



Article

# PSMA-Oriented Target Delivery of Novel Anticancer Prodrugs: Design, Synthesis, and Biological Evaluations of Oligopeptide-Camptothecin Conjugates

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**Abstract:** Clinical applications of camptothecin (CPT) have been heavily hindered due to its non-targeted toxicity, active lactone ring instability, and poor water solubility. Targeted drug delivery systems may offer the possibility to overcome the above issues as reported. In this research, a series of prostate-specific membrane antigen (PSMA)-activated CPT prodrugs were designed and synthesized by coupling water-soluble pentapeptide, a PSMA hydrolyzing substrate, to CPT through an appropriate linker. The cytotoxicity of CPT prodrugs was masked temporarily until they were hydrolyzed by the PSMA present within the tumor sites, which restored cytotoxicity. The in vitro selective cytotoxic activities of the prodrugs were evaluated against PSMA-expressing human prostate cancer cells LNCaP-FGC and non-PSMA-expressing cancer cells HepG2, HeLa, MCF-7, DU145, PC-3 and normal cells MDCK, LO2 by standard methylthiazol tetrazolium (MTT) assay. Most of the newly synthesized CPT prodrugs showed excellent selective toxicity to PSMA-producing prostate cancer cells LNCaP-FGC with improved water solubility. From among the library, CPT-HT-J-ZL<sub>12</sub> showed the best cytotoxic selectivity between the PSMA-expressing and the non-PSMA-expressing cancer cells. For example, the cytotoxicity of CPT-HT-J-ZL<sub>12</sub> (IC<sub>50</sub> = 1.00 ± 0.20 μM) against LNCaP-FGC (PSMA<sup>+</sup>) was 40-fold, 40-fold, 21-fold, 5-fold and 40-fold, respectively, higher than that against the non-PSMA-expressing cells HepG2 (IC<sub>50</sub> > 40.00 μM), HeLa (IC<sub>50</sub> > 40.00 μM), MCF-7 (IC<sub>50</sub> = 21.68 ± 4.96 μM), DU145 (IC<sub>50</sub> = 5.40 ± 1.22 μM), PC-3 (IC<sub>50</sub> = 42.96 ± 3.69 μM) cells. Moreover, CPT-HT-J-ZL<sub>12</sub> exhibited low cytotoxicity (IC<sub>50</sub> > 40 μM) towards MDCK and LO2 cells. The cellular uptake experiment demonstrated the superior PSMA-targeting ability of the CPT-HT-J-ZL<sub>12</sub>, which was significantly accumulated in LNCaP-FGC (PSMA<sup>+</sup>), while it was minimized in HepG2 (PSMA<sup>-</sup>) cells. Further cell apoptosis analyses indicated that it showed a dramatically higher apoptosis-inducing activity in LNCaP-FGC (PSMA<sup>+</sup>) cells than in HepG2 (PSMA<sup>-</sup>) cells. Cell cycle analysis indicated that CPT-HT-J-ZL<sub>12</sub> could induce cell cycle arrest at the S phase.

**Keywords:** camptothecin; oligopeptide derivatives; prostate-specific membrane antigen; structure-activity relationships; apoptosis

## 1. Introduction

Cancer remains one of the major diseases that severely threatens human life and health. Chemotherapy is still one of the main clinical treatments for cancer, either alone or in combination with

other treatment options [1,2]. The low selectivity of conventional chemotherapeutic drugs induces serious adverse events and systemic toxicities [3–6]. Therefore, it is of great importance to develop targeted anti-cancer drug delivery.

Camptothecin (CPT), a natural alkaloid from *Camptotheca acuminata*, possesses remarkable antitumor properties against a variety of cancers via inhibition of DNA enzyme topoisomerase I (topo I) [7–9]. Even though CPT exhibits potent anticancer activities in vitro and vivo, its therapeutic application is severely hindered due to its poor aqueous solubility and high lipophilicity, active lactone ring instability, and non-targeted toxicity [10–12]. Previous research has shown that structural modifications at the 20-OH of camptothecin could enhance the stability of the lactone ring, and prodrugs derivatized at this site have been vigorously pursued [13–16].

The prostate-specific membrane antigen (PSMA) is a 750-amino acid type II transmembrane peptidase enzyme that is encoded by the folate hydrolase 1 (*FOLH1*) gene. Previous studies demonstrated that PSMA was highly expressed in prostate cancer cells and in tumor endothelial cells (ECs) of a variety of non-prostatic solid tumor types but was not expressed by ECs in normal tissues. In addition, PSMA is also known as glutamate carboxypeptidase II, *N*-acetyl-L-aspartyl-L-glutamate peptidase I, and *N*-acetyl-aspartylglutamate peptidase. It contains a binuclear zinc site and is active as a glutamate carboxypeptidase, catalyzing the hydrolytic cleavage of  $\alpha$ - or  $\gamma$ -linked glutamates from peptides or small molecules [17–20]. Based on the above, PSMA is becoming one of the current research hotspots and is an excellent target for the anti-angiogenesis targeted therapy [19–23]. The enzymatic activity of PSMA can be exploited for the design of prodrugs, in which an inactive form of the cytotoxic drug is selectively cleaved and thereby activated only at cells that express PSMA within the tumor microenvironment.

