



Bioactivation of carbamate-based 20(S)-camptothecin prodrugs

Neta Pessah,^a Mika Reznik,^b Marina Shamis,^a Ferda Yantiri,^c Hong Xin,^c
Katherine Bowdish,^c Noam Shomron,^b Gil Ast^b and Doron Shabat^{a,*}

^a*School of Chemistry, Faculty of Exact Sciences, Tel-Aviv University, Tel Aviv 69978, Israel*

^b*Department of Human Genetics and Molecular Medicine, Tel Aviv University Medical School,
Tel-Aviv University, Tel Aviv 69978, Israel*

^c*Alexion Antibody Technologies, Inc., 3985 Sorrento Valley Blvd., Suite A, San-Diego, CA 92121, USA*

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Abstract—Two new prodrugs of CPT were synthesized, based on carbamate linkages between the 20-hydroxy group of CPT and a linker designed to be enzymatically removed by either Penicillin-G-Amidase or catalytic antibody 38C2. Cell growth inhibition assays showed an up-to-2250-fold difference in toxicity between the prodrugs and the active drug. A significant increase in toxicity was observed upon incubation of the enzyme or the catalytic antibody with the corresponding prodrug. The described derivatives of CPT further our knowledge in the design of prodrugs for use in selective approaches for targeted chemotherapy.

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1. Introduction

Chemotherapy remains the major systemic treatment of malignant diseases. However, it is not very effective against tumors, especially once they have metastasized, mainly because of insufficient drug concentrations inside the tumors, systemic toxicity, development of resistance, and lack of selectivity for tumor cells over normal cells. The clinical efficacy of chemotherapy can be improved by selective delivery of the available drugs to malignant cells, thus reducing toxicity, and permitting much higher drug doses and more frequent treatments.^{1,2} One approach to overcome these drawbacks is the development of relatively non-toxic anticancer agents, in a prodrug form, specifically activated in or near the tumor tissue. The ideal prodrug should be stable in vivo, far less toxic than its parent form, and activated specifically in or within the microenvironment of the tumor cells. However, at this point, a major problem with the prodrug strategy in vivo is the enzymatic moiety: High background reactions by endogenous enzymes result in loss of selectivity.³

The antitumor activity of 20(S)-camptothecin (CPT), a pentacyclic plant alkaloid, was recognized 20 years ago. It was first isolated from the Asian tree *Camptotheca acuminata* by Wall et al. in 1966.⁴ CPT exerts its antitumor activity mainly through inhibition of topoisomerase I.⁵ This enzyme, which is found in mammals, binds preferentially to double-stranded DNA, cleaving one strand and forming an enzyme-DNA covalent bond between a tyrosine residue and the 3' phosphate of the cleaved DNA. Drug-induced accumulation of topoisomerase I-DNA complexes was identified as an essential step, ultimately leading to cell death by apoptosis.⁶

The most common problem associated with CPT is the stability of the 20-hydroxy lactone, which is easily hydrolyzed at neutral pH to provide the inactive carboxylate. It was shown that both the lactone ring stability and the 20-hydroxy group of CPT are critical for its antitumor activity. It was found that masking the 20-hydroxy group of CPT, results in reduced activity of the drug and increased stability of the lactone ring.⁷ The 20-hydroxyl generates an intramolecular hydrogen bond with the carbonyl moiety of the lactone, which accelerates the hydrolysis of the otherwise stable lactone ring. Therefore, masking this hydroxyl by a chemical linker that can be selectively removed, is a convenient approach for generation of a prodrug from CPT.⁸ The masking linker serves two purposes: (1) removal of the intramolecular hydrogen bond, resulting with a

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* Corresponding author. Tel.: +972-3640-8340; fax: +972-3640-9293; e-mail: chdoron@post.tau.ac.il

stable lactone and (2) increasing solubility of CPT, which is almost insoluble in aqueous and organic media without the linker.

The low chemical reactivity of the 20-hydroxy group results from a strong steric hindrance, due to its functionality as a tertiary alcohol. Esterification of this hydroxy group under standard conditions is the easiest method for masking it. Therefore, most of the prepared prodrugs of CPT were ester derivatives.^{9,10} The major disadvantage of an ester prodrug, which needs to be activated specifically, is its poor stability to hydrolysis in physiological pH (exceptional example is of the CPT-PEG ester, which was reported by Greenwald¹⁰). Furthermore, endogenous enzymes with esterase activity can also hydrolyze such an ester prodrug. The relative instability of the ester CPT prodrug results in a small difference of the toxicity levels between the prodrug and the free drug; for example, CPT prodrugs with ester linkage are only 10–20-fold less toxic than their parent drug.¹¹ Here we report on the synthesis and characterization of new CPT prodrugs that are masked through a stable carbamate linkage and are more than 1000-fold less toxic than the parental CPT in cell growth inhibition assays. This CPT prodrugs could be activated by *Escherichia coli* penicillin-G amidase^{8,12} (PGA) or by catalytic antibody 38C2,^{11,13,14} as was shown by in vitro studies on three different cancer cell lines.

