6{,}13\cdot10^{-6}$	2,30±0,25

Table 5. Absorption rate coefficients, Ka, and permeability values obtained *in situ* in rat. Metoprolol is used as model compound of high oral permeability. Data correspond to values of six independent experiments

3. Conclusion

The hexacyclicCPT derivative, 9, 10-[1,3]-Dioxocamptothecin (CDiox) has been synthesized and evaluated *in vitro* for its antitumor activity and oral biodisponibility. CDiox showed citotoxicity comparable to other topoisomerase I inhibitors reference compounds as CPT, Topotecan and Rubitecan producing DNA synthesis inhibition and apoptosis induction. Transport studies, carried out using Caco-2 cell monolayers and *in situ* perfusion in rat, indicated that the permeability is clearly better than for Topotecan, thus ensuring a higher oral fraction absorbed. Moreover, mechanistic studies showed that CDiox is not a Pgp substrate, which is an important goal for an anticancer drug as it ensures less variable drug absorption and broad tissue distribution as well as much better penetration to cancer cells. CDiox showed a promising antitumor profile and improvement as an effective drug for oral administration

4. Experimental protocols

4.1 Chemistry

4.1.1 Materials and instruments

All reagents and solvents were reagent grade. 10-Hydroxycamptothecin was purchase by AKScientific. Formaline, triflic acid, H_2SO_4, and standard solvents were purchase from Sigma Aldrich. Column chromatography was carried out on silica gel (Merck 200-300 mesh). TLC analysis was elaborated on silica gel plates (Merck $60F_{254}$). ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 at 300MHz with a Bruker instrument. Mass spectra were recorded using an Acquity UPLC system (Waters Corp.) The separation was carried out on a C18 column (50 mm × 2.1 mm i.d., 1.7μm). The analysis was achieved with gradient elution using methanol and water (containing 0.01% formic acid) as the mobile phase. The

injection volume was 2µL. The spectrometer was connected to the UPLC system via electrospray ionization (ESI) interface. The ESI source was operated in positive ionization mode with the capillary voltage at 3.0 kV

4.1.2 Chemical synthesis

To a neat mixture of solid 10-hydroxycamptothecin and formaldehyde solution (36%) was added drop wise triflic acid at 0ºC. After being stirred for 12h at room temperature, ice water was added and the precipitated was purified by column chromatography to give CDiox

Silica gel chromatography (eluent: CH_2Cl_2/MeOH 95:5).Yellow powder. Yield: 95%. ^{1}H-NMR (300 MHz; DMSO-d_6) δ: 0.88 (t, 3H, -CH_3, J=7.4 Hz); 1.80-1.93 (m, 2H); 5.19 (s, 2H); 5.30 (s, 2H); 5.41 (s, 4H); 6.49 (s, 1H, -OH); 7.27 (s, 1H); 7.43 (d, 1H, J=9.2Hz); 7.80 (d, 2H, J= 9.1Hz); 8.39 (s, 1H). ^{13}C NMR (300 MHz, DMSO-d_6) δ- 7.76, 30.2, 50.35, 53.41, 65.22, 72.37, 95.78, 118.08, 118.94, 122.55, 127.19, 128.96, 129.60, 130.03, 143.53, 145.90, 148.88, 150.02, 153.71, 156.85, 172.51. HRMS (m/z): calcd. for $C_{22}H_{18}N_3O_6$ (M + Na): 429.1063, found: 429.1063. Anal. Calcd. for $C_{22}H_{18}N_2O_6$ (429.10): C, 61.54; H, 4.23; N, 6.52. Found: C, 61.54; H, 4.23; N, 6.52.

4.2 Biological assays

Cell culture conditions attached to Supp. Inf.

