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Oxidative stimuli-responsive nanoprodrug of camptothecin kills glioblastoma cells

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ABSTRACT

The purpose of this study was to prepare and characterize nanometer-sized prodrug (nanoprodrug) of camptothecin. The camptothecin prodrug was synthesized using tetraethylene glycol spacer linked via carbonate bond to camptothecin and via ester bond to α-lipoic acid. The nanoprodrug was prepared through the spontaneous emulsification mechanism using the mixture of camptothecin prodrug and α-tocopherol which served as a structural matrix. The nanoprodrug was activated readily by porcine liver esterase and, with a much slower rate, by hydrolytic degradation. Upon longterm storage, the α-lipoic acid moiety of the camptothecin prodrug gradually oxidized without loss of structural integrity and therapeutic efficacy. Interestingly, the hydrolytic activation was negligible before the oxidation, but was significantly accelerated after the oxidation of the α-lipoic acid moiety, suggesting an oxidative stimuli-responsive activation of the prodrug. The camptothecin nanoprodrug was found to possess significant inhibitory effect on the proliferation of U87-MG glioma cells with an IC_{50} of 20 nM.

Since the isolation and identification of the chemical structure in 1966,[1] the pentacyclic alkaloid camptothecin (CPT), an inhibitor of DNA topoisomerase I,[2] has been attracting considerable attention in the oncology community. After demonstrating tumoricidal activity in vitro and efficacy in animal model of cancer, the initial enthusiasm faded quickly when it showed no substantial efficacy in human clinical studies.[3,4] The major problem of CPT for clinical studies was its low water-solubility. As the open ring carboxylate form (Fig. 1a) of CPT was used to overcome the poor water-solubility, it was shown that the carboxylate form possessed much lower therapeutic efficacy along with unpredictable severe side effects.[5] It was also demonstrated that the intact lactone ring is necessary for the anticancer efficacy of CPT. Due to the intrinsic instability of CPT resulting from the rapid hydrolysis of the lactone ring, a significant loss of therapeutic activity was observed.

After the discovery that the primary cellular target of CPT is DNA topoisomerase,[2] the interest in the drug was revived and systematic approaches were undertaken to improve the therapeutic efficacy based on the previous results. The developments were focused to improve the water-solubility and the stability of lactone ring through chemical modification of CPT.

Two water-soluble CPT derivatives were developed and have been approved by the FDA for clinical use. Topotecan (9-[(dimethylamino)methyl]-10-hydroxy-camptothecin is a semi-synthetic CPT analogue and the increased water-solubility is attributed to the basic tertiary amino side chain at carbon 9 of the A ring (Fig. 1b). In the same manner, irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin, CPT-11) is a synthetic analogue which is modified by the addition of the basic

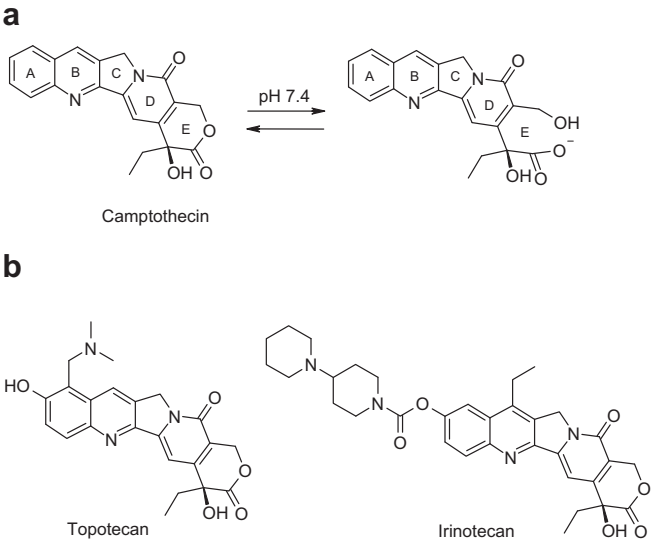

Figure 1. (a) Camptothecin with closed and open lactone ring; (b) camptothecin analogues.

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dipiperidine side chain at carbon 10 of the A ring (Fig. 1b). Irinotecan is relatively inactive, but after the cleavage of the dipiperidino side chain by carboxyl esterase, the biologically active metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38) is formed.[6] This prodrug approach has been applied widely in the development of CPT analogues to increase the water-solubility and stability of the lactone ring. Several other approaches have been developed to synthesize water-soluble camptothecin derivatives[7–9] and to improve the stability of labile lactone ring of CPT,[10,11] both of which can be activated enzymatically and/or chemically in physiologic condition.