2. Results

2.1. Synthesis

CPT prodrug **7** was synthesized by a coupling reaction between compounds **3** and the mono-Boc-*N,N'*-ethylene-diamine derivative of camptothecin **6** after removal of the Boc protecting group (Fig. 1A). CPT prodrug **7a** was synthesized by direct reaction between linker **3a** (prepared in situ from compound **2a** and trifluoroacetic acid) and the *p*-nitrophenyl-carbonate derivative of camptothecin **5** in the presence of triethylamine (Fig.

1B). Both prodrugs were purified by standard flash chromatography on silica gel and characterized by NMR, MS and HPLC chromatograms. The carbamate prodrugs were obtained as a yellowish powder and found to be soluble in most of the organic solvents. The prodrugs were used for further studies by preparing stock solutions in DMSO.

2.2. Prodrugs activation

The masking linker of prodrug **7** was designed for removal by PGA. This enzyme catalyzes the amide bond cleavage of phenylacetamide **7** to generate intermediate **8** (Fig. 2A), which goes through a 1,6-quinone-methide like rearrangement, followed by decarboxylation to generate intermediate **9**. The latter is self-cyclized spontaneously to release the free drug. Activation of the prodrug by PGA was performed by incubating the prodrug with the enzyme at 37 °C in cell-culture-medium. The conversion of the CPT prodrug to its parental drug was monitored by HPLC as presented in Figure 3A. It is clearly shown that upon incubation of prodrug **7** with PGA, intermediates **8** and **9** immediately start to appear along with small amounts of CPT. After a while, the intermediates are decreased and converted completely to CPT.

Similarly, the masking linker of prodrug **7a** was designed to be removed by catalytic antibody 38C2. This aldolase antibody catalyzes a retro-aldol retro-Michael cleavage reaction, followed by spontaneous decarboxylation to generate intermediate **9**. The latter goes through a self-cyclization reaction to release the free drug (Fig. 2B). The conversion of the CPT prodrug **7a** to the parental drug was monitored by HPLC, and the results are presented in figure 3B. It is shown that upon incubation of antibody 38C2 with the prodrug intermediate **9** begins to appear, along with small amounts of CPT. After a while, both the prodrug and the intermediate are decreased and converted to CPT. When prodrug **7a** is incubated in PBS 7.4 at 37 °C only, no background reaction is observed for at least a week.

Table 1. Concentration of CPT, proCPT **7**, and proCPT **7** + PGA, which inhibited cell proliferation of cancer cells by 50% after 72 h of incubation time

Cell line	Tissue	IC 50 (μM)		
		Drug	Prodrug	Prodrug + Enzyme PGA
293T	Embryonic kidney	0.04	56.23	0.50
HeLa	Cervix carcinoma	0.05	18.62	5.01
HepG2	Hepatocellular carcinoma	0.06	35.48	0.49

Table 2. Concentration of CPT, proCPT **7a**, and proCPT **7a** + antibody 38C2, which inhibited cell proliferation by 50% after 72 h of incubation time

Cell line	Tissue	IC 50 (μM)			
		Drug	Prodrug	Prodrug + 2.4 μM 38C2	Prodrug + 24 μM 38C2
Du145	Prostate cancer	0.012	11.55	0.311	0.061
A375	Malignant melanoma	0.003	3.32	0.121	0.05
MCF-7	Breast adenocarcinoma	0.082	47.5	1.75	1.1
BxPc-3	Pancreatic adenocarcinoma	0.02	45	1.5	ND

2.3. Cell growth inhibition

The cytotoxicities of CPT and pro-CPTs were monitored by the number of living cells in the presence of drug and prodrug at a range of concentrations. Three different human tumor cell lines were evaluated for prodrug **7**: embryonic kidney 293T, cervix carcinoma HeLa, and hepatocellular carcinoma HepG2. Comparison of IC_{50} values demonstrated that pro-CPT **7** was 372–1405-fold less toxic than CPT (Table 1). As was shown previously, PGA catalyzed the cleavage of the relatively non-toxic pro-CPT **7** into active CPT in vitro in three of these cell-based assays. Figure 4A shows experimental plots measured for embryonal kidney 293T cell line.

Another four human tumor cell lines were evaluated for prodrug **7a**: breast adenocarcinoma cell line MCF-7, prostate cancer cell line Du 145, malignant melanoma cell line A375, and primary pancreatic adenocarcinoma cell line BxPc-3. Comparison of IC_{50} values demonstrated that pro-CPT **7a** was 500–2250-fold less toxic than CPT (Table 2). To evaluate the efficacy of antibody 38C2-mediated pro-CPT activation, the activity of pro-CPT **7a** was monitored in the absence and presence of catalytic antibody 38C2 (at two different concentrations) with cultured human Du 145 prostate cancer cell line (Fig. 4B). In this cell line, pro-CPT **7a** demonstrated around 1000-fold less toxicity than CPT. In the presence of catalytic antibody 38C2, pro-CPT **7a** was only 25-fold and 5-fold less toxic than CPT, respectively (Table