In view of the unique carboxypeptidase activity and restricted expression of the PSMA, we accomplished the synthesis of several PSMA-activated camptothecin pentapeptide prodrugs **CPT-HT-J-ZLn**, by introducing an oriented oligopeptide **HT-J** (Glu\*Glu\*Glu\*Asp-Glu) (“\*” mean the  $\gamma$ -glutamyl linkage; “-” mean the  $\alpha$ -glutamyl linkage) to the 20-OH of CPT via carbonate linkers with different lengths, in order to improve their tumor targeting, stability, and aqueous solubility. All newly synthesized compounds were fully characterized by  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ , and HRMS; they were also tested for selective cytotoxic activity against PSMA-expressing human prostate cancer cell LNCaP-FGC and non-PSMA-expressing cells HepG2, Hela, MCF-7, DU145, and PC-3, respectively [24–28] and normal cells MDCK, LO2 by standard MTT assay. Meanwhile, the preliminary anti-tumor mechanisms of the most selectivity compound **CPT-HT-J-ZLn** were also investigated by fluorescence staining observation and flow cytometric analysis in the present study. In addition, the structure-activity relationships of these derivatives are briefly discussed.

## 2. Results

### 2.1. Chemistry

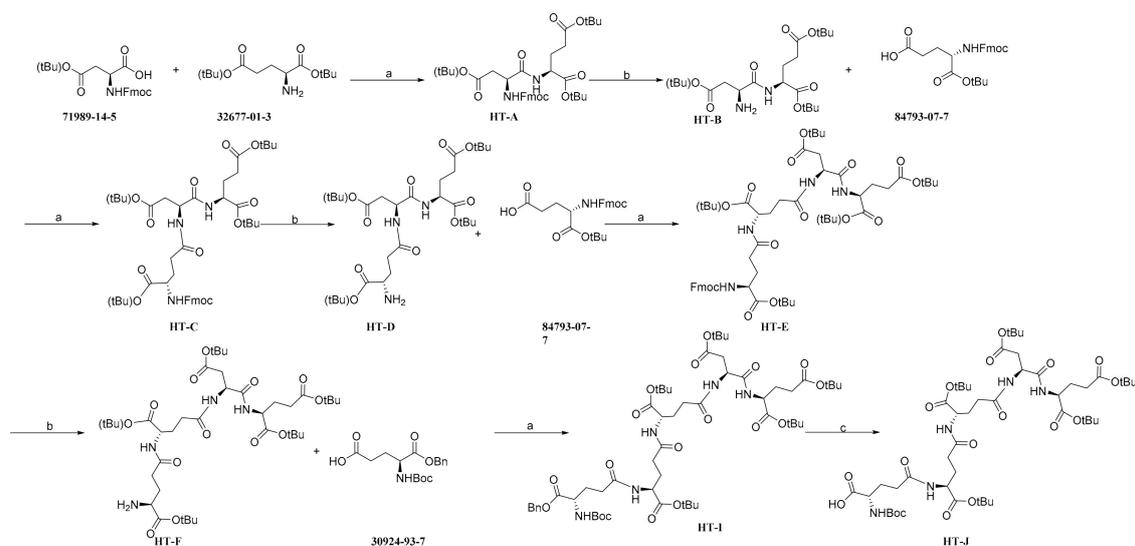
The designed derivatives were prepared following the procedures in Schemes 1–3. **HT-J** (Glu\*Glu\*Glu\*Asp-Glu), the pentapeptide used to target PSMA, was synthesized by liquid-phase peptide chemistry starting with Fmoc-Asp(OtBu)-OH (Scheme 1).

The intermediate **CPT-A-Ln** was subsequently prepared by 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDCI)-mediated esterification from CPT. Then **CPT-A-Ln** was further treated with trifluoroacetic (TFA) in dry dichloromethane (DCM) to remove the Boc-protective group, and the key intermediate **CPT-B-Ln** was successfully obtained (Scheme 2).

