Delivery of Camptothecin to Cancer Cells with Silica Nanoparticles

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ABSTRACT

We present a novel and robust delivery platform for camptothecin (CPT) based in silica nanoparticles (SNP) of 5-15 nm diameter that transport the drug attached by ester bond in 20-OH position. The surface of these SNP is functionalized with carboxylic groups (SNP-COOH). These are ionized at physiological pH, thus, provoking electrostatic repulsion and avoiding particle aggregation, which allows the preparation of stable colloids in aqueous medium. Camptothecin-loaded silica nanoparticles (SNP-CPT, 7.5 wt% CPT) show high stability in human serum, with less than 15% hydrolysis in 24 h. Citotoxic activity of CPT, SNP-COOH and SNP-CPT by MTT assay over HeLa, U87MG, HCT-116 and MDA-MB-231 cell-lines, indicates that SNP-CPT induces cell death at slightly higher concentration than the free drug in the four lines tested, whereas CPT-free SNP-COOH do not alter cell proliferation or morphology.

Keywords: cancer treatment, drug delivery, camptothecin, silica nanoparticles

1 INTRODUCTION

Although the 20(S)-camptothecin (CPT) has shown a broad range of anticancer activity [1], the clinical application has not been achieved due to the poor aqueous solubility and low stability at physiological pH of the lactone form, the only one effective against carcinomas (see Figure 1) [2]. At this point, it has been reported that substitution on 20-OH of CPT can substantially reduce the tendency for lactone ring opening [3]. For this reason, covalent attachment of CPT via 20-OH has been done on different water-soluble polymers as polyethylenglycol [4], poly-*N*-(2-hydroxypropyl)methacrylamide [5], and poly-L-glutamic acid [6]. However, only in some cases these conjugates allow solubilizing the therapeutic agent, as well as preparation of pharmaceutical formulations able to perform a controlled release of the drug. Particularly relevant is the work of Davis' group, who synthesized linear, water-soluble, high molecular weight (over 50 kDa),

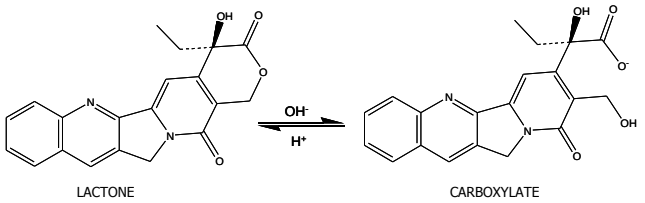

Figure 1: CPT structure and pH-equilibrium between the lactone (active form) and the carboxylate (non-active form).

cyclodextrin-based polymers that contain pendant carboxylate groups and covalently conjugated CPT to them in 20-OH position. These systems have shown high efficiency as antitumor therapeutics both *in vitro* and *in vivo* [7-10], and their main limitations include a lower activity than the free drug and a dramatic accelerated release of CPT in plasma.

Despite its recent development, the use of inorganic nanoparticles in biomedical applications has found rapid progress in the last decade, in particular, as contrast agents for MRI [11], and as vehicles for delivery of drugs and biomolecules [12]. In this sense, mesoporous silica nanoparticles (MSN) have found application for intracellular delivery and controlled release of small therapeutic molecules [13]. Recently, the group of Zink presented a system for the intracellular delivery of CPT based in MSN that incorporated the drug into the pores and delivered it into a variety of cancer cells to induce cell death [14-15]. The protection of the surface of nanoparticles with phosphonate groups provides hydrophillicity to the submicron structure, thus, leading to a stable colloid in aqueous medium. However, as CPT release does not depend on a specific intracellular stimuli (just molecular diffusion through the pores), premature release of the therapeutic agent in plasma is expected.

Here we propose a novel and robust delivery platform for CPT based in SNP of 5-15 nm diameter that transport the drug attached by ester bond in 20-OH position, according to the sequence shown in Fig. 2. The surface of these SNP is functionalized with carboxylic groups. These are ionized at physiological pH, thus, provoking electrostatic repulsion and avoiding particle aggregation, which allows the preparation of stable colloids

in aqueous medium.

2 EXPERIMENTAL

Commercial SNP of 5-15 nm diameter, CPT, PBS, human serum, anhydrous solvents, HPLC grade solvents, and other common organic reagents were all supplied by Sigma-Aldrich.

Citotoxic activity of CPT, CPT-COOH and SNP-CPT was tested by MTT assay over 4 cell lines: HeLa (cervix carcinoma), U87MG (glioblastoma), HCT-116 (colon cancer) and MDA-MB-231 (breast cancer). Cells were cultured in RPMI (HeLa, HCT-116 and MDA-MB-231) or DMEM media (U87MG) supplemented with 10% fetal bovine serum and 200 mM L-glutamine, all from Lonza Ltd.