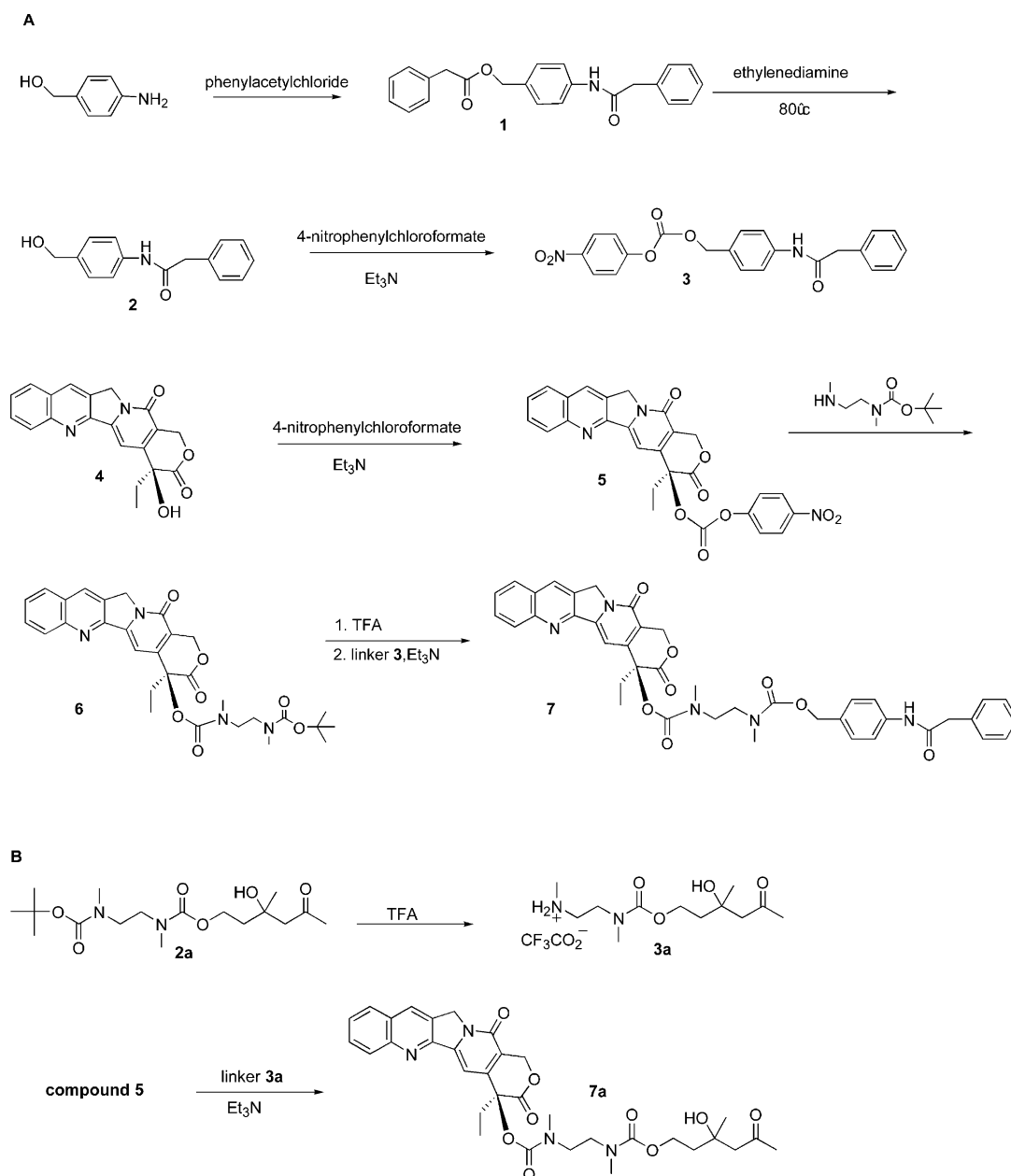


Figure 1. (A) Synthesis of a camptothecin prodrug, designed for activation by penicillin-G amidase. (B) Synthesis of a camptothecin prodrug, which can be activated by catalytic antibody 38C2.