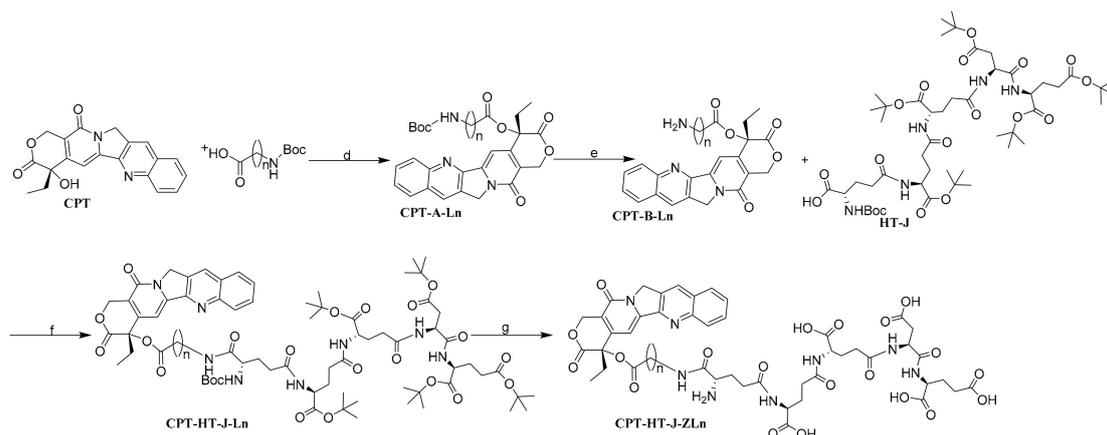
For the synthesis of the PSMA-activated prodrug **CPT-HT-J-ZLn**, reaction of **CPT-B-Ln** with **HT-J** in the presence of EDCI, *N,N*-Diisopropylethylamine (DIPEA), and 1-hydroxybenzotriazole (HOBT) in DCM afforded the corresponding intermediate **CPT-HT-J-Ln**, which was further treated with TFA to remove the protective groups, yielding the final prodrug **CPT-HT-J-ZLn** (Scheme 2). It is worth noting that the tert-butyl cation released in the decomposition of the tert-butyl ester was a powerful

electrophile that might react with the substrate. To avoid the formation of undesirable by-products, the nucleophilic scavenger thioanisole was added to TFA.

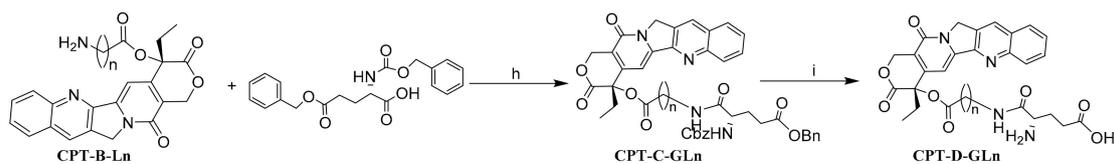
Previous studies have shown that PSMA is an exopeptidase, and hydrolytic processing of any prodrug would result in a product consisting of a cytotoxin coupled to the acidic amino acids glutamate or aspartate [19]. To better study the PSMA-targeting ability of the prodrug **CPT-HT-J-ZL<sub>12</sub>**, the PSMA hydrolysate **CPT-D-Ln** was prepared as well, according to the procedures in Scheme 3 in this study. In brief, the intermediate **CPT-B-Ln** underwent coupling reaction with *N*-Cbz-L-glutamic acid 5-benzyl ester in the presence of EDCl, HOBt, and DIPEA in dry DCM to afford the intermediate **CPT-C-GLn**, which was further treated with Pd/C (10%) in methanol (MeOH) to give the final compound **CPT-D-GLn**. The structures of all target derivatives were confirmed by spectral (<sup>1</sup>H-NMR, <sup>13</sup>C-NMR, and HRMS) analysis (Figures S1–S122).



**Scheme 1.** Synthesis of the pentapeptide **HT-J**. Reagents and Conditions: (a) EDCl, HOBt, DIPEA, DCM, 25 °C, 3 h; (b) Piperidine, dimethylformamide (DMF), 1 h; (c) Wet Pd/C (5%), MeOH, 12 h.



**Scheme 2.** Synthesis of the PSMA-activated prodrugs **CPT-HT-J-ZLn**. Reagents and Conditions: (d) EDCl, DMAP, DCM, 25 °C, 12 h; (e) TFA, DCM, 25 °C, 2 h; (f) EDCl, HOBt, DIPEA, DCM, 25 °C, 3 h; (g) Trifluoroacetic (TFA):Thioanisole:H<sub>2</sub>O = 95:2.5:2.5, 25 °C, 3 h.



