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Bioreduction activated prodrugs of camptothecin: molecular design, synthesis, activation mechanism and hypoxia selective cytotoxicity†

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Several water-soluble derivatives (**CPT3, CPT3a–d**) of camptothecin (**CPT**) were synthesized, among which **CPT3** bearing an *N*,*N*′-dimethyl-1-aminoethylcarbamate side-chain was further conjugated with reductively eliminating structural units of indolequinone, 4-nitrobenzyl alcohol and 4-nitrofuryl alcohol to produce novel prodrugs of camptothecin (**CPT4–6**). All **CPT** derivatives were of lower cytotoxicity than their parent compound of **CPT**. In contrast, **CPT4** and **CPT6** showed higher hypoxia selectivity of cytotoxicity towards tumor cells than **CPT**. A mechanism by which a representative prodrug **CPT4** is activated in the presence of DT-diaphorase to release **CPT** was also discussed. The bioreduction activated **CPT** prodrugs including **CPT4** and **CPT6** are identified to be promising for application to the hypoxia targeting tumor chemotherapy.

Introduction

20(*S*)-Camptothecin (**CPT**) is a potent inhibitor of DNA topoisomerase I (topo I) first isolated by Wall and coworkers from *Camptotheca acuminate*.[1,2] **CPT** has been identified to stabilize covalent binding of topo I to DNA, thereby resulting in irreversible and lethal strand breaks of DNA during its replication.[2] The clinical application of **CPT** to cancer treatment was however suspended because of its unfavorable properties such as non-specific toxicity and negligible water solubility.[3] Another key problem is the structural instability of the 20-hydroxy lactone ring moiety, which is easily hydrolyzed into an inactive *E*-ring-opened carboxylate form under physiological conditions (Scheme 1).[4] To improve these drawbacks, a great deal of effort has been made to develop various modifications such as **CPT** analogues,[5,6] **CPT** prodrugs,[7,8] and combination with drug delivery systems,[9,10] some of which have recently been subjected to clinical trials.[6,11]

In this work, an attempt was made to develop a new class of water-soluble bioreduction activated prodrugs of **CPT** that are subject to metabolism by intracellular reductases and thereby release a potent topo I inhibitor of **CPT** selectively under hypoxic conditions. The strategy employed herein is closely related to tumor hypoxia targeting therapy,[12] antibody-directed enzyme prodrug therapy (ADEPT)[13] and gene-directed enzyme prodrug therapy (GDEPT).[14] Firstly, we synthesized a series of water-soluble **CPT** derivatives (**CPT3, CPT3a–d**) to explore appropriate linker structures for constructing efficient bioreduction activated **CPT** prodrugs. Based on this exploration, **CPT3** bearing an *N*,*N*′-dimethyl-1-aminoethylcarbamate side-chain was conjugated with three types of well documented structural units with bioreduction reactivity (indolequinone,[15] 4-nitrobenzyl[16] and 4-nitrofuryl motifs[17]) to obtain the corresponding prodrugs **CPT4–6**, as shown in Fig. 1. We also evaluated *in vitro* cytotoxicity of the **CPT** derivatives towards tumor cells under both aerobic and hypoxic conditions, and bioreductive activation of a representative prodrug **CPT4** by

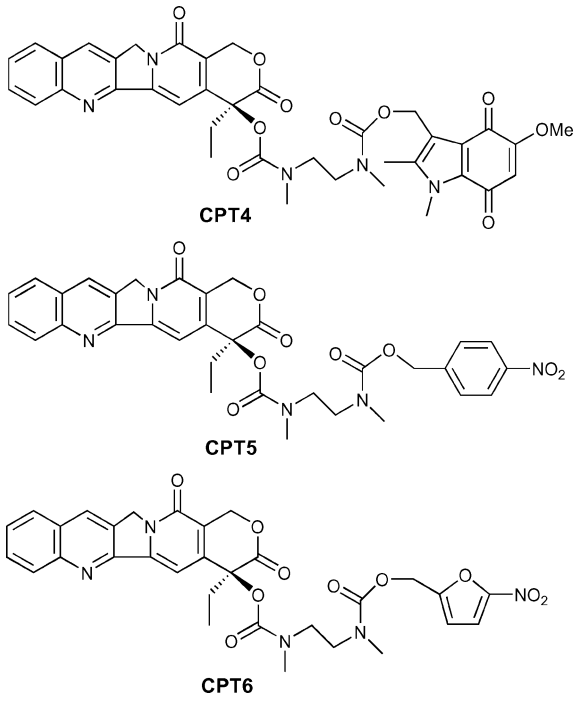
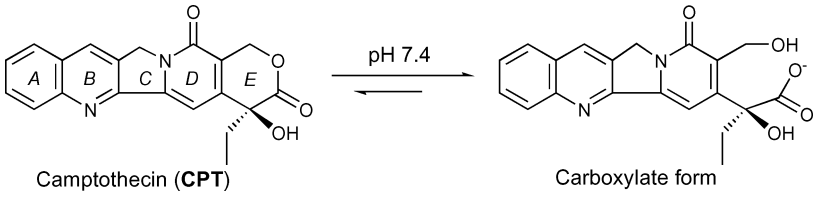


Fig. 1 Structures of bioreduction activated **CPT** prodrugs **CPT4–6**.