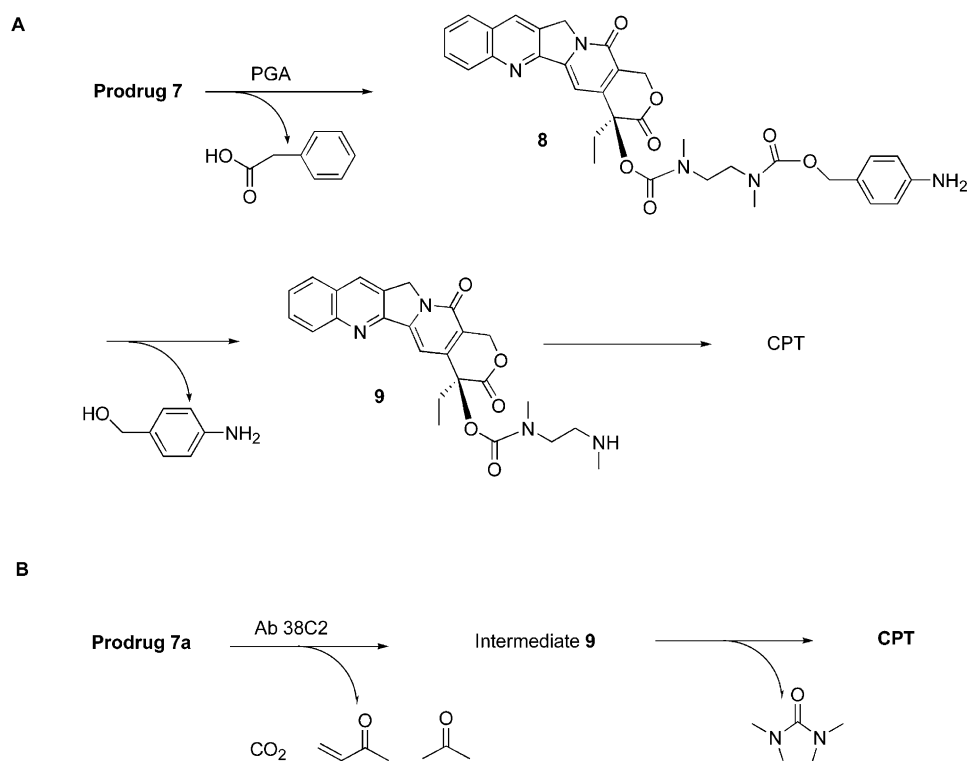


Figure 2. (A) General description of the prodrug activation strategy by PGA catalyzed phenylacetamide cleavage, followed by 1,6-quinone methide rearrangement, spontaneous cyclization and release of the free drug. (B) Prodrug activation strategy by antibody 38C2 catalyzed the retro-aldol-retro-Michael reaction sequence, followed by spontaneous decarboxylation, cyclization and release of the free drug.

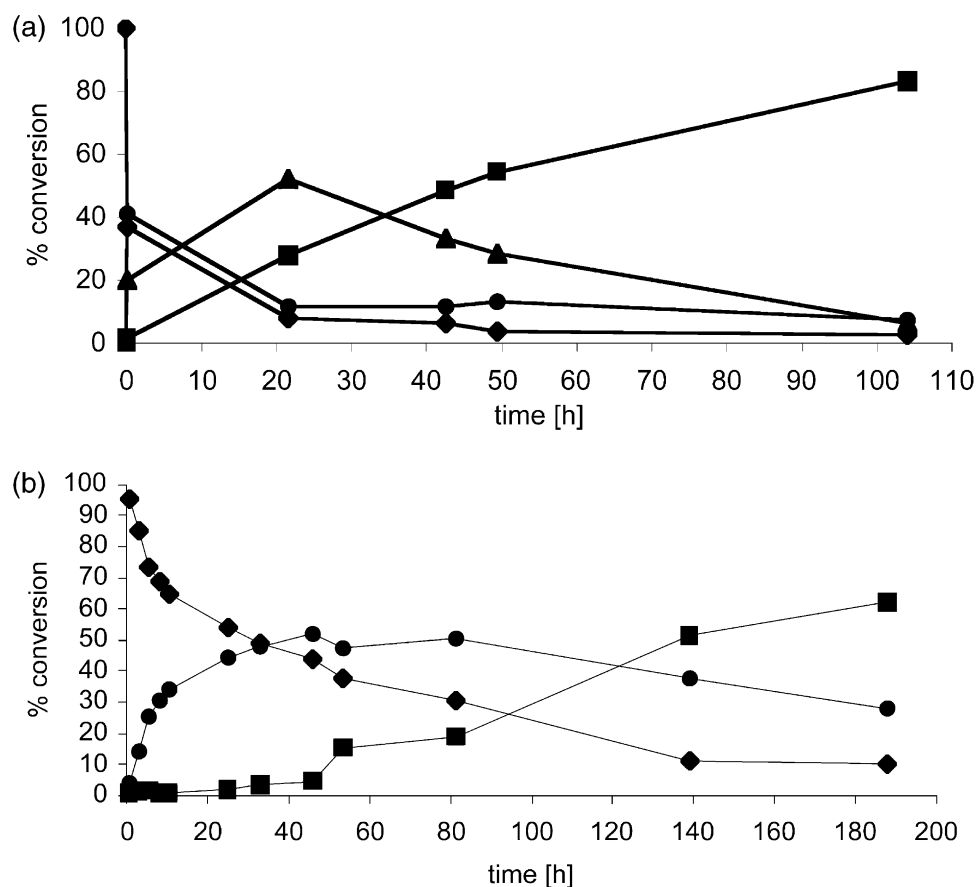


Figure 3. (A) Monitoring of CPT release over time, upon incubation of prodrug **7** (500 μ M) with penicillin-G amidase (5 μ M) in PBS 7.4 at 37°C. (B) Monitoring of CPT release over time, upon incubation of prodrug **7a** (500 μ M) with catalytic antibody 38C2 (50 μ M) in PBS 7.4 at 37°C.