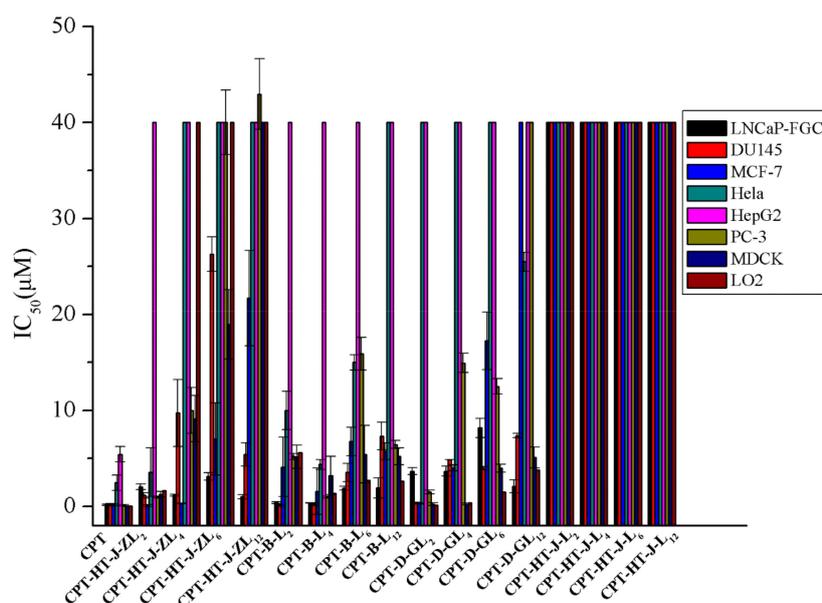
**Scheme 3.** Synthesis of the prostate-specific membrane antigen (PSMA) hydrolysates **CPT-D-GLn**.  
Reagents and Conditions: (h) EDCI, HOBT, DIPEA, DCM, 25 °C, 3 h; (i) Wet Pd/C (5%), MeOH, 12 h.

## 2.2. Cytotoxicity

The cytotoxicities of PSMA-activated prodrug **CPT-HT-J-ZLn** and PSMA hydrolysate **CPT-D-GLn** were evaluated by MTT assays on PSMA-expressing (PSMA<sup>+</sup>) cancer cell LNCaP-FGC, non-PSMA-expressing (PSMA<sup>-</sup>) cancer cells HepG2, MCF-7, HeLa, DU145, and PC-3, and normal cells MDCK, LO2, respectively, using **CPT** as a positive control. The IC<sub>50</sub> values are summarized in Table 1 and Figure 1.

**Table 1.** The anti-proliferative effects and ClogP values of the **CPT-HT-J-ZLn** and the prostate-specific membrane antigen (PSMA) hydrolysate **CPT-D-GLn**.

Compound	IC <sub>50</sub> (μM)								CLogP
	MCF-7	PC-3	DU145	LNCaP-FGC	HepG2	HeLa	LO2	MDCK	
<b>CPT</b>	0.16 ± 0.10	0.13 ± 0.09	0.21 ± 0.09	0.18 ± 0.17	5.43 ± 0.81	2.48 ± 0.80	0.04 ± 0.01	0.02 ± 0.01	0.9
<b>CPT-HT-J-ZL<sub>2</sub></b>	0.11 ± 0.08	1.00 ± 0.10	1.16 ± 0.28	2.04 ± 0.30	>40.00	3.54 ± 2.54	1.68 ± 0.45	1.27 ± 0.30	-7.45
<b>CPT-HT-J-ZL<sub>4</sub></b>	0.32 ± 0.01	9.98 ± 2.38	9.73 ± 3.49	1.18 ± 0.10	>40.00	>40.00	>40.00	9.13 ± 2.40	-6.86
<b>CPT-HT-J-ZL<sub>6</sub></b>	7.03 ± 3.76	40.00 ± 3.37	26.28 ± 1.81	3.13 ± 0.40	>40.00	>40.00	>40.00	18.96 ± 3.60	-6.60
<b>CPT-HT-J-ZL<sub>12</sub></b>	21.68 ± 4.96	42.96 ± 3.69	5.40 ± 1.22	1.00 ± 0.20	>40.00	>40.00	>40.00	>40.00	-3.42
<b>CPT-B-L<sub>2</sub></b>	4.11 ± 3.09	5.19 ± 0.30	0.12 ± 0.09	0.38 ± 0.10	>40.00	10.00 ± 2.00	5.61 ± 1.40	5.16 ± 1.20	0.54
<b>CPT-B-L<sub>4</sub></b>	1.58 ± 2.42	1.00 ± 0.10	0.25 ± 0.13	0.37 ± 0.02	>40.00	4.37 ± 0.50	1.36 ± 0.30	3.20 ± 2.00	1.26
<b>CPT-B-L<sub>6</sub></b>	6.76 ± 1.47	15.89 ± 1.73	3.55 ± 0.95	1.89 ± 0.20	>40.00	15.0 ± 0.80	2.71 ± 0.38	5.44 ± 3.00	1.76
<b>CPT-B-L<sub>12</sub></b>	5.76 ± 0.86	6.45 ± 0.42	7.32 ± 1.43	1.93 ± 1.03	>40.00	>40.00	2.63 ± 0.84	5.2 ± 0.88	4.94
<b>CPT-D-GL<sub>2</sub></b>	0.30 ± 0.05	1.5 ± 0.20	0.40 ± 0.05	3.68 ± 0.36	>40.00	>40.00	0.16 ± 0.07	0.26 ± 0.14	-2.88
<b>CPT-D-GL<sub>4</sub></b>	4.03 ± 0.30	14.93 ± 1.00	4.89 ± 0.01	3.68 ± 0.50	>40.00	>40.00	0.40 ± 0.15	0.23 ± 0.10	-2.29
<b>CPT-D-GL<sub>6</sub></b>	17.25 ± 3.00	12.50 ± 0.80	3.97 ± 0.16	8.17 ± 1.00	>40.00	>40.00	1.5 ± 0.60	4.0 ± 0.42	-2.03
<b>CPT-D-GL<sub>12</sub></b>	>40.00	>40.00	7.38 ± 0.20	2.10 ± 0.68	>40.00	25.47 ± 1.0	3.8 ± 0.80	5.1 ± 1.10	1.15
<b>CPT-HT-J-L<sub>2</sub></b>	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	4.04
<b>CPT-HT-J-L<sub>4</sub></b>	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	4.62
<b>CPT-HT-J-L<sub>6</sub></b>	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	4.89
<b>CPT-HT-J-L<sub>12</sub></b>	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	>40.00	8.07