4.2.1 Cytotoxic studies

The in vitro antiproliferative activity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[26]. Cells were seeded in a total volume of 200 µL of culture medium in 96-well microtiter plates with flat-bottomed wells at a density of 25000 cells per well. Cultures were incubated at 37 ºC in a 5% carbon dioxide

atmosphere. 24 h later, the medium was replaced with a fresh medium and compounds at increasing concentrations were added. Samples solutions were prepared in DMSO 0.1% or miliQ water in the case of Topotecan. Plates were incubated for 24, 48, or 72 h and then, 20 µL of MTT solution (5 mg/mL) were added to each well. The samples were incubated at 37ºC for 3 h to allow reaction for the formazan formation. Absorbance was measured at 570 nm with a Labsystems Multiskan EX plate reader and the values were corrected with reference to the measurement at 630 nm.

4.2.2 Flow cytometry assay

The nature of compounds effects on the cells was determined with the aid of flow cytometry, after the double staining with propidium iodide (PI) and annexin V–fluorescein isothiocyanate (annexinV) [27]. Cells were treated with CDiox or the references compounds (CPT, Topotecan and Rubitecan) at 0.05 or 0.5 µM and then were stained with annexinV and PI. AnnexinV has high affinity for the membrane phospholipid phosphatidylserine and, for this reason, is useful to identify apoptotic cells which exposed phosphatidylserine. PI is a standard staining compound that allows distinguishing viable from nonviable cells. Viable cells exclude PI, whereas the membranes of dead or damaged cells are permeable to PI. The double staining allows us to identify four groups of cells: a) cells that exclude both staining molecules are alive, b) cells that stain positive for annexinV and negative for PI are undergoing apoptosis, c) cells that stain positive for both annexinV and PI are in the end stage of apoptosis and d) cells that stain negative for annexinV and positive for PI undergoing necrosis or are already dead.

The flow cytometry analyses were carried out by using a Beckman Coulter EPICS XL-MCL cytometer. Cells were stained using the annexin V–

fluoresceinisothiocyanate apoptosis detection kit (Roche) that provides a double staining with PI and annexinV [27].

4.2.3 Measurement DNA synthesis

Proliferation of cell lines was determined with the "Cell proliferation ELISA BrdU colorimetric assay" from Roche. Cells were cultured in 96 wells plates at density of 25,000 cells/well. 24 h after seeding, the compounds were added to the corresponding wells. After 24 h of incubation the cell lines were fixed and the immunoassay was performed according the manufacturer's protocol. The reaction was measured with the spectrophotometer Spectra MAXPLUS 384 from Molecular Devices, at wavelengths of 370-492 nm and 3 intervals of 5 min. Differences in absorbance values are proportional to the incorporation of BrdU.

4.2.4 Total protein extraction

Total protein extracts were extracted from approximately 10^6 cells lysed in ice for 15 min using 100 µL of lysis buffer (20 mM Hepes, pH 7.4, 1% Triton X-100, 100 mM NaCl, 50 mM, NaF, 10 mM, β-glycerophosphate, 1 mM activated sodium orthovanadate, 1 mM PMSF and 2 µL/mL protein proteases inhibitor cocktail). Then, the suspension was spun-down at 13,000 xg for 10 min at 4 ºC, and supernatants were collected and stored at -80 ºC until their use. Protein content was determined by a modified Lowry method [28].

4.2.5 Western blot analysis

Western blotting is an important technique used for identifying specific proteins from a complex mixture of proteins obtained from cells. Aliquots of cell lysates (30 µg protein) were added to sample buffer containing 10% β-mercaptoethanol and the mixture was immediately boiled during 5 min.

Proteins were separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide (12%) gels (SDS-PAGE), at 100 V during 2 h. After that, the proteins were electroblotted (Bio-Rad) onto nitrocellulose membranes. Membranes were blocked with 0.05 g/ml non-fat milk or with 0.05 g/ml BSA in TBS containing 0.2% Tween 20 (TBST), depending on the antibody, washed three times at room temperature, and incubated for 2 h at room temperature with primary antibodies diluted in TBST with 0.01 g/ml non-fat milk as follows, hsp70 (1:1000, Stressgene), phopho-p53 (1:1000, Abcam), p53 (1:1000, Calbiochem), and bax (1:1000, GeneTex). As loading controls, antibodies that recognize α-tubulin or α-actin (1:1000, Santa Cruz BioTech. USA) antibodies were used. The blots were washed again with TBST and further incubated for 1 h with a secondary mouse, rabbit or goat antibody conjugated with horseradish peroxidase. Blots were incubated with ECL™ Western Blotting Detection Reagents as indicated the manufacturer protocol (Amersham GE HealthcareBio-Sciences AB, Uppsala, Sweden). Chemioluminescent signals were assessed using a General Electric´s scanning densitometer (LAS-4000).