2). This shows clearly that catalytic antibody 38C2 cleaves pro-CPT into active CPT in vitro in cell culture.

We also evaluated the minimum concentration of PGA required to activate prodrug **7**. Embryonic kidney 293T cells were incubated with constant concentration of prodrug **7** and different concentrations of PGA (Fig. 5). Activation was clearly observed with an enzyme concentration as low as 0.001 mg/mL.

3. Discussion

Prodrugs **7** and **7a** are new reported carbamate derivatives between a masking enzymatic substrate and the 20-hydroxy group of CPT. This carbamate linkage is relatively stable at physiological pH. After 7 days of

incubation at 37 °C in PBS-7.4, the prodrugs remain unchanged with no background decomposition (data not shown). We further evaluated the stability of prodrug **7** by incubating it with the extracted cells-supernatant and with 100% mouse serum. In both cases, the prodrug remained unchanged for 72 hr, meaning that there is no natural enzyme in the cells and in the serum that is capable of cleaving the masking linker in a manner similar to PGA.

The CPT prodrugs were found to have increased solubility in aqueous media with values of 2-fold for prodrug **7** and 3-fold for prodrug **7a** (measurements were performed by preparing saturated solutions of the compounds in water and reading their concentration by HPLC with a UV detector). As was indicated above, by masking the 20-hydroxy group, it is possible to