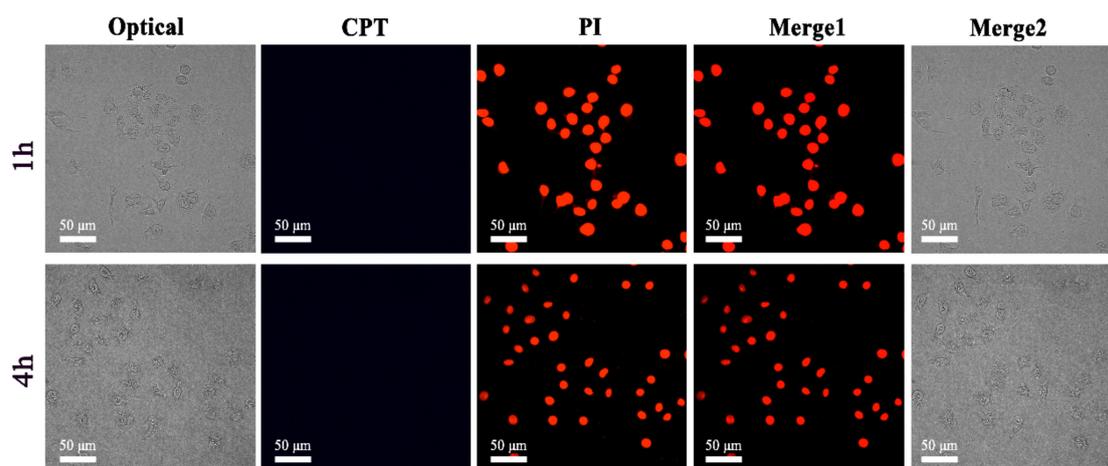
**Figure 1.** The cytotoxicities of the **CPT-HT-J-ZLn** and the PSMA hydrolysate **CPT-D-GLn** to different tumor and normal cells.



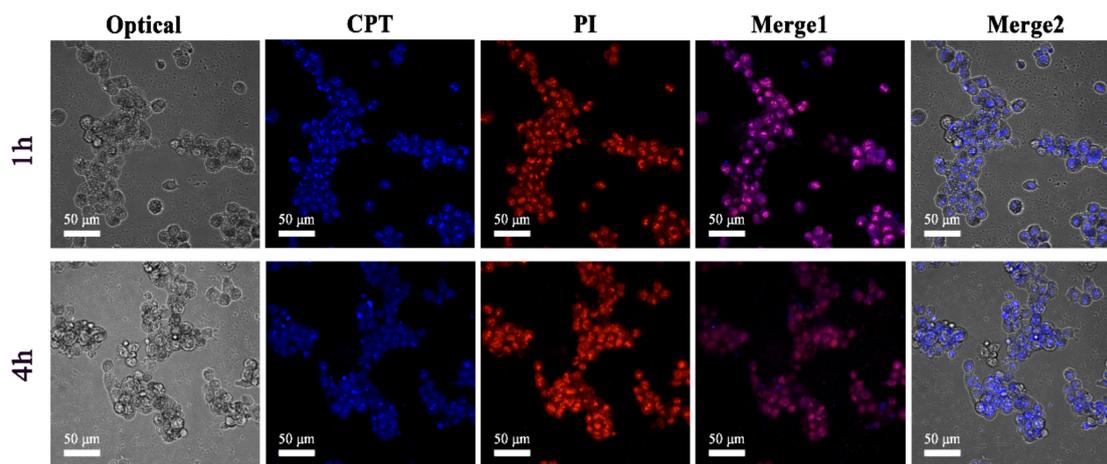
an important role in the drug discovery process on account of their being closely associated with the absorption, distribution, metabolism, and excretion (ADME) properties of the compounds [30–32]. In this work, the  $\log P$  values for these derivatives were calculated using the Sybyl-X 2.0 software (Tripos, Certara Inc., St. Louis, MO, USA). As shown in Table 1, all the prodrug **CPT-HT-J-ZLn** showed lower  $\text{ClogP}$  than **CPT**. Meanwhile the aqueous solubility of the prodrug **CPT-HT-J-ZLn** (19.00 mg/mL in PBS, pH = 7.4), which was much higher than **CPT** (0.003 mg/mL in PBS, pH = 7.4).