4.3 Transport studies

4.3.1 Cell monolayer cultures

Cell monolayers were prepared by seeding 400,000 cells on each polycarbonate membrane placed in the base of transwell inserts (MILLICEL®-PCF, surface area 4.2 cm^2, 0,4 μm pore size) [29, 30]. Cultures were grown in Dulbecco's modified Eagle's media, as described previously until confluence during 19-22 days maintaining the cultures at standard conditions of 37°C under 90% humidity and 5% CO_2 [31]. The integrity of each cell monolayer was tested by measuring its transepithelial

electrical resistance (TEER) value before and after each transport assay. Normal TEER values for Caco-2 monolayers were ranging 500-750 $\Omega \cdot cm^2$ [32]. Cell monolayers with TEER values less than 420 $\Omega \cdot cm^2$ were discarded. Hank's balanced salt solution (HBSS) (9.8 g/L) supplemented HEPES (5.96 g/L) was used for all the experiments after adjusting pH to the desired value.

4.3.2 In vitro permeability determination

The *in vitro* transport study was developed using the Caco-2 cell monolayers (ATCC) with an appropriate trans-epithelial electrical resistance (TEER) value.

Tested compound solutions were loaded into the donor side and buffer was added to the receiver chamber of each well. The six-well plate containing the inserts with the cell monolayers was put into an orbital environmental shaker maintained at a constant temperature (37 °C) and agitation rate (50 rpm) during all the experiments. To carry out transport assays in presence of SAz, it was always placed on both sides of the cell monolayer. Four samples of 200 µL each were collected at 15, 30, 60 and 90 minutes in the receiver chamber and the volume was replaced each time with fresh buffer. Moreover, two samples of 200 µL each were taken at the start and the end of the assay from the donor chamber.

HPLC analysis and pharmacokinetics parameters are described in Supp. Inf.

4.3.3 In situ assays: rat perfusion studies

Male Wistar rats were weighted after two hours fast with access to water. Rats were anesthetized using a mixture of diazepam (Valium, Roche) (1.67 mg/kg), ketamine (Ketolar; Parke-Davis) (50 mg/kg) and atropine (atropine

sulfate; Braun) (1 mg/kg) and placed on heated surface to be maintained at 37˚C.

The surgical procedure consists on a midline abdominal incision in order to create a compartment in the small intestine with the aid of two syringes and two three-way stopcock valves. The bile duct was closed before the perfusion to prevent enterohepatic recycling and the presence of bile salts in lumen. Studies employed the entire small intestine. The drug solution is placed in the intestinal segment with the aid of the syringes and samples of the intestinal fluid are withdrawn at predefined times to analyze the remaining drug concentration. Test solutions at 50 µM of the reference and test compounds were prepared freshly before each use. Sampling of the perfusate into glass tubes was carried out at fixed times, after 5 min at intervals of 5 min up to 30 min.

Water reabsorption studies and permeability values are described in Supp. Inf.

Acknowledgement

G.R.-B. ackowledges Instituto de Tecnología Química for a scholarship; Consolider-Ingenio 2010 (proyecto MULTICAT), Subprograma de Apoyo a Centros de Excelencia Severo Ochoa, and Agencia de Gestió d´Ajuts Universiteris i de Recerca (2009 SGR 758, to S.S.) I.G.-A. , V.M.-S., M.G.-A. and M. B. acknowledge financial support from the European Financial Commission (Red bioFarma DCI-ALA/19.09.01/10/21526/245-297/ALFA III (2010). J.L.G.-G. acknowledges the financial support of the CIBERER (Biomedical Network Research Center for Rare Diseases) ISCIII.

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