2.1 Synthesis of Camptothecin-Loaded Silica Nanoparticles (SNP-CPT)

Before incorporation to SNP, CPT was modified in 20-possition by synthesis of 20-*O*-trifluoroglycinylcamptothecin (Gly-CPT) according to a previous procedure [16] with slight modification (Figure 2).

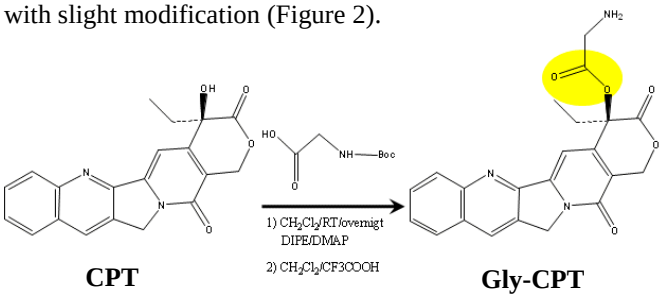

Figure 2: Synthesis of Gly-CPT according to ref. [16].

For the synthesis of SNP-CPT 1.00 g of preformed SNP (5-15 nm diameter) were dried at 350 ºC and vacuum for 3 h in a 100-mL round bottom flask, with a magnetic stir bar, a condenser and septum. Afterwards, 10 mL of anhydrous toluene was added and the mixture was heated to reflux. Then, 78 µL of (3-cyanopropyl)trichlorosilane (0.50 mmol) was added and the mixture was stirred for 3 h. The obtained product was filtered off, washed with toluene and methanol and dried at room temperature and vacuum for 16 h. This solid was dispersed in 150 mL of sulfuric acid (60%) and heated at 150 ºC for 3 h in a reflux system. Then, the acid suspension was diluted with 200 mL of distilled water, filtered off, washed with toluene and metanol and finally dried at 60 ºC and vacuum for 16 h, to obtain SNP with a coating of carboxylic moieties attached to surface (SNP-COOH, Figure 3a).

SNP-COOH (500 mg) were dehydrated at 60 ºC and vacuum for 6 h in a 100 mL round bottom flask, with a magnetic stir bar, a condenser and septum. Then, 10 mL of dichloromethane, 122 mg of EDCI (0.64 mmol) and 130 mg of HOBT (0.96 mmol) were added and the mixture was stirred at 0 ºC for 30 min. Next, 65 mg of Gly-CPT (0.125 mmol) and 87 mL of DIPEA (0.50 mmol) were introduced, and the system was allowed to warm to room temperature and remain stirring for 16 h. The resulting SNP-CPT were centrifuged (8000 rpm, 30 min) and washed with methanol. The last step was repeated until complete removal of physically adsorbed Gly-CPT on the inorganic support (as determined by UV-Vis, A_{368}, in a Nanodrop ND1000 spectrophotometer). The amount of attached CPT was calculated by subtracting the CPT content in the supernatant from the initial concentration, whereas nanoparticle size measurements were conducted using a Zetasizer Nano ZS (Malvern Instruments Ltd.). The full sequence for the preparation of SNP-CPT is presented in Figure 3.

2.2 Stability of SNP-CPT in PBS and Human Serum

SNP-CPT (7.5 wt% CPT) was prepared at 5 mg mL^{-1} in PBS (1x, pH=7.4). A 500 µL aliquot was transferred to a 1.5 mL Eppendorf tube and incubated at 37 ºC. Afterwards, the sample was centrifuged (14.000 rpm, 15 min) and the supernatant was freeze-dried. The residue was reconstituted with 1 mL of a solution methanol/HCl (95:5 v/v). 20 mL of