† Electronic supplementary information (ESI) available: Experimental procedures for syntheses of **CPT** derivatives, **CPT1–3, CPT3a–d, CPT3a**′ and **CPT3b**′. See http://www.rsc.org/suppdata/ob/b5/b502813b/



Scheme 1 Hydrolytic ring-opening reaction of **CPT** under physiological conditions.

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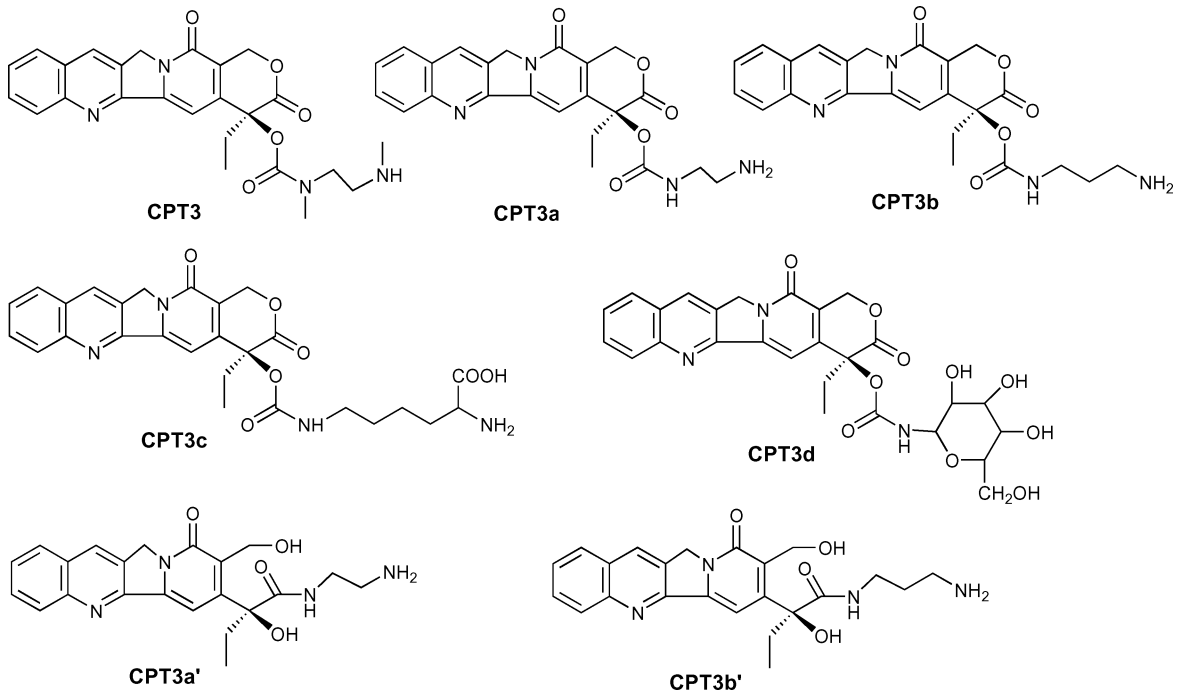


Fig. 2 Structures of water-soluble **CPT** derivatives **CPT3** and **CPT3a–d**, and the representative carboxylate forms **CPT3a′**,**3b′**.

DT-diaphorase (DTD). These *in vitro* assays demonstrated that the bioreduction activated prodrugs such as **CPT4** and **CPT6** have higher hypoxia selectivity of cytotoxicity towards tumor cells than **CPT**.

Results and discussion

Synthesis of CPT prodrugs

For screening of water-soluble linker structures appropriate for construction of bioreduction activated **CPT** prodrugs, several **CPT** derivatives listed in Fig. 2 were synthesized. According to the previous reports,[18] modification of the 20-hydroxy group in the lactone ring moiety has the merit of enhancing the stability of **CPT**. In this light, reaction of **CPT** at the 20-hydroxy group with 4-nitrophenyl choroformate was performed to obtain 4-nitrophenyl camptothecin carbonate (**CPT1**),[7] which was readily converted to water-soluble **CPT** derivatives, **CPT3** and **CPT3a–c**, upon treatment by mono-Boc-*N*,*N*′-dimethylethylenediamine, mono-Boc-ethylenediamine, *N*-Boc-1,3-diaminopropane, and *N*-α-Boc-L-lysine, respectively, followed by deprotection: a representative synthesis route of **CPT3** is outlined in Scheme 2. Similarly, treatment of **CPT1** by 1-amino-1-deoxy-β-D-galactose gave **CPT3d**. For reference, *E*-ring opened analogues of **CPT3a,b** (**CPT3a′**,**b′**) were also derived from reactions of **CPT1** with ethylenediamine and 1,3-diaminopropane, respectively.