The development of nanostructures with physically or chemically encapsulated CPT or CPT analogues have received significant attention in recent years. Koizumi et al. developed a polymeric micelle system where the biologically active metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38) was covalently attached to the carboxylate side chains of a poly(ethylene glycol)–poly(glutamic acid) block copolymer which forms a polymeric micelle by self-assembling of the hydrophobized block copolymer.[12] Another approach by Noble et al. used nanometer-sized liposome to deliver CPT-11 resulting nearly 65 times higher drug residence time in tissue. They showed an increase of median survival time from 28.5 days for free drug to >100 days for liposomal CPT-11 in the intracranial U87 glioma xenograft model.[13]

To combine the two concepts of prodrug and nanometer-sized drug delivery system we have developed nanometer-sized prodrug (nanoprodrug) of CPT by spontaneous emulsification of CPT prodrug using α-tocopherol as a structural matrix. α-Tocopherol was used as structural matrix due to its highly stabilizing and size-reducing effects.[14] Furthermore, α-tocopherol can serve as an excellent solvent for the water-insoluble oily CPT prodrug due to its compatibility with other solvents, oils and surfactants.[15]

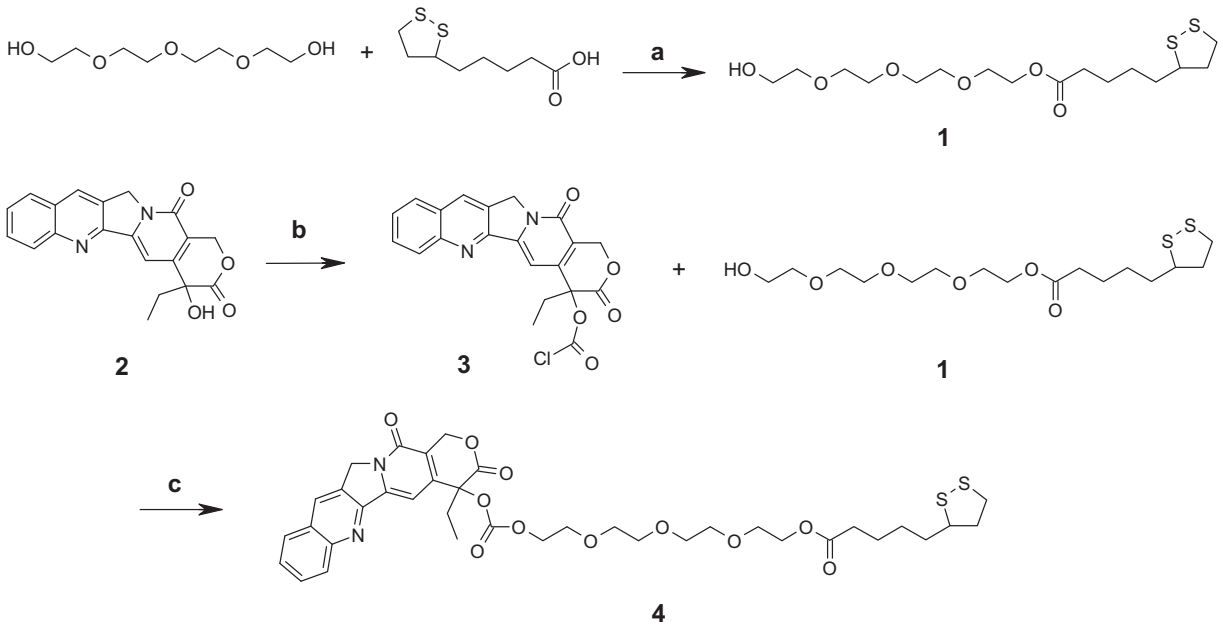
The problem associated with CPT's incorporation in the matrix was its low solubility in organic solvents that are commonly used for the spontaneous emulsification process. The critical characteristic of those solvents, most notably acetone and acetonitrile, is their complete and instantaneous water-miscibility (spontaneity of emulsification).[16–19] We found that the solubility of CPT is approximately 0.1 and 0.2 mg/mL in acetonitrile and acetone, respectively, which were too low to be incorporated in the nanoprodrug (data not shown). In addition, CPT was precipitated during the emulsification process, suggesting its incompatibility with α-tocopherol.

Thus, our goal was to increase the solubility of the CPT in acetone by means of chemical modification via reversible bonds, and at the same time, to maintain or increase the hydrophobicity of CPT. This is because more stable nanoprodrug can be formed from more hydrophobic compound, mainly due to the stronger hydrophobic interaction between the molecules. In our previous report we have shown that the increase in hydrophobicity leads to a more stable and compact nanoprodrugs.[14]

On the other hand, the possible applicability of the more hydrophobic CPT prodrugs depend on whether they are sufficiently susceptible to hydrolytic and/or enzymatic activation. As previous demonstrated in our study, hydrophobically modified NSAID molecules can be transformed into stable nanostructures and, despite the highly hydrophobic nature of the derivatives, NSAIDs were readily hydrolyzed enzymatically from the nanoprodrugs.[20,21]

The CPT prodrug was synthesized using tetraethylene glycol spacer (TEG) linked to CPT via a carbonate bond and to α-lipoic acid (ALA) via an ester bond (Scheme 1). The activation of the CPT-20-OH via carbonate was performed as described.[10] The monoesterification of ALA with tetraethylene glycol has been reported in our previous study.[20] The synthesis of the prodrug CPT-TEG-ALA by the coupling of CPT to the hydroxyl group of ALA-TEG-OH via carbonate bonds was performed as described.[22] The purity of the prodrug was confirmed by RP-HPLC,[23] and the structure was confirmed by ^{1}H and ^{13}C NMR spectroscopy.[24] The solubility of CPT-TEG-ALA in acetone was >200 mg/mL, whereas it was approximately 0.2 mg/mL for CPT.