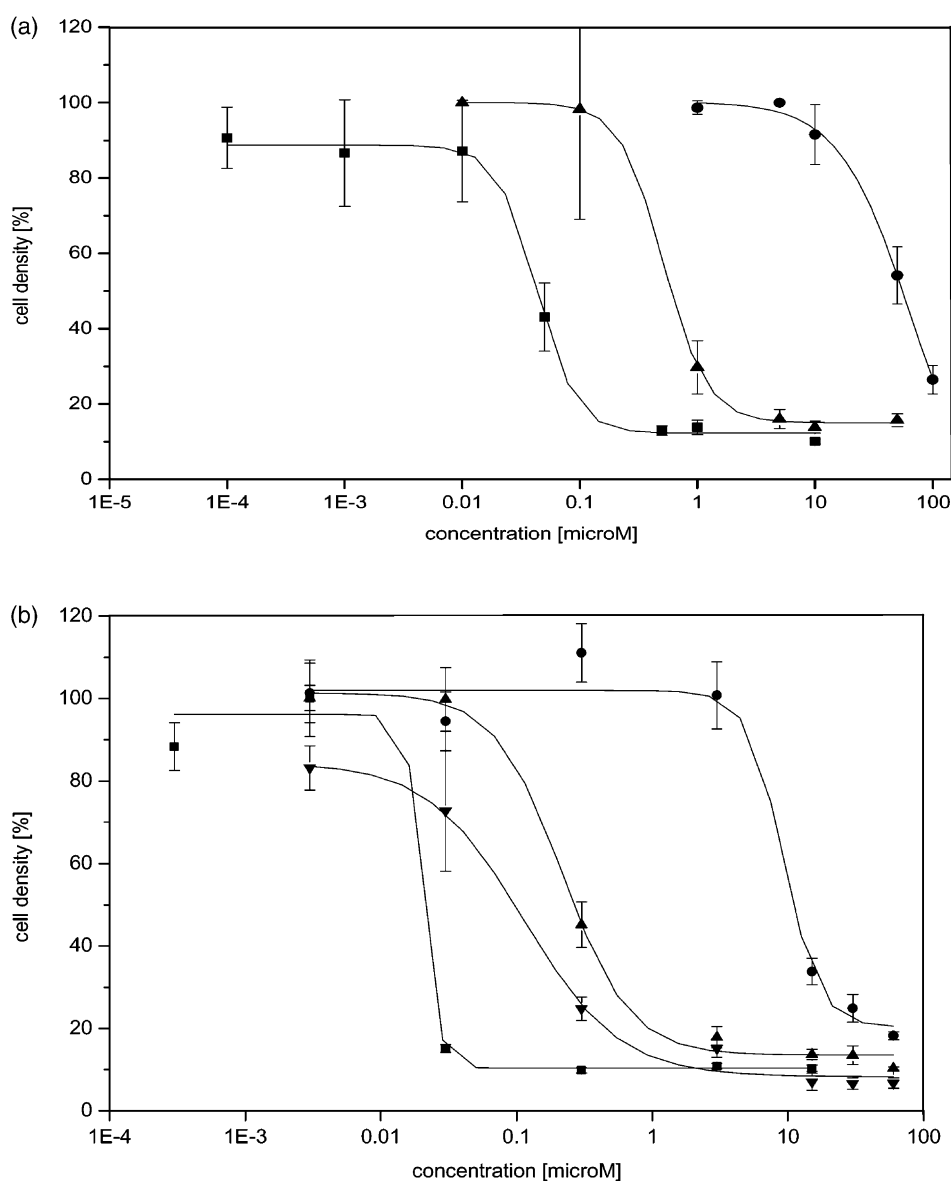


Figure 4. (A) Cytotoxicity of prodrug **7** with PGA on human embryonic kidney 293T cell line. The cells were incubated with the drug and prodrug, with or without 0.005 μg/μL PGA. The viability of the cells was monitored 72 hr after drug/prodrug addition by XTT cell proliferation assay and detected by the absorbance at 470 nm, (■) CPT, (●) pro-CPT, (▲) pro-CPT + 1 μM PGA. (B) Cytotoxicity of drugs to Du 145 human prostate cancer cells. Du 145 cells were exposed to drug and pro-drug in the absence and presence of catalytic antibody 38C2. (■) CPT, (●) pro-CPT, (▲) pro-CPT + 2.4 μM 38C2, (▼) pro-CPT + 24 μM 38C2.

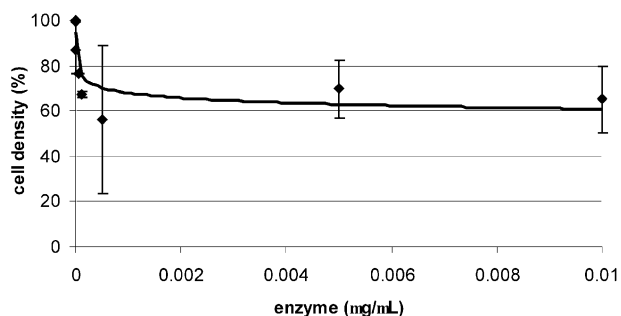


Figure 5. The effect of different concentrations of PGA on the cytotoxicity level of prodrug 7. Human embryonal kidney 293T cells were incubated with 0.5 μ M prodrug (IC₅₀ values were calculated as shown in Fig. 4A) and the indicated concentration of PGA. Cell density was measured as in Figure 4A.

prevent the internal hydrogen bond of the hydroxyl with the neighbored oxygen of the carbonyl, thus leaving the hydroxyl available to a generate hydrogen bond with water molecules.

Although there is a slow activation rate of the prodrugs, cytotoxicity studies performed with several cancer cell lines clearly demonstrated the significantly reduced toxicity of the pro-CPTs, when compared with their parent drug. The best results for prodrug 7 were obtained with human embryonic kidney cell line 293T, in which the pro-CPT was 1405-folds less toxic than CPT (Fig. 4A). When the studies were performed in the presence of PGA, the toxicity of the pro-CPT increased 100-fold, compared with a control experiment in the absence of PGA. The 10-fold cytotoxicity difference between the prodrug+PGA and the drug alone is derived from the slow cyclization of intermediate 9 to free CPT (Fig. 2). Therefore, by increasing the rate of cyclization, it is possible to design an improved prodrug. Addition of substitutions on the *N,N'*-dimethylethylenediamine spacer should speed up the desired cyclization, by constraining the conformation of the five-member ring. Recent studies in our lab have confirmed this assumption. The best results for prodrug 7a were obtained for primary pancreatic adenocarcinoma cell line BxPc-3, in which the pro-CPT was 2250 fold less toxic than CPT. When the studies were performed in the presence of catalytic antibody 38C2, the toxicity of the pro-CPT increased 27–189-fold.

Recently a new approach for selective chemotherapy has been developed (Polymer Directed Enzyme Prodrug Therapy^{15–17} (PDEPT)). This method is a two-step antitumor approach in which both the prodrug and the enzyme are targeted to the tumor site with a polymer molecule. In the first step, a polymer-prodrug conjugate is administered and trapped in tumor tissues through the EPR (enhanced permeability and retention) effect. A conjugate of an HPMA-copolymer and catalytic antibody 38C2 has already been reported.¹⁸ In this chemotherapeutic approach, the relatively slow release of the free drug may well be advantageous in vivo. It will prolong the duration of the drug delivery, already targeted to the tumor tissue by the carrier HPMA-copolymer molecule.

In conclusion, we have designed and synthesized two new camptothecin prodrugs that are suitable for activation with the enzyme PGA and catalytic antibody 38C2. The prodrugs are based on a carbamate linkage between the 20-hydroxy group of CPT and an amine masking linker. These are the first reported carbamate prodrugs of 20(*S*)-CPT, and they exhibit a more than 1000-fold difference between the drug and the prodrug activities. The masking molecule is constructed of two carbamate-functional groups, which make the prodrug more hydrophilic and, therefore, more soluble in water. Further studies toward faster cyclization linkers may lead to more practical carbamate CPT prodrugs.