In view of the stability and hydrolysis reactivity of the above water-soluble **CPT** derivatives (see below), **CPT3** was identified as a favorable lead compound that was readily conjugated with well-known bioreductive motifs of indolequinone, 4-nitrobenzyl alcohol and 4-nitrofuryl alcohol in their *p*-nitrophenyl carbonate forms to obtain the corresponding prodrugs **CPT4–6** (see also Scheme 2 for the synthesis of **CPT4**).

Stability and hydrolysis reactivity of CPT derivatives

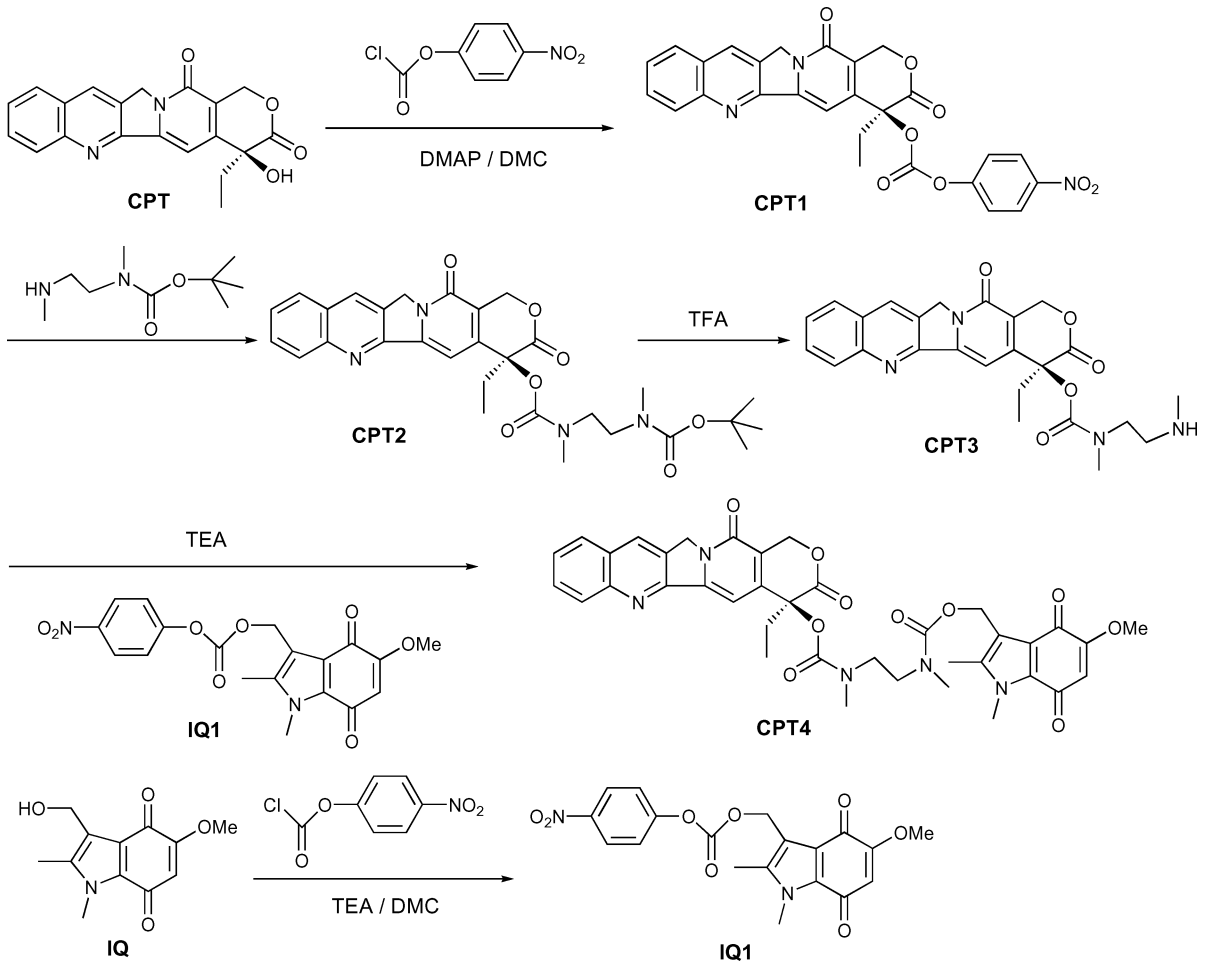
All the modified **CPT** derivatives except **CPT5** showed higher water-solubility than **CPT**. These **CPT** derivatives underwent hydrolysis in tris buffer (pH 7.4) at 37 °C, following the pseudo-first-order kinetics with their characteristic reactivity. **CPT3a** was the most unstable with a half life time ($t_{1/2}$) of 3.6 hours. The results of analytical HPLC and FAB-MS indicated that **CPT3a** was readily converted into the corresponding *E*-ring opened carboxylate form **CPT3a′** at the lactone ring moiety in tris buffer, accompanying the release of free **CPT**. **CPT3b** was similarly less stable in tris buffer ($t_{1/2}$ = 10.2 h) and was partly converted into its carboxylate form **CPT3b′**, along with the release of free **CPT**. In contrast, **CPT3** was relatively more stable ($t_{1/2}$ = 57.2 h), undergoing slow and spontaneous cyclization to release **CPT** with the highest selectivity, as shown in Scheme 3.[7,8a] The other derivatives **CPT3c** ($t_{1/2}$ = 67.3 h), **CPT3d** ($t_{1/2}$ = 36.5 h) and **CPT4** ($t_{1/2}$ = 67.3 h) were also slowly hydrolyzed by a mechanism different from cyclization to release **CPT** in tris buffer, in which several unknown products were simultaneously produced.