In order to take the advantage of nanostructured biomaterials, we prepared nanometer-sized prodrugs from the synthesized CPT prodrug CPT-TEG-ALA according to a method which utilizes spontaneous emulsification.[25] First, experiments were performed to assess the amount of CPT-TEG-ALA resulting in the highest recovery yield. In this study, formulation parameters were kept constant except for the amount of CPT-TEG-ALA. The recovery yield was calculated as follows:



Scheme 1. Synthesis of the α-lipoic acid-containing camptothecin prodrug CPT-TEG-ALA. Reagents and conditions: (a) 3 equiv tetraethylene glycol, 1.5 equiv DMAP, 1.0 equiv EDCI, CH_2Cl_2, rt, 5 h; (b) 0.35 equiv triphosgene, 3.5 equiv DMAP, CH_2Cl_2, rt, 10 min; (c) CH_2Cl_2, rt, 16 h.

$$\text{Recovery yield } (\%) = \frac{\text{Amount of prodrugs found in nanoprodrugs}}{\text{Amount of prodrugs used}} \times 100$$

The yield reached a maximum of 72% when approximately 3.3 mg of CPT-TEG-ALA was used (nanoprodrug 2) and decreased when the amount was further increased (Fig. 2b). Notably, when the amount exceeded 7 mg, a formation of macroscopic floating flakes of CPT-TEG-ALA was observed, suggesting the existence of a critical ratio of the amounts of CPT-TEG-ALA and α-tocopherol, which was confirmed by the fact that the amount of CPT-TEG-ALA could be increased directly proportional to the increase of α-tocopherol (data not shown).

The size was within a close range of 160–170 nm and practically no differences in the size were observed between the nanoprodrugs with varying amount of CPT-TEG-ALA (Fig. 2a). This is critically advantageous because differences in the therapeutic efficacy can be attributed directly to the amount of prodrugs in the nanoprodrugs, eliminating the interfering influence of the nanoprodrug size.

To assess the physical and chemical stability of CPT-TEG-ALA in the nanoprodrug upon longterm storage, the three nanoprodrugs were stored as suspension in water at 4 °C for four weeks and the size as well as the amount of the intact CPT-TEG-ALA were measured.

The recovery yield was calculated as follows:

$$\text{Recovery yield } (\%) = \frac{\text{Amount of prodrugs after storage}}{\text{Amount of prodrugs before storage}} \times 100$$

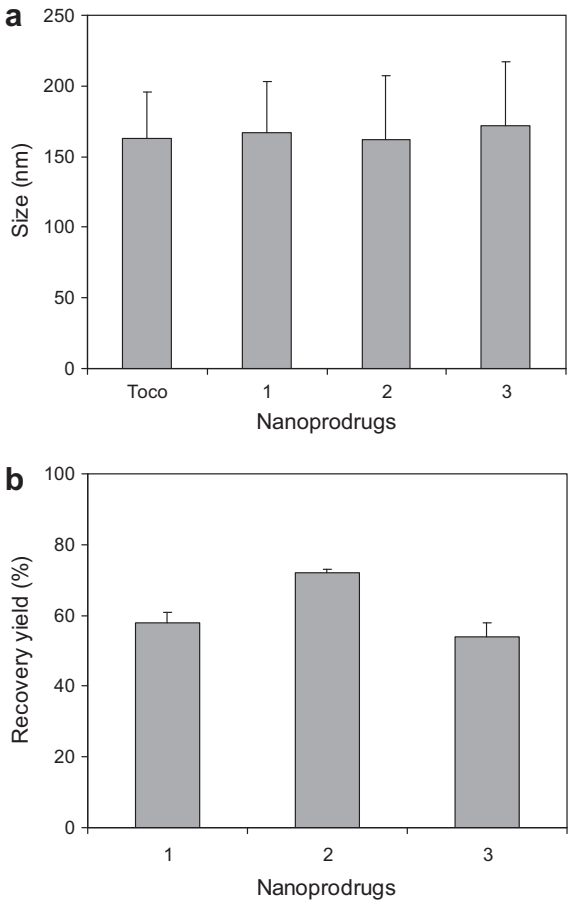


Figure 2. Size and recovery yield of camptothecin nanoprodrugs. Content of CPT-TEG-ALA in the nanoprodrugs: 1.64 mg (1), 3.28 mg (2), and 6.56 mg (3). The results are the mean ± S.D. of three experiments.

The size remained almost constant and the recovery was the highest for the nanoprodrugs 2 with 90% (Fig. 3).

For further investigation we chose the nanoprodrug 2 and assessed the stability by measuring the size variation and the recovery yield after 12 weeks-storage at 4 °C. In this study, the size of the nanoprodrug remained almost unchanged (Fig. 4a), whereas the amount of the intact prodrug molecule was decreased to 88%, 72%, and 63% after 4, 8, and 12 weeks, respectively (Fig. 4b). Notably, the polydispersity index (P.I) was lower after 8 and 12 weeks, which means that the particle size distribution (PSD) became narrower. The chromatogram (Fig. 5a) was obtained from the same samples after up to 12 weeks of storage. It shows that CPT-TEG-ALA (peak p3) in the nanoprodrug oxidized gradually upon storage, resulting in the oxidized CPT-TEG-ALA (peak p2) which did not further degrade to CPT (peak p1). Considering the combined results of the size measurement in Figure 4a and the oxidation of the prodrug in Figure 5a, it can be concluded that the PSD becomes lower with increasing degree of the prodrug oxidation.