#### 2.4. In Vitro Cellular Uptake

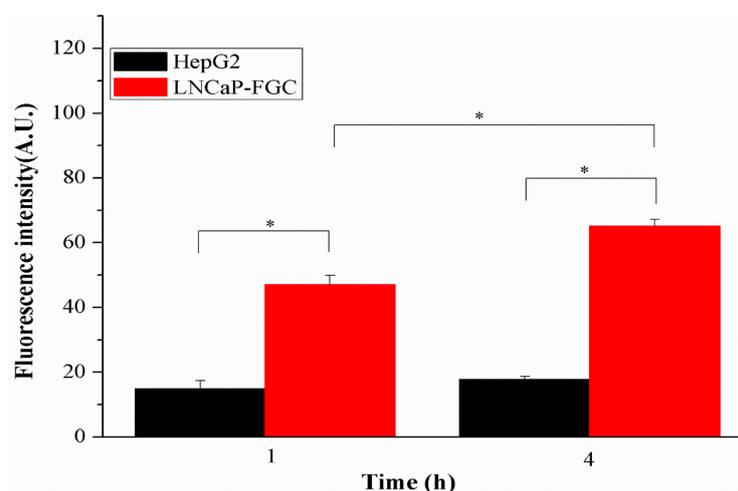
In addition to solubilization, the pentapeptide serves to mask the cytotoxicity by preventing nonspecific uptake of drug into cells due to the presence of negative charged amino acids. Upon cleavage of the prodrug by PSMA within the PSMA-expressing cells, the active compound was able to enter cells. To prove our assumption, the cellular uptake behavior of **CPT-HT-J-ZL<sub>12</sub>** in LNCAP-FGC (PSMA<sup>+</sup>) and HepG2 (PSMA<sup>-</sup>) was investigated by confocal laser scanning microscope (CLSM). The nucleus was stained with PI (red) and the fluorescence of **CPT** was pseudo-colored with blue. As shown in Figure 3, there was almost no blue fluorescence when treated with **CPT-HT-J-ZL<sub>12</sub>** in HepG2 (PSMA<sup>-</sup>) cells for 1 h and 4 h, demonstrating the poor uptake of **CPT-HT-J-ZL<sub>12</sub>**. On the contrary, the blue fluorescence intensity in LNCAP-FGC (PSMA<sup>+</sup>) for 1 h was stronger, indicating that the active compound was able to enter the cancer cells. In addition, the blue fluorescence in LNCAP-FGC (PSMA<sup>+</sup>) increased with the incubation time (4 h) (Figures 4 and 5), implying that prolonged treatment facilitated cellular uptake of the subsequent active compound.



**Figure 3.** Cellular uptake of **CPT-HT-J-ZL<sub>12</sub>** in HepG2 (no expression of PSMA) cells. CLSM images of HepG2 cells incubated with **CPT-HT-J-ZL<sub>12</sub>** (10  $\mu\text{M}$ ) for 1 and 4 h. Nuclei were stained by PI (red), the blue color was indicative of **CPT**. **CPT**: false-color green. For **CPT**:  $\lambda_{\text{ex}}$  = 405 nm, band-pass filter  $\lambda$  = 500–550 nm. For PI:  $\lambda_{\text{ex}}$  = 561 nm, band-pass filter  $\lambda$  = 575–625 nm.