Bioreductive activation of CPT4 by DT-diaphorase

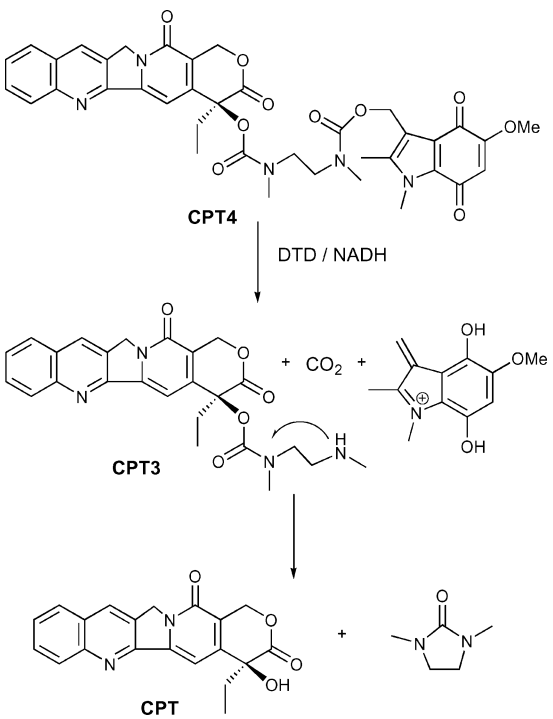
We further characterized a mechanism by which a representative **CPT** prodrug **CPT4** is bioreductively activated by DT-diaphorase (DTD) in tris buffer. It is well known that DTD can two-electron reduce various quinoid compounds, including indolequinones, which have been applied to bioactivated chemotherapy.[12a,19] In the presence of DTD and 2-nicotinamide adenine dinucleotide disodium salt (NADH), **CPT4** was quickly reduced to eliminate the conjugated indolequinone moiety and produce **CPT3** in tris buffer, as shown in Fig. 3a (see also Scheme 3). As characterized in a separate experiment, the resulting **CPT3** effectively underwent intramolecular cyclization to release **CPT**. In a control solution without DTD, **CPT4** was quite slowly hydrolyzed to release **CPT3** followed by formation of **CPT** in considerably low yield, as shown in Fig. 3b.

Cytotoxicity of CPT and its derivatives

In order to evaluate the anticancer ability, the cytotoxicity (IC_{50}; 50% inhibitory concentration) of **CPT** derivatives towards three kinds of tumor cell lines of HT-1080, EMT6/KU and HT-29 were determined by an MTT method.[20] The IC_{50} value of **CPT5** was failed to be measured because of its very weak solubility. As summarized in Table 1, the bioreduction activated prodrugs **CPT4** and **CPT6** showed at least one order of magnitude (about 10∼27 times) lower cytotoxicity than the parent anticancer agent **CPT**, while the water-soluble derivatives **CPT3** and **CPT3a–d** were of slightly reduced cytotoxicity: exceptionally **CPT3c,d**



Scheme 2 Synthesis of bioreduction activated prodrug **CPT4**.



Scheme 3 Mechanism of **CPT4** activation by a reductase of DTD.