To confirm that the oxidized CPT-TEG-ALA resulting from long-term storage is the same as chemically oxidized prodrug, we treated CPT-TEG-ALA dissolved in 70% acetone with hypochlorous acid (HOCl) and compared the chromatograms. Figure 5b showed the gradual increase of the oxidized prodrug (p2) upon treatment with increasing amount of HOCl, indicating that the oxidation in both stored and HOCl-treated samples occurred in the dithiolane ring of the α-lipoic acid moiety (Scheme 2).[20,21]

Next, the nanoprodrug was treated with increasing amount of HOCl and immediately analyzed with HPLC. As expected, the prodrug was oxidized instantly after the addition of HOCl, which was confirmed by the absence of changes in chromatograms measured after further incubation for 30 min and 1 h at room

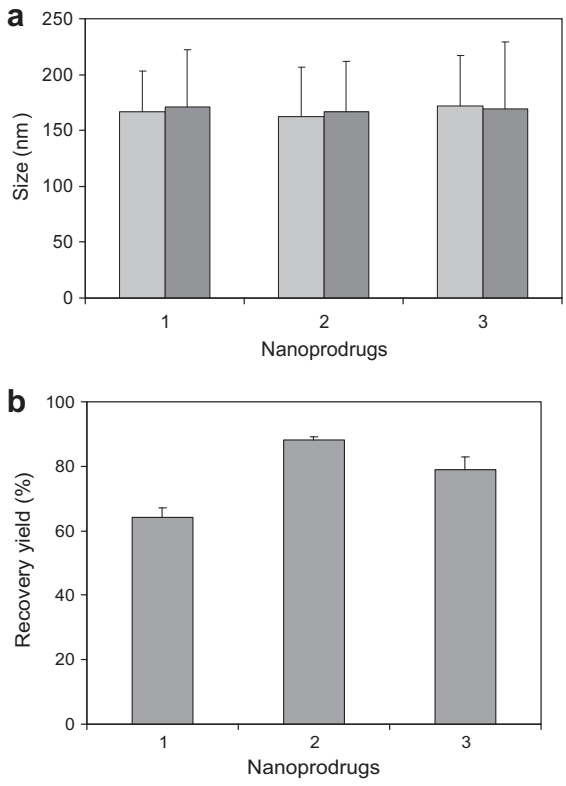


Figure 3. Long term stability of camptothecin nanoprodrugs. Content of CPT-TEG-ALA in the nanoprodrugs: 1.64 mg (1), 3.28 mg (2), and 6.56 mg (3). The results are calculated as the percentage of size (a) and prodrugs (b) with 100% equal to the size and amount of prodrugs before incubation. The results are the mean ± S.D. of three experiments.

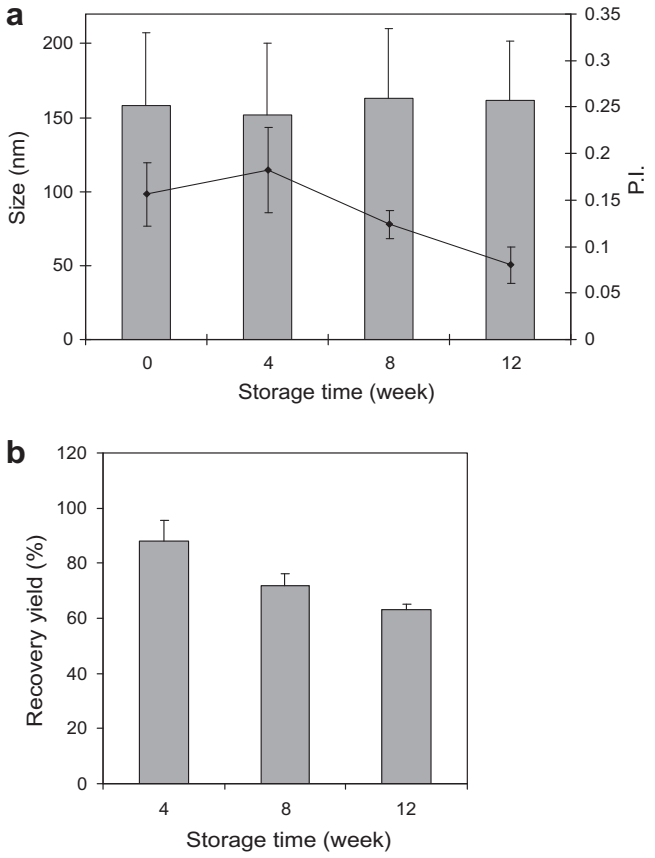


Figure 4. Long term stability of camptothecin nanoprodrug. Content of CPT-TEG-ALA in the nanoprodrug: 3.28 mg. The results are calculated as the percentage of size (a) and prodrugs (b) with 100% equal to the size and amount of prodrugs before incubation. The results are the mean ± S.D. of three experiments.