4. Experimental

4.1. General

All reactions requiring anhydrous conditions were performed in oven-dried glassware under an Ar or N₂ atmosphere. Chemicals and solvents were either *puriss p.A.* or purified by standard techniques. THF was distilled from sodium-benzophenone. Thin layer chromatography (TLC): silicagel plates Merck 60 F₂₅₄; compounds were visualized by irradiation with UV light and/or by treatment with a solution of 25 g phosphomolybdic acid, 10 g Ce(SO₄)₂·H₂O, 60 mL concd. H₂SO₄ and 940 mL H₂O, followed by heating and/or by staining with a solution of 12 g 2,4-dinitrophenylhydrazine in 60 mL concd. H₂SO₄; 80 mL H₂O and 200 mL 95% EtOH, followed by heating and/or immersing into an iodine bath (30 g I₂, 2 g K_i, in 400 mL EtOH/H₂O 1:1) and warming. Flash chromatography (FC): silica gel Merck 60 (particle size 0.040–0.063 mm), eluent given in parentheses. ¹H NMR: Bruker AMX 200. The chemical shifts are expressed in δ relative to TMS (δ =0 ppm) and the coupling constants *J* in Hz. The spectra were recorded in CDCl₃ as solvent at room temperature unless stated otherwise. HR-MS: liquid secondary ionization (LSI-MS): VG ZAB-ZSE with 3-nitrobenzyl-alcohol matrix. All general reagents, including salts and solvents, were purchased from Aldrich (Milwaukee, MN).

4.2. Synthesis of camptothecin prodrug 7

Linker 3 was prepared by the following method: Phenylacetic acid was dissolved in thionyl chloride and the reaction mixture was refluxed for 1 h. The excess of thionyl chloride was removed under reduced pressure, yielding phenylacetyl chloride. The product (3.14 g, 0.02 mol) was then dissolved in dry THF and added dropwise to a solution of 4-aminobenzylalcohol (1 g, 8.12 mmol) and DMAP (2.47 g, 0.02 mole) at 0°C. The mixture was stirred overnight and then washed with dichloromethane and NaOH 1M. The organic layer was dried over magnesium sulfate. The solvent was removed under reduced pressure, and the product was purified by column chromatography on silica gel (ethylacetate/hexanes 40:60), yielding compound 1 (2.17 g, 74.5%). Compound 1 (2 g, 5.6mmol) was heated to 80°C with ethylenediamine for 1.5 h and then washed with HCl 1M and Ethyl acetate to give pure compound 2 (1.207 g, 89%).

^1H NMR (200MHz, MeOD): δ = 7.52 (2H, d, J = 8.5); 7.3 (2H, d, J = 8.5); 4.37 (2H, s); 3.49 (2H, s).

The *p*-nitrophenyl-carbonate of compound **2** was prepared by the following procedure. Compound **2** (1.2g, 4.97 mmol), triethylamine (0.706 mL, 9.95 mmol) and DMAP (cat. amount) were dissolved in dry THF. *p*-Nitrophenyl-chloroformate (2 g, 9.95 mmol) was added, and the mixture was stirred for 1.5 h under Argon. The mixture was washed with ethylacetate and 1M HCl and then with saturated NaHCO₃. The organic layer was dried. The solvent was removed under reduced pressure, and the product was purified by column chromatography on silica gel (ethylacetate/hexanes 30:70), yielding compound **3** (1.18g, 82%).

^1H NMR (200MHz, CDCl₃): δ = 8.26 (2H, d); 7.36 (11H, m); 5.23 (2H, s); 3.76 (2H, s);

Compound **5**. Camptothecin (1.5 g, 4.3 mmol) and PNP-chloroformate (2.6 g, 12.9 mmol) were dissolved in methylene-chloride (45 mL) at 0° C, followed by the addition of DMAP (3.18 g, 25.8 mmol). The resulting clear solution was stirred at room temperature for 1 h. The reaction was monitored by TLC (EtOAc 100%). After completion, the mixture was diluted with 75 mL of methylene-chloride and washed with 30 mL HCl 0.1 N. The organic layer was dried over magnesium sulfate, concentrated under reduced pressure to 10 mL, and precipitated with ether. The precipitated solid was filtered and dried to give crude compound **5** in the form of yellow powder (2.5 g).

^1H NMR (200MHz, CDCl₃): δ = 8.39 (1H, s); 8.25 (1H, d, J = 8); 7.94 (1H, d, J = 8); 7.85–7.81 (1H, m); 7.71–7.67 (1H, m); 7.48 (2H, d, J = 7); 7.14 (2H, d, J = 7); 5.76 (1H, d, J = 16); 5.35–5.2–32 (3H, m); 1.90–2.86 (2H, m); 1.11–1.03 (3H, m).

Compound **6** was prepared by the following procedure. *p*-nitrophenyl camptothecin carbonate **5** (710 mg, 1.4 mmol) was dissolved in DMF, and mono-Boc-*N,N'*-dimethyl ethylene-diamine (400 mg, 2.13 mmol) was added. The mixture was stirred for 30 min, and the DMF was removed under reduced pressure. The remaining residue was purified by column chromatography on silica gel (ethyl acetate, 100%), yielding Compound **6** (256 mg, 32%).