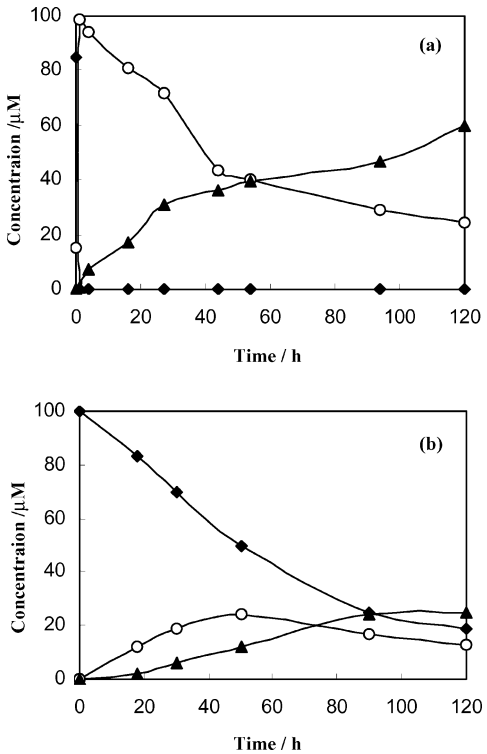


Fig. 3 (a) Time course profiles of **CPT4** (♦), **CPT3** (○) and **CPT** (▲) in the hydrolysis of 0.1 mM **CPT4** in 0.1 M tris buffer (pH 7.4) containing DTD (2.5 units mL^{-1}) and NADH (1.25 mM) at 37 °C; (b) time course profiles of **CPT4** (♦), **CPT3** (○) and **CPT** (▲) in the hydrolysis of 0.1 mM **CPT4** in 0.1 M tris buffer (pH 7.4) containing NADH (1.25 mM) but not DTD at 37 °C.

showed one order of magnitude lower cytotoxicity towards EMT6/KU cells.

Table 1 also compares the IC_{50} values of**CPT, CPT4** and **CPT6** towards HT-29 cells without and with 90 min hypoxia

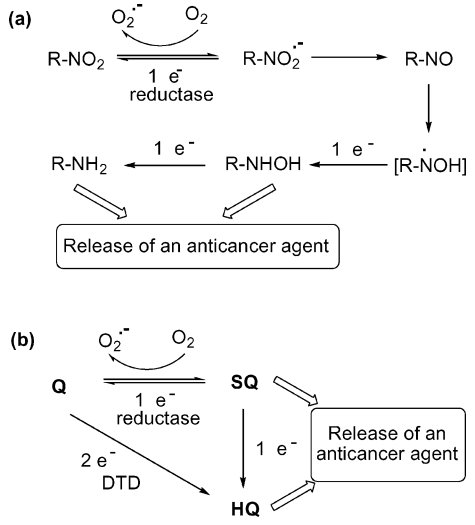
Table 1 Cytotoxicity of **CPT** and its derivatives towards various tumor cells

Compound	IC_{50}/μM, mean ± SD, $n = 5$			
	HT-1080	EMT6/KU	HT-29	HT-29 with hypoxia treatment for 90 min
CPT	0.07 ± 0.01	0.13 ± 0.02	0.09 ± 0.01	0.06 ± 0.01
CPT3	0.15 ± 0.02	0.14 ± 0.02	0.10 ± 0.01	–[a]
CPT3a	0.10 ± 0.02	–[a]	0.10 ± 0.02	–[a]
CPT3b	–[a]	–[a]	0.20 ± 0.02	–[a]
CPT3c	0.30 ± 0.01	1.5 ± 0.1	0.30 ± 0.02	–[a]
CPT3d	0.50 ± 0.05	1.9 ± 0.1	0.70 ± 0.10	–[a]
CPT4	0.75 ± 0.05	2.1 ± 0.2	2.5 ± 0.2	1.40 ± 0.02
CPT6	1.0 ± 0.1	1.4 ± 0.1	2.1 ± 0.2	0.50 ± 0.01

[a] Not determined.

treatment. A trend is obvious that the bioreduction activated prodrugs **CPT4** and **CPT6** caused higher hypoxia selectivity of cytotoxicity (the enhancement ratio of hypoxia selective cytotoxicity at 90 min were 1.7 and 4.1, respectively) than their parent **CPT**. The higher cytotoxicity of **CPT** under hypoxia is attributed to environment acidification induced by tumor cell glycolysis,[21] in accord with the recent reports that cytotoxicity of **CPT** and its analogues is enhanced at acidic pH.[22] The HT-29 cells employed for the assay are among various tumor cell lines over-expressing bioreductive enzymes of DTD[23] and NADPH cytochrome P450 reductase.[24] It is predictable that the apparent hypoxia selective cytotoxicity of **CPT4** and **CPT6** will be more enhanced, as the concentration of released **CPT** increases upon prolonged hypoxic incubation.