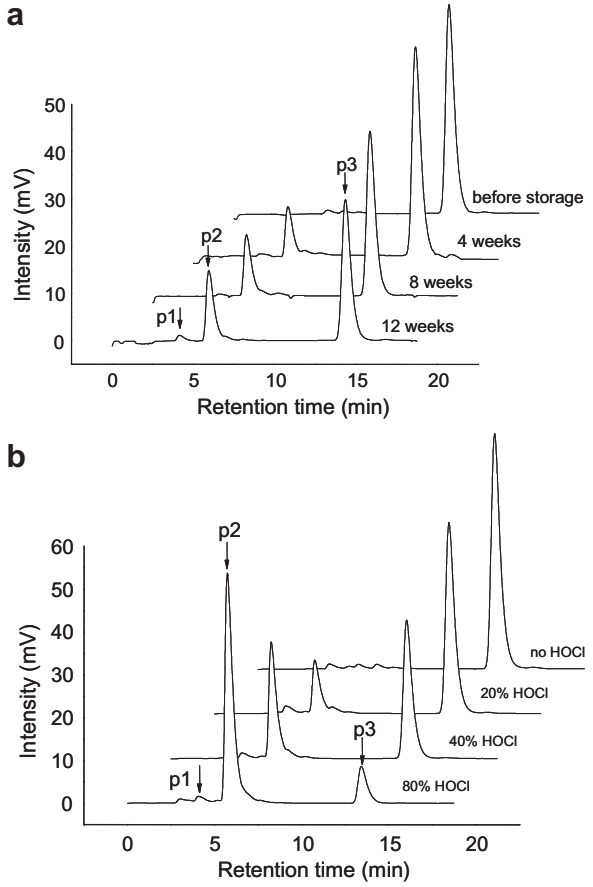
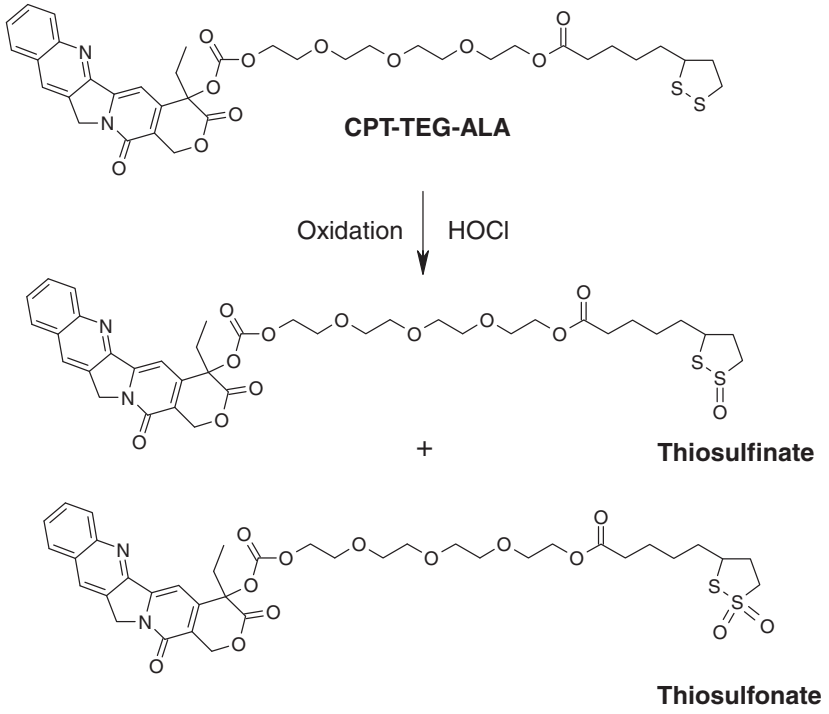


Figure 5. Oxidation of camptothecin prodrug CPT-TEG-ALA: camptothecin (p1), oxidized CPT-TEG-ALA (p2), intact CPT-TEG-ALA (p3). (a) Oxidation of camptothecin prodrug embedded in nanoprodrug during 12-weeks storage; (b) oxidation of camptothecin prodrug by HOCl in solution. The chromatograms were taken as described.[23]



Scheme 2. Oxidation of camptothecin prodrug CPT-TEG-ALA by HOCl.

temperature. Interestingly, the oxidized prodrug was further degraded to camptothecin when the amount of HOCl increased. Upon complete oxidation with molar excess of 120%, approximately 50% of the oxidized CPT-TEG-ALA was degraded to CPT and oxidized ALA-TEG-OH.

To further elucidate the hydrolytic degradation of the oxidized and intact prodrugs, the HOCl-treated samples were further incubated for two days at room temperature. Figure 6b shows that the oxidized prodrug was completely degraded (disappearance of p2 peaks), while practically no degradation occurred in control nanoprodrug (no HOCl treatment). Only 10% of the intact prodrugs were degraded in the samples treated with 6% and 12% of HOCl, whereas 16% and 58% of the intact prodrugs were degraded in the sample treated with 30% and 60% of HOCl, suggesting that the oxidation and degradation caused some changes in the nanoprodrug morphology, which in turn promoted hydrolytic degradation of the intact prodrug molecules. Obviously, the hydrolytic degradation occurred at higher rate after the oxidation of the prodrugs, indicating that the oxidation of dithiolane ring rendered the molecules more hydrophilic, which made them more susceptible to hydrolytic degradation.