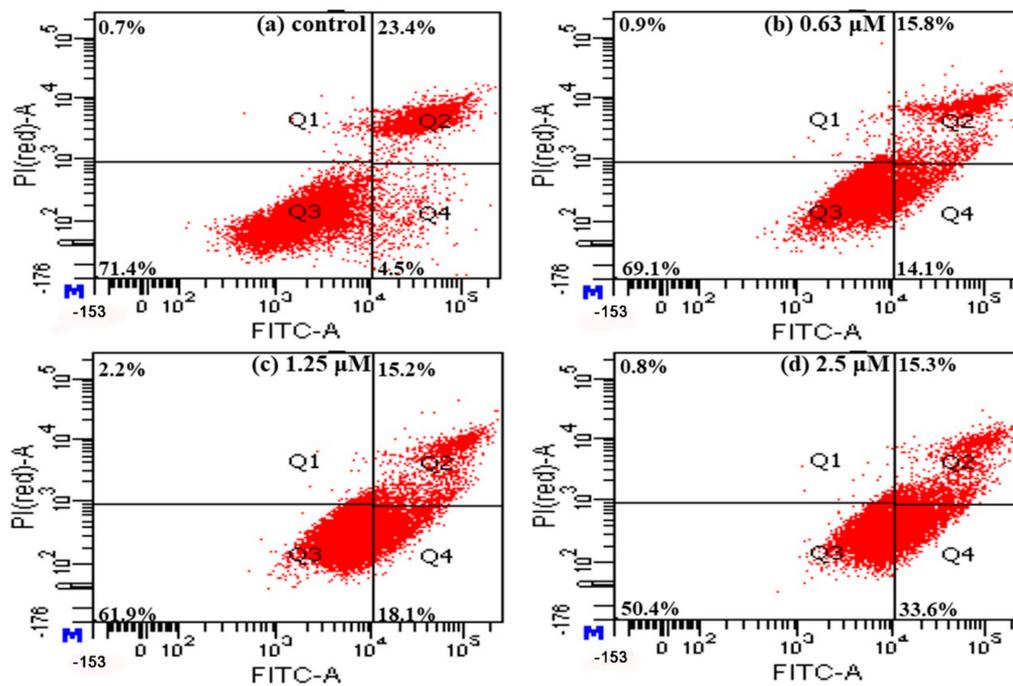
**Figure 4.** Cellular uptake of CPT-HT-J-ZL<sub>12</sub> in LNCaP-FGC (high expression of PSMA) cells. CLSM images of LNCaP-FGC cells incubated with CPT-HT-J-ZL<sub>12</sub> (10 μM) for 1 and 4 h. Nuclei were stained by PI (red); the blue color was indicative of CPT. CPT: false-color green. For CPT:  $\lambda_{ex}$  = 405 nm, band-pass filter  $\lambda$  = 500–550 nm. For PI:  $\lambda_{ex}$  = 561 nm, band-pass filter  $\lambda$  = 575–625 nm.



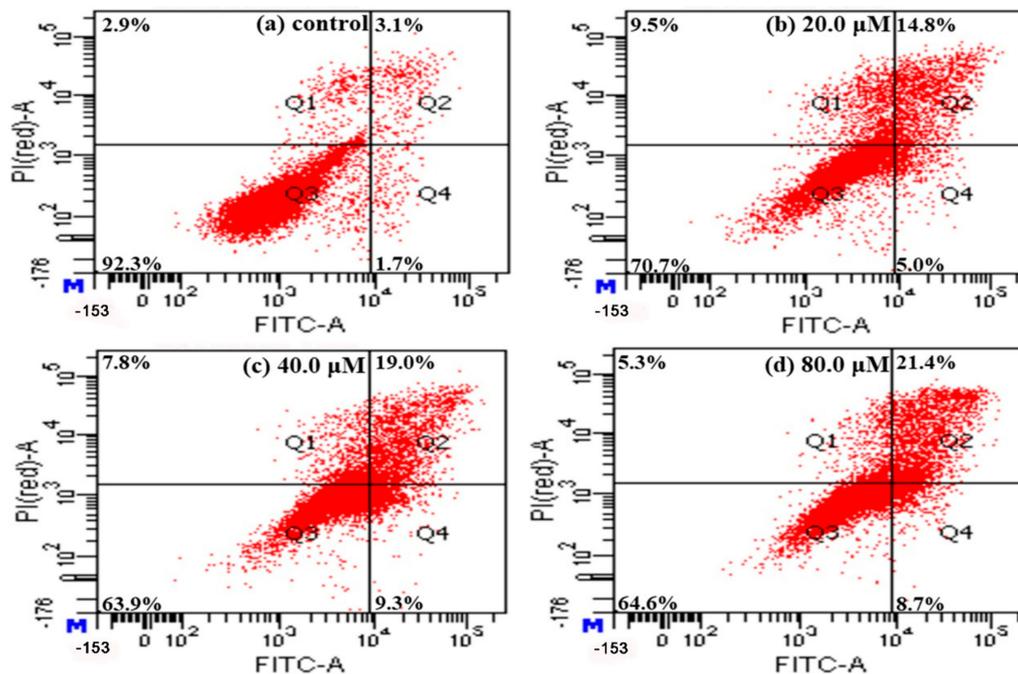
**Figure 5.** Mean fluorescence intensity of CPT-HT-J-ZL<sub>12</sub> internalized by LNCaP-FGC (high expression of PSMA) and HepG2 (none expression of PSMA) cells after incubation for 1 and 4 h. \*  $p < 0.05$ .