These behaviors demonstrate that bioreductive motifs involved in the **CPT4** and **CPT6** are responsible for the hypoxia selective cytotoxicity. Evidently, nitroaromatics as in **CPT6** are more effective motifs than indolequinones as in **CPT4** for enhancing hypoxia selective cytotoxicity. This is in accord with previously reported bioreductive activation mechanisms for prodrug systems with nitroaromatics and quinines including indolequinone. In the nitroaromatics prodrug system (see Scheme 4),[12a,16,17] the first reduction step from nitro to nitroso anion may be inhibited by back-oxidation in the presence of oxygen. On the other hand, the quinone (**Q**) prodrug system (Scheme 4) is activated through either a pathway of semiquinone (**SQ**) intermediate (one-electron reduction process such as bioreduction by NADPH cytochrome P450 reductase) or a direct pathway to hydroquinone (**HQ**) (two-electron reduction process such as bioreduction by DTD) (Scheme 4).[12a,15,19] Whereas the former one-electron reduction pathway may be inhibited by oxygen, the latter two-electron reduction pathway is independent of oxygen effect.



Scheme 4 Bioreductive activation mechanisms of (a) the nitroaromatics prodrug system and (b) the quinone prodrug system releasing anticancer agent.

Conclusion

A series of water-soluble **CPT** derivatives **CPT3** and **CPT3a–d** were synthesized. **CPT3** was a useful lead compound that could be readily conjugated with three kinds of typical bioreductive motifs to produce the bioreduction activated prodrugs **CPT4–6**, respectively. All the modified **CPT** derivatives, except **CPT5**, had greater water-solubility than their parent **CPT**, thus undergoing hydrolysis to release free **CPT** in tris buffer at pH 7.4 according to the pseudo-first-order kinetics with their characteristic reactivity. A mechanism by which a representative **CPT** prodrug, **CPT4** is bioreductively activated by DTD in tris buffer was characterized and discussed. As determined by *in vitro* studies, the bioreduction activated prodrugs **CPT4** and **CPT6** had at least one magnitude of lower cytotoxicity (measured by IC_{50}) than **CPT**, showing hypoxia selective cytotoxicity. **CPT4** and **CPT6** are identified as promising bioreduction activated anticancer prodrugs that may be applicable to the hypoxia targeting tumor chemotherapy and the gene-directed enzyme prodrug therapy (GDEPT).

Experimental

Materials

20(*S*)-Camptothecin (**CPT**) was obtained from Tokyo Kasei (Tokyo, Japan). 4-Dimethylaminopyridine (DMAP), acetonitrile, trifluoroacetic acid (TFA), 2-propanol, triethylamine (TEA), β-nicotinamide adenine dinucleotide disodium salt (NADH), culture medium PRMI-1640, and Eagle's minimal essential medium (MEM) were purchased from Nacalai Tesque (Kyoto, Japan). Indolequinone was a gift from Mr Arimichi Okazaki. *N*-α-Boc-L-lysine was purchased from Watanabe Chemical Ind. (Hiroshima, Japan). Fetal bovine serum (FBS) was obtained from Thermo Trace (Melbourne, Australia). Thiazolyl blue tetrazolium bromide (MTT), 1-amino-1-deoxy-β-D-galactose, 4-nitrophenyl choroformate and *E. coli* DT-diaphorase (DTD) were obtained from Sigma (Steinheim, Germany). Dimethyl sulfoxide (DMSO), 4-nitrofuryl alcohol, 4-nitrobenzyl alcohol and other chemicals were purchased from Wako (Osaka, Japan). The cell line of HT-1080 human fibrosarcoma was supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The cell line of EMT6/KU murine mammary carcinoma was subcultured in Kyoto University. The cell line of HT-29 human colorectal adenocarcinoma was obtained from the American Type Culture Collection (www.attc.org).

General methods

Thin-layer chromatography (TLC) was performed on a silica gel coated glass sheet (Silica Gel 60 F_{254} TLC plates, Merck & Co., Germany). Preparative layer chromatography (PLC) was

performed on a Silica Gel 60 F_{254} PLC plate (Merck & Co. Germany). A Wakogel C-300 silica gel (45–75 μm, Wako Pure Chemical Industries Ltd.) was used for column chromatography. Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a JEOL JNM-EX270J (270 MHz) spectrometer and a JNM-AL300 (300 MHz) spectrometer, respectively. Fast atom bombardment mass spectrometry (FAB-MS) was performed on a JEOL JMS-SX102A mass spectrometer, using a glycerol or 3-nitrobenzyl alcohol (NBA) matrix. High performance liquid chromatography (HPLC) was performed on a Hitachi D-7000 HPLC system equipped with an Interstil® ODS-3 column (4.6ϕ × 250 mm, GL Science Inc., Japan). The samples were eluted with an aqueous CH_3CN (30%) solution containing 0.1 M TEEA buffer (pH 6.4) at a flow rate of 0.6 mL min^{-1} and detected by UV absorbance at wavelength of 360 nm.