In order to assess the rate of enzymatic prodrug activation, the aqueous suspension of the nanoprodrug was incubated with porcine liver esterase. Due the molecular structure based on carbonate and ester bonds, the CPT prodrug molecules were expected to be degraded by enzymatic ester hydrolysis. As shown in our previous investigation, the ester bond between tetraethylene glycol and α-lipoic acid was hydrolyzed with different hydrolysis rates, which depend on the hydrophobic nature of the entire molecule.[14,20,21] It has also been reported that the carbonate bond to the camptothecin can be readily cleaved by porcine liver esterase.[26]

As shown in Figure 7, the camptothecin prodrugs embedded in the nanoprodrugs were completely hydrolyzed within 4 h. In our previous study we demonstrated that the oxidation of α-lipoic acid promoted the enzymatic hydrolysis of NSAID nanoprodrugs, which was attributed to the decreased hydrophobicity of the oxidized prodrugs and increased interaction between the oxidized prodrugs and hydrolytic enzymes. In this study, practically no differences in the hydrolysis rate of the intact and oxidized camptothecin nanoprodrugs could be detected (data not shown), suggesting that the camptothecin prodrug could be readily hydrolyzed by esterase despite of its highly hydrophobic nature and water-insolubility. Because of the water-insolubility of CPT-TEG-ALA, the enzymatic hydrolysis was negligible in an aqueous solution (data not shown).

The highly reactive nature of CPT-TEG-ALA towards the hydrolytic enzymes can be explained as follows. First, the formation of the nanometer-sized prodrug may generate a large surface area on which the interaction between hydrolytic enzymes and prodrugs can take place.[27,28] This interaction would be otherwise impossible due to the insolubility of the prodrugs in aqueous media. Second, although it is not yet proven, the observed reactivity suggests a highly ordered structure of the CPT-TEG-ALA on the surface of the nanoprodrugs, while α-tocopherol mainly forms the core of the nanoprodrugs. This defined special distribution could be accomplished by the self-assembling or self-organizing process drived by noncovalent forces[29] between the CPT prodrugs and the more hydrophobic α-tocopherol during the spontaneous emulsification process.

In order to evaluate the effect of CPT nanoprodrugs on tumor cell growth, we studied the inhibitory effect on the cell growth of U87-MG glioma cells.[30,31] Glioma cells were treated with camptothecin nanoprodrugs and free camptothecin in the concentration range from 5 to 1000 nmol for 6 days. The nanoprodrugs used in the cell culture study contained less than 0.5% of oxidized CPT-TEG-ALA molecules, but no free camptothecin was detected by RP-HPLC (Fig. 5a). Control cells were also treated with nanospheres prepared from α-tocopherol only by exposing to an equimolar concentration of α-tocopherol. Compared with cell culture with culture medium only, the control cells with α-tocopherol did not show any effect on the cell growth (data not shown). As shown in Figure 8, the nanoprodrugs of camptothecin and free camptothecin inhibited the cell growth completely at the concentration of 250 nM and above. Both drugs were potent at reducing the cell

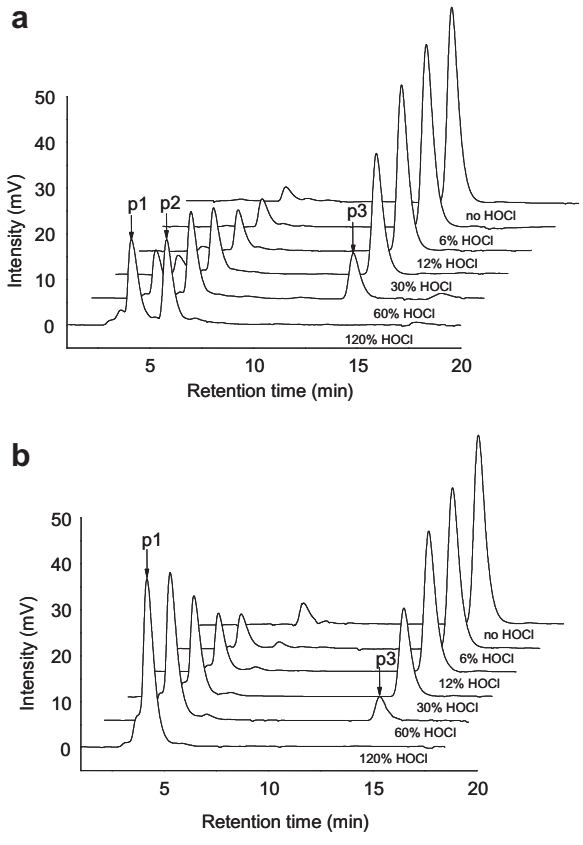


Figure 6. Oxidation of camptothecin prodrugs CPT-TEG-ALA by HOCl: camptothecin (p1), oxidized CPT-TEG-ALA (p2), intact CPT-TEG-ALA (p3). (a) Oxidation of camptothecin prodrug embedded in nanoprodrug. The chromatograms were taken after 30 min of incubation with HOCl; (b) hydrolytic degradation of the oxidized camptothecin prodrugs. The chromatograms were taken after 2 days of incubation at room temperature.