^1H NMR (200 MHz, CDCl₃): δ = 8.40 (1H, s); 8.20 (1H, d, J = 8); 7.97–7.67 (3H, m); 5.65 (1H, d, J = 10); 5.29 (4H, m) 3.8–2.83 (10H, m); 2.02–1.0 (14H, m).

MS(FAB): C₃₀H₃₄N₄O₇ [M + Na]⁺ 585.2

4.3. Camptothecin prodrug **7**

Trifluoroacetic acid (2 mL) was added to compound **6** (50 mg, 0.089 mmol). After 2 min of stirring, the excess of TFA was removed under reduced pressure. The crude product was used directly for the next reaction and was dissolved in 3 mL of DMF. Compound **3** (37.43 mg, 0.089 mmol) was added to the stirred solution, followed by the addition of 0.5 triethylamine. The mixture was

stirred for 15 min, and the DMF was removed under reduced pressure. The remaining residue was purified by thin-layer chromatography on silica gel (ethylacetate/methanol 98:2) yielding prodrug **7** (45.1 mg, 68%).

M.S. FAB MNa⁺ expected: 752.28, found: 752.2

4.4. Synthesis of Camptothecin prodrug **7a**

Linker **2a** was synthesized as published previously (7). Linker **3a** was prepared in situ by the following procedure. Trifluoroacetic acid (2 mL) was added to compound **2a** (360 mg, 1.0 mmol). After 2 min of stirring, the excess of TFA was removed under reduced pressure, resulting of linker **3a**. The crude product was used directly for the next reaction. Thus, compound **5** (754 mg, 1.0 mmol) was added to a stirred solution of linker **3a** in 5 mL of DMF, followed by the addition of 1 mL triethylamine. The mixture was stirred for 1 h, and the DMF was removed under reduced pressure. The remaining residue was purified by column chromatography on silica gel (ethyl acetate/methanol 95:5), yielding prodrug **7a** (711 mg, 81%).

M.S. MALDI-FTMS MNa⁺ expected: 657.2531, found: 657.2505.

4.5. HPLC assay for pro-CPT activation

(Prodrug **7**) A stock solution of 3.5 mg/mL Penicillin-G-Amidase in DMEM cell medium stored at 4 ° C was used. All enzyme reactions were performed in DMEM cell medium at 37° C in microfuge tubes. The reactions were performed at a concentration of 250 μM of substrate and 43 μM enzyme. Enzyme-catalyzed reactions were monitored at 360 nm for CPT by RP-HPLC using a Gradient of acetonitrile:water at 1 mL/min.

(Prodrug **7a**), A stock solution of 5 mg/mL antibody 38C2 in PBS (pH 7.4) stored at 4° C was used. All antibody reactions were performed in PBS (pH 7.4) at 37° C in microfuge tubes, typically at concentrations of 20–200 μM of substrate and 5 μM antibody. Antibody-catalyzed reactions were monitored at 360 nm for CPT by RP-HPLC using a ratio of 35:65 of acetonitrile:water at 1 mL/min.

4.5.1. Cell lines. Human embryonic kidney (293T), human cervix carcinoma (HeLa) cells and human hepatocellular carcinoma (HepG2) were grown in DMEM supplemented with 10% foetal calf serum and were purchased from Biological industries, Bet-Haemek. Breast adenocarcinoma (MCF-7), prostate cancer (Du 145), malignant melanoma (A375), and primary pancreatic adenocarcinoma (BxPc-3) cell lines were obtained from American Type Culture Collection (ATCC). EMEM (2mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential aminoacid, 1.5 g/l sodium bicarbonate, and 10% fetal bovine serum) medium was used for these cell lines.

4.5.2. In vitro growth inhibition assays. The activity of pro-CPTs against carcinoma cell lines was analyzed in the present and absence of PGA and catalytic antibody

38C2. Cells were plated in 96-well plates at 2×10^3 cells per well. CPT and pro-CPT were added 24 h later at various concentrations in the presence and absence of the enzymes. CPT was dissolved in DMSO and pro-CPT in acetonitrile, diluted in 200 μ L of EMEM or DMEM. Quadruped wells were prepared for each drug concentration and for the controls. Plates were incubated at 37 C 5% CO₂ for 72 h. After 72 h, the medium was removed, and 200 μ L of fresh medium was added to the cells. The number of living cells was determined by Cell-Titer 96[®] AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega). Briefly, 2.0 mL of novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt MTS, and 100 μ L of an electron coupling reagent (phenazine methosulfate, PMS) were mixed. 20 μ L of MTS/PMS solution was added to cells, which then were incubated at 37 C 5% CO₂ for 3 h. The conversion of MTS into aqueous soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product was measured by the intensity of 490 nm absorbance on ELISA plate reader. IC₅₀ values were calculated from interpolation of logarithmic dose-A₄₉₀ curves. Cell proliferation assay was repeated twice for most of the cell lines, and the mean value and standard deviation were calculated.

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