Camptothecin–indolequinone conjugate CPT4

Indolequinone (58 mg, 0.25 mmol), 4-nitrophenyl choroformate (50 mg, 0.25 mmol) and triethylamine (0.04 mL, 0.5 mmol) were dissolved in dichloromethane (5 mL) at 0 °C. The resulting solution was stirred for 2 hours at room temperature under nitrogen to produce *p*-nitrophenyl indolequinone carbonate (**IQ1**). **CPT2** (50 mg, 0.089 mmol) was treated by 1 mL of trifluoroacetic acid for 15 min at room temperature. After removal of trifluoroacetic acid the residual crude product **CPT3** was added to the solution containing **IQ1**, followed by the addition of 0.5 mL triethylamine. The reaction mixture was kept under stirring for another 15 min, and concentrated *in vacuo*. The residue was purified by PLC (ethyl acetate) to give camptothecin–indolequinone conjugate **CPT4** (60 mg, 93%) as a red powder. λ_{max} (CH_3CN)/nm 253 (ε/dm^3 mol^{-1} cm^{-1} 27 300), 288 (20 500), 360 (17 300), 440 (2300). δ_H (300 Hz, $CDCl_3$) 0.92 (m, 3 H), 2.06–2.24 (m, 5 H), 2.71–3.79 (3 m, 16 H), 5.08–5.60 (m, 7 H), 7.16 (m, 2 H), 7.56–8.29 (m, 4 H). δ_C (75 MHz, $CDCl_3$) 7.7, 8.4, 29.6, 31.9, 32.3, 35.4, 36.0, 45.6, 47.4, 47.7, 49.9, 57.5, 66.9, 75.9, 96.3, 106.6, 116.4, 127.9, 128.1, 128.2, 128.5, 129.5, 130.5, 131.1, 138.0, 145.9, 148.7, 152.5, 157.4, 159.6, 168.1, 178.8. FAB-HRMS (positive mode, NBA as matrix): *m/z* 724.2631 [MH$^+$], $C_{38}H_{38}N_5O_{10}$ requires 724.2619.

Camptothecin–nitrobenzyl conjugate CPT5

4-Nitrobenzyl alcohol (50 mg, 0.3 mmol), 4-nitrophenyl choroformate (60 mg, 0.3 mmol) and triethylamine (0.05 mL, 0.6 mmol) were mixed in dichloromethane (5 mL) at 0 °C. The resulting solution was stirred for 2 hours at room temperature under nitrogen to produce *p*-nitrophenyl nitrobenzyl carbonate (**NB1**). **CPT2** (40 mg, 0.071 mmol) was treated by 1 mL of trifluoroacetic acid for 15 min at room temperature. After removal of trifluoroacetic acid the crude product **CPT3** was added to the solution containing **NB1**, followed by the addition of 0.2 mL triethylamine. The reaction mixture was kept under stirring for 20 min and concentrated *in vacuo*. The residue was purified by PLC (ethyl acetate) to give camptothecin–nitrobenzyl conjugate **CPT5** (40 mg, 87%) as a yellow powder. λ_{max} (CH_3CN)/nm 217 (ε/dm^3 mol^{-1} cm^{-1} 31 800), 250 (25 100), 362 (14 500). δ_H (270 MHz, $CDCl_3$) 0.98 (t, J = 7.3 Hz, 3 H), 2.04–2.25 (m, 2 H), 2.80–3.51 (m, 10 H), 5.17–5.70 (m, 6 H), 7.21–8.37 (m, 10 H). δ_C (68 MHz, $CDCl_3$) 7.8, 32.0, 35.0, 46.5, 46.9, 49.9, 65.7, 67.2, 68.4, 96.2, 120.1, 123.5, 123.8, 125.1, 127.7, 127.9, 128.1, 128.2, 128.4, 129.3, 130.6, 131.1, 144.0, 145.8, 147.1, 148.7, 150.7, 152.5, 154.6, 157.2, 167.9. FAB-HRMS (positive mode, NBA as matrix): *m/z* 642.2201 [MH$^+$], $C_{33}H_{32}N_5O_9$ requires 642.2200.