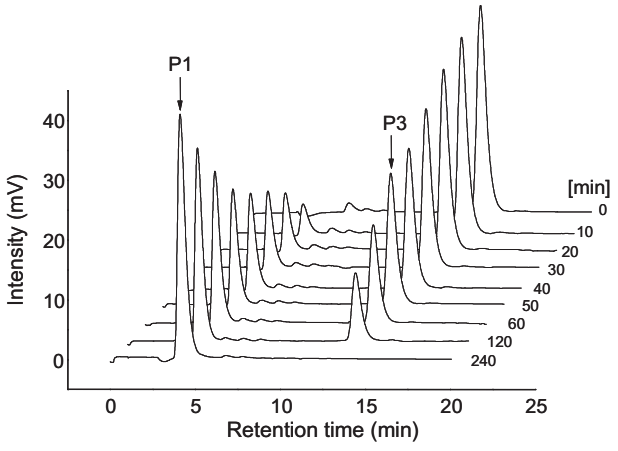


Figure 7. Enymatic hydrolysis of camptothecin prodrug CPT-TEG-ALA from nanoprodrugs: camptothecin (p1), intact CPT-TEG-ALA (p3).

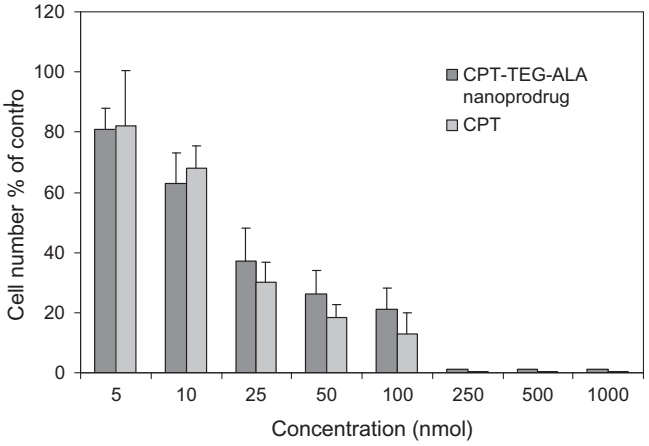


Figure 8. Effect of camptothecin nanoprodrugs and free camptothecin on glioma cell proliferation.

proliferation with a LD_{50} value of 20 nM. Free camptothecin showed slightly higher effect in the concentration range from 25 nM to 100 nM, but the differences were not statistically significant.

In summary, a novel camptothecin prodrug molecule was synthesized and nanometer-sized prodrugs (nanoprodrug) were prepared by a spontaneous emulsification process. We demonstrated that the physicochemical properties of the CPT prodrug could be changed substantially in favor of the formation of stable nanostructures, including increased hydrophobicity, increased solubility in acetone, and its oily nature which all together enabled the preparation of stable nanoprodrugs using spontaneous emulsification. Embedded in α-tocopherol matrix, the oxidation of CPT prodrug proceeded gradually without loss of the active CPT component and structural integrity. The CPT prodrug can be activated through hydrolytic, as well as enzymatic degradation. Despite the highly hydrophobic nature of the CPT prodrug, it was readily hydrolyzed enzymatically from the nanoprodrugs. Whereas the hydrolytic activation of the intact prodrug was negligible, it proceeded faster after the prodrug oxidation, suggesting that the nanoprodrug can be used as an oxidative stimuli-responsive CPT prodrug. Furthermore, the water-insoluble CPT prodrug can be transformed into stable nanostructures obviating the need to dissolve the compounds in an excessive amount of co-solvents and thus eliminating the interference of toxic side effects caused by co-solvents. In addition, the formation into the compact nanostructures confers an additional advantage of higher drug loading per volume, which is of crucial importance when high dosing is required.