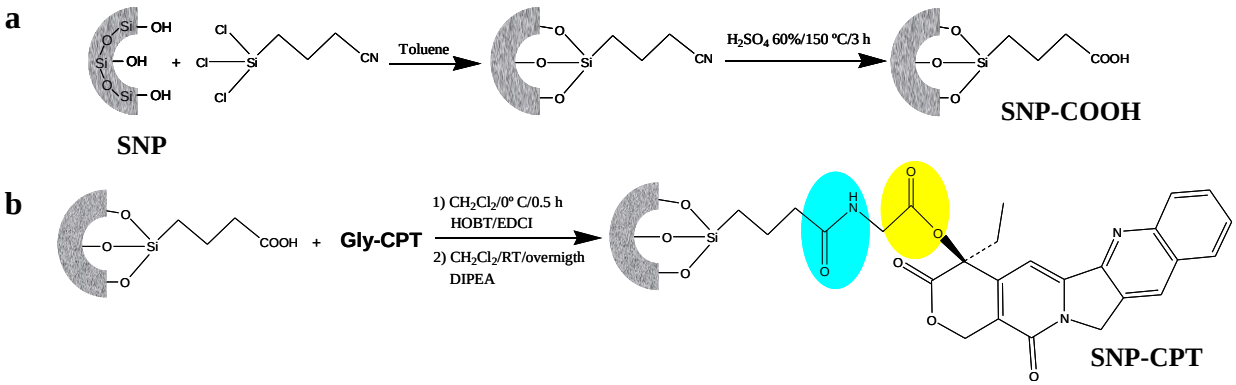

Figura 3: Sequence for the functionalization of SNP with carboxylic groups (a) and the covalent attachment of CPT through O-substitution on 20-OH (b).

this solution was injected into a HPLC system, with a C-18 reverse phase column (MEDITERRANEA SEA18, 5 μm 25 x 0.46 mm, Teknokroma) equipped with a UV-Vis detector (Varian ProStar 310), using a potassium phosphate buffer (pH=4.3) and acetonitrile. Absorption wavelength was set at 368 nm. The peak area from the CPT lactone form was integrated and compared to a standard curve.

A 5 mg mL^{-1} suspension of SNP-CPT (7.5 wt% CPT) was also prepared in human serum and incubated as before. After incubation, the sample was centrifuged at 4 ºC and 250 μL of supernatant was transferred to a sterile tube and diluted with 10 mL of methanol at 0 ºC. The solution was shaken for 3 min and further centrifuged at 4 ºC (10000 rpm, 30 min). An aliquot of 500 μL was treated with 500 μL of a 5% aqueous solution of trifluoroacetic acid at 0 ºC, mixed for 60 seconds and centrifuged at 4 ºC (10000 rpm, 30 min). The supernatant was freeze-dried and the residue was reconstituted with 1 mL of a solution methanol/HCl (95:5 v/v). 20 μL of this solution was injected into the HPLC. The peak area from the CPT lactone form was integrated and compared to a standard curve.

2.3 IC_{50} via MTT Assay

Cells were cultured at 37 ºC, under humidified atmosphere of 95% air and 5% CO_2. Cells were seeded in 96-well plates, 18 h before nanoparticles addition at 2000 cells/well (HeLa), 6000 cells/well (U87MG) and 10000 cells/well (HCT-116 and MDA-MB-231).

Cell cultures were treated with SNP-CPT or CPT with final doses ranging from 2.5 x 10^{-5} μg mL^{-1} to 2.5 μg mL^{-1} (in CPT equivalents) during 72 h. At the end of the incubation period, 5 mg mL^{-1} of MTT solution was added to the wells and 4 h later formazan crystals were dissolved with HCl 0.1 N in anhydrous isopropanol or DMSO, and spectrophotometrically measured at 590 nm. Cell viability for each concentration was calculated by the normalization of optical densities to the negative control. Toxicity of CPT-free SNP-COOH was also tested in HeLa cells, using the same dosing range.

3 RESULTS AND DISCUSSION

SNP-CPT with 7.5 wt% CPT were obtained as small aggregates of the original SNP, with an average particle size of 110 nm. Here, CPT is expected to be released inside the cells by hydrolysis of the ester bond with the glycine linker (Figure 3b) by cytoplasmatic esterases. Nevertheless, the moderate alkaline pH of body fluids could provoke some non-specific release before the intracellular delivery. In this sense, this material showed high stability in PBS and human serum, respectively with less than 4% and 9% hydrolysis in 3 h. Furthermore, SNP-CPT releases less than 15% of its total CPT content after a 24 h-incubation at 37 ºC in human serum. In general, the ester bond in 20-OH position looks pretty stable at pH below 8. It is worth noting that our system is more stable than those based in cyclodextrins, which present a dramatic, accelerated release of CPT [7], in a considerable shorter time (50% of the transported CPT is released in human plasma in less than 2 h). Hence, the high solubility of these polymers probably favors the enzymatic activity, e.g., decomposition by plasmatic carboxylases.

Table 1 and Figure 4 show that SNP-CPT induces cytotoxicity at slightly higher concentration than the free drug in the four lines tested. In this sense, IC_{50} values for CPT and SNP-CPT in HeLa, U87MG and HCT-116 cell lines indicate that CPT-loaded SNP are 2 to 3 times less effective than the naked drug. This difference seems to be even higher in the MDA-MB-231 cell line. This is probably due to uncompleted hydrolysis of the ester linking by intracellular esterases, as the accessibility of the ester bonds to the active sites of enzymes could present some steric restrictions on the surface of nanoparticles. Moreover, the sensitivity to CPT and SNP-CPT varies among cell lines, HeLa being the most sensitive and MDA-MD-231 the one with the lowest sensitivity. These results are in the line of those obtained by Davis *et al.* for the intracellular delivery of CPT with cyclodextrins-based polymers [7], and Zink *et al.* with MSN [14].