Camptothecin–nitrofuryl conjugate CPT6

4-Nitrofuryl alcohol (50 mg, 0.34 mmol), 4-nitrophenyl choroformate (60 mg, 0.3 mmol) and triethylamine (0.05 mL, 0.6 mmol) were dissolved in dichloromethane (3 mL) at 0 °C. The resulting solution was stirred for 2 hours at room temperature under nitrogen to produce *p*-nitrophenyl nitrofuryl carbonate (**NF1**). **CPT2** (50 mg, 0.089 mmol) was treated by 1 mL of trifluoroacetic acid for 15 min at room temperature. After removal of trifluoroacetic acid the crude product **CPT3** was added to the solution containing **NF1**, followed by the addition of 0.2 mL triethylamine. The reaction mixture was kept under stirring for 20 min and concentrated *in vacuo*. The residue was purified by PLC (ethyl acetate) to give camptothecin–nitrofuryl conjugate **CPT6** (45 mg, 80%) as a yellow powder. λ_{max} (CH_3CN)/nm 247 (ε/dm^3 mol^{-1} cm^{-1} 19 700), 310 (15 500), 360 (12 500), 425 (6800). δ_H (300 MHz, $CDCl_3$) 0.93 (t, J = 5.4 Hz, 3 H), 2.02–2.24 (m, 2H), 2.75–3.50 (m, 10 H), 5.05–5.64 (m, 6 H), 6.58 (m, 1 H), 7.15–8.34 (m, 7 H). δ_C (75 MHz, $CDCl_3$) 7.7, 31.9, 34.9, 35.1, 46.6, 46.8, 50.0, 66.8, 67.0, 96.8, 112.1, 112.2, 112.8, 115.6, 126.1, 128.2, 128.3, 129.2, 130.8, 131.4, 140.9, 145.9, 147.1, 148.7, 152.2, 153.3, 153.6, 155.6, 157.5, 162.7, 168.0. FAB-MS (positive mode, NBA as matrix): *m/z* 632.1991 [MH$^+$], $C_{31}H_{30}N_5O_{10}$ requires 632.1993.

Stability and hydrolyses of CPT derivatives

Solutions of **CPT** derivatives **CPT3** and **CPT3a–d** (0.1 μmol) in 0.1 M tris buffer containing 5% acetonitrile (1 mL, pH 7.4) were incubated at 37 °C. Aliquots (10 μL) were sampled at various time intervals, and the concentrations of **CPT** derivatives and hydrolysates during hydrolysis were quantified by analytical HPLC, using the respective calibration curves prepared by reference to the authentic samples. Since the hydrolytic decompositions of **CPT** derivatives were well represented in terms of the first-order kinetics, the hydrolysis rate constants (k) were evaluated from eqn. (1):

$$C_t = C_{t=0}\exp(-kt) \quad (1)$$

Accordingly, the half-life periods ($t_{1/2}$) of **CPT** derivatives were then evaluated as the measures of their stability from eqn. (2):

$$t_{1/2} = \ln 2/k \quad (2)$$

Bioreductive activation of CPT4 by DT-diaphorase

A solution of **CPT4** (0.1 mM), NADH (1.25 mM), and DT-diaphorase (2.5 units mL^{-1}) in 0.1 M tris buffer containing 5% acetonitrile (1 mL, pH 7.4) was incubated at 37 °C under aerobic conditions. Aliquots were sampled for analytical HPLC at appropriate time intervals. A control solution of **CPT4** (0.1 mM) and NADH (1.25 mM) in 0.1 M tris buffer was similarly incubated and analyzed.

In vitro cytotoxicity assays

HT-1080 and EMT6/KU cells were cultured in MEM containing 1% non-essential amino acids and 10% FBS, while HT-29 cells were cultured in RPMI-1640 medium containing 10% FBS. All the cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37 °C. Inhibition of the cellular growth was evaluated using a method of MTT assay described by Mosmann.[20] Suspensions of 5000 cells (50 μL) were seeded in each well of a 96-well cell culture microplate and treated by either 50 μL fresh drug–medium solution at various concentrations from 10^{-10} to 10^{-3} mol L^{-1} or 50 μL control medium solution. These plates were incubated for 72 hours at 37 °C in an atmosphere of humidified 5% CO_2, and then 10 μL of 5 mg mL^{-1} MTT in PBS was added to each well. After MTT cleavage for 4 hours at 37 °C, 100 μL of 0.04 N HCl in 2-propanol was added to each well to dissolve the dark blue crystals. The absorbance at a test wavelength of 570 nm was measured on a Model-550 Microplate Reader (Bio-Rad Laboratories, Hercules, California). The concentration of drug to reduce the cell survival to half of the control value (IC_{50})

was calculated from a regression analysis of cell survival *vs* drug concentration. Hypoxia selective cytotoxicity of **CPT4** and **CPT6** towards HT-29 cells were determined by a similar MTT assay, except that the microplates containing HT-29 cells were kept under N_2 for 90 min after incubation with **CPT4**,6 for 5 hours.

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