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22. Unless otherwise noted, solvents and chemicals were obtained at highest purity from Sigma–Aldrich Chemical Co. (St Louis, MO, USA) and used without further preparation. A suspension of camptothecin (139 mg, 0.4 mmol), triphosgene (44 mg, 0.147 mmol) and DMAP (156 mg, 1.28 mmol) in anhydrous DCM (20 mL) was stirred fro 10 min at room temperature. ALA-TEG-ALA (0.153 g, 0.4 mmol) was added and the reaction mixture was stirred fro 16 h at room temperature under light protection. The solvent was evaporated until dryness and the residue was dissolved in acetone (5 mL) and insoluble matter was removed by filtration. H_2O (5 mL) was added to the clear acetone solution and the precipitate was collected and dried under vacuum. The precipitate was dissolved in acetone (2 mL). The solution was stored at −20 °C overnight and the insoluble matter was removed by filtration. The solvent was evaporated to dryness to yield the camptothecin prodrug as a light brown colored thick oil (136 mg, 45%). The purity of the camptothecin prodrug was assessed by HPLC to be >99.5%.
23. HPLC analysis was performed on a Merck-Hitachi analytical LaChrom D-7000 HPLC/UV detector system (Merck, Darmstadt, Germany) with a CAPCELL PAK, Type SG 120 (phenomenex, Torrance, CA, USA) C_{18} reversed phase column (250/4.6 mm, 5 μm). The composition of the mobile phase (acetonitrile/water mixture containing 0.1% (v/v) trifluoroacetic acid) was adjusted for camptothecin and camptothecin prodrug in order to provide an appropriate retention time and separation. Linearity of the calibration curves was tested in the range 3–100 μg/mL for CPT-TEF-ALA and 1–30 μg/mL for CPT with good linear relationships ($r^2 > 0.99$). Within this concentration range the amount of the prodrug and camptothecin could be determined reproducibly. To determine the concentration of the prodrugs or camptothecin from the nanoprodrugs, the aqueous nanoprodrugs suspension (100 μL) was added to acetonitrile (400 μL) and analyzed using RP-HPLC. The separation was performed under isocratic condition with a 60:40 mixture of acetonitrile/water at a flow rate of 1 mL/min. The detection was carried out at 360 nm.
24. ^1H and ^{13}C NMR spectra were conducted on a Varian 400 MHz spectrometer and chemical shifts (δ) were given in ppm relative to TMS. The spectra were recorded with the solvent $CDCl_3$ at room temperature.
CPT-TEG-ALA: ^1H NMR (400 MHz, $CDCl_3$): δ = 1.0 (t, 3 × H), 1.49 (m, 2 × H), 1.70 (m, 4 × H), 1.90 (m, 1 × H), 2.15 (m, 2 × H), 2.30 (t, 2 × H), 2.42 (m, 1 × H), 3.09 (m, 1 × H), 3.12 (m, 1 × H), 3.52 (m, 1 × H), 3.62 (m, 12 × H), 4.18 (m, 4 × H), 5.24 (s, 2 × H), 5.38 (d, 1 × H), 5.65 (d, 1 × H), 7.39 (s, 1 × H), 7.70 (t, 1 × H), 7.85 (t, 1 × H), 7.95 (d, 1 × H), 8.21 (d, 1 × H), 8.40 (s, 1 × H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 7.64, 24.59, 28.70, 31.90, 33.91, 34.57, 38.47, 40.20, 50.00, 56.32, 61.73, 63.46, 67.06, 67.97, 68.68, 69.13, 70.51, 70.57, 70.74, 96.00, 120.28, 128.08, 128.18, 128.22, 128.47, 129.64, 130.70, 131.20, 146.05, 153.01, 173.44.
25. Camptothecin nanoprodrug was prepared according to the method using spontaneous emulsification as follows. 25 mg of camptothecin prodrug CPT-TEG-ALA and 5 mg of α-tocopherol were dissolved in acetone (5 mL) containing polysorbate 80 (0.1% w/v). The organic solution was poured under moderate stirring on a magnetic plate into an aqueous phase prepared by dissolving 25 mg of Pluronic F68 in 10 mL distilled water (0.25% w/v). Following 15 min of magnetic stirring, the acetone was removed under reduced pressure at room temperature. The suspension was dialyzed in cellulose membrane tube (Sigma, code D9777) overnight in distilled water and filtered through 0.8 μm hydrophilic syringe filter (Corning, Part No. 431221, Fisher Scientific Co., Pittsburgh, PA, USA). As control, nanospheres were prepared with 25 mg of α-tocopherol in the absence of CPT-TEG-ALA using the same procedure as described above. The size measurement was performed as described.[14]
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30. The U87-MG human glioma cell line was obtained from American Type Culture Collection (ATCC, Bethesda, MD, USA). The cells were grown and maintained in Minimum Essential Medium (MEM, Invitrogen) containing antibiotics penicillin (100 U/mL) and streptomycin (100 μg/mL) and supplemented with 10% fetal bovine serum (FBS, Invitrogen). Cells were grown at 37 °C at an atmosphere of 5% CO_2 in humidified air.

31. The glioma cells were seeded at 5×10^4 cells per well in 6-well plates and grown for 24 h. The cells were treated with free camptothecin and camptothecin nanoprodrugs at a final concentration ranging from 5 to 1000 nM of CPT-TEG-ALA. Free camptothecin was prepared as stock solutions in DMSO, having concentrations ranging from 5 μM to 1000 μM. 2 μL of each stock solution were added to the 2 mL cell culture in each well, resulting in the final concentrations ranging from 5 nM to 1000 nM. After 6 days of treatment, the culture medium was removed and cells were washed with PBS. 0.5 mL of 0.25% trypsin/EDTA was added to each well and the detached cells were counted immediately in a hemocytometer. The antiproliferative effect of the nanoprodrugs was presented as a cell number% of control, which was calculated as follows:

$$\text{Cell number\%of control} = (\text{Cell number}_{\text{treated}} \,/\, \text{Cell number}_{\text{control}}) \times 100$$

where $\text{Cell number}_{\text{treated}}$ is the number of cells after treatment with nanoprodrugs and $\text{Cell number}_{\text{control}}$ is the number of cells of control culture which was incubated with culture medium only. The cells were also treated with nanospheres prepared from α-tocopherol only.