Cell line	CPT	SNP-CPT	n
HeLa	0.011±0.001	0.020±0.002	3
U87-MG	0.061±0.024	0.144±0.035	3
HCT-116	0.039±0.012	0.120±0.011	3
MDA-MD-231	0.161±0.008	0.739±0.091	2

Table 1: IC50 values (mean ± SE, in μg mL^{-1}) for free CPT and SNP-CPT in HeLa, U87-MG, HCT-116 and MDA-MD-231 cells (n=number of experiments).

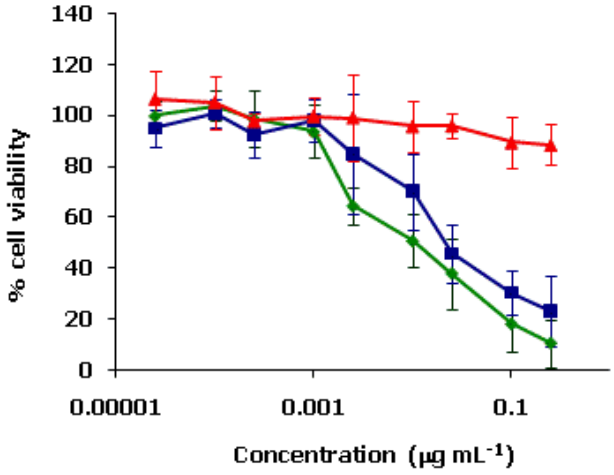

Figure 4: Effect of CPT, SNP and SNP-CPT in HeLa cells. Concentration corresponds to CPT equivalents

Finally, it has been proved that cytotoxicity was due only to CPT. Here, the observation under the inverted

microscope during the incubation period indicated that cells treated with CPT and SNP-CPT were dying and did not proliferate, whereas cells treated with SNP-COOH showed a normal morphology and proliferation rate (Figure 5).

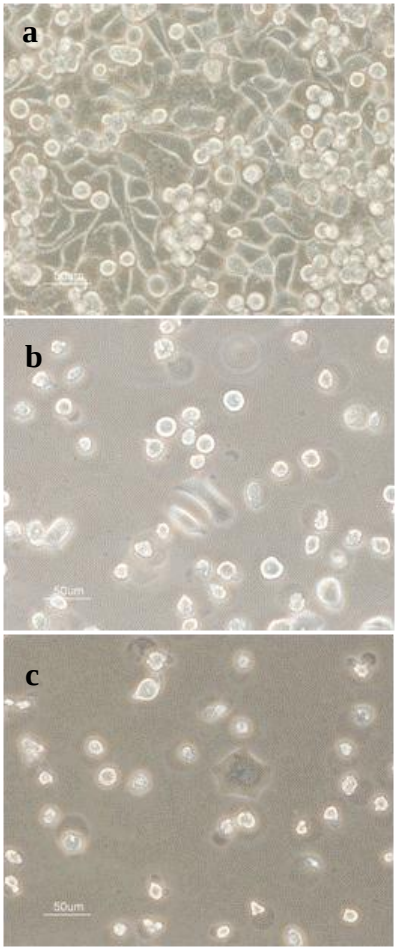

Figure 5: Morphology of HeLa cells after incubation with SNP-COOH (a), SNP-CPT (b) or CPT (c).

5 CONCLUSIONS

Novel systems for delivery and release of therapeutic molecules with low solubility in biological fluids as CPT are strongly needed. Here, we present a robust model for delivery of CPT into cancer cells based in very small silica nanoparticles functionalized with carboxylic groups on surface (SNP-COOH). These carboxylic groups are ionized at physiological, thus, provoking electrostatic repulsion and avoiding particle aggregation, which allows the preparation of stable colloids in aqueous medium. Moreover, SNP-CPT show high stability in simulated body fluid (PBS) and in human serum, thus, avoiding an undesired premature release of the drug before targeting the target cells. SNP-CPT are able to induce cell death in all cell lines tested with IC_{50} values in the same order than the drug. Conversely CPT-free SNP do not alter cell proliferation or morphology.

The biodistribution of SNP-CPT conjugates labeled with the fluorochrome Cy5.5 is currently being evaluated in athymic mice with subcutaneous xenograft tumors.

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