### 2.5. Detection of Apoptosis Using Annexin V-FITC/PI Staining

To further evaluate the selective cytotoxicity on LNCaP-FGC (PSMA<sup>+</sup>) and HepG2 (PSMA<sup>-</sup>) after treatment with CPT-HT-J-ZL<sub>12</sub>, apoptotic rates were analyzed by flow cytometry using an Annexin V-FITC/PI staining. It was observed that the LNCaP-FGC (PSMA<sup>+</sup>) cell demonstrated a remarkable response to the apoptotic effect of CPT-HT-J-ZL<sub>12</sub> in a dose-dependent manner. The apoptotic effect was determined by counting the apoptosis ratios (including the early and late apoptosis ratios). Following different concentrations (0.63, 1.25, 2.50 μM) of CPT-HT-J-ZL<sub>12</sub> treatment, the apoptosis ratios increased from 29.90% to 33.30% and 48.90%, respectively (Figure 6). On the contrary, the drug-induced apoptosis for HepG2 (PSMA<sup>-</sup>) cell demonstrated a totally different trend. The percentage of apoptotic HepG2 (PSMA<sup>-</sup>) cell was evaluated to be 19.80%, 28.30%, and 30.10% after treatment with 20.00 μM, 40.00 μM and 80.00 μM of CPT-HT-J-ZL<sub>12</sub>, respectively (Figure 7). These results demonstrated CPT-HT-J-ZL<sub>12</sub> showed PSMA-oriented targeting activity.



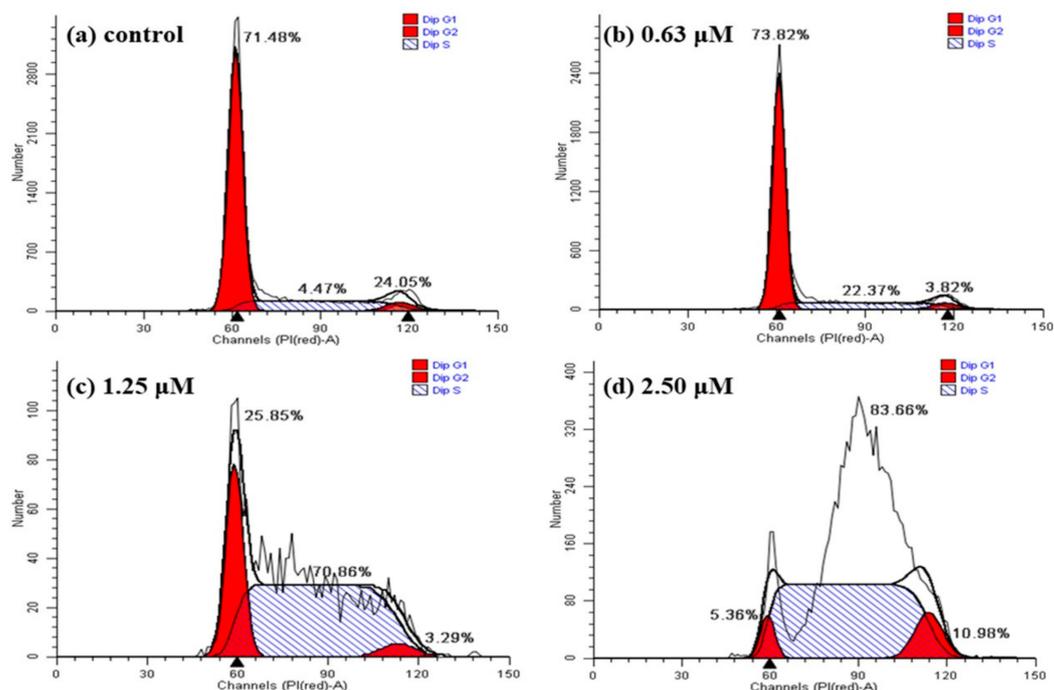
**Figure 6.** Flow cytometry analysis for apoptosis of LNCaP-FGC cells (high expression of PSMA) induced by CPT-HT-J-ZL<sub>12</sub>: (a) control group; (b) 0.63  $\mu$ M; (c) 1.25  $\mu$ M; and (d) 2.5  $\mu$ M.



**Figure 7.** Flow cytometry analysis for apoptosis of HepG2 (none expression of PSMA) cells induced by CPT-HT-J-ZL<sub>12</sub>: (a) control group; (b) 20.0  $\mu$ M; (c) 40.0  $\mu$ M; and (d) 80.0  $\mu$ M.

## 2.6. Detection of Cell Cycle Using PI Staining

To further study CPT-HT-J-ZL<sub>12</sub>-induced growth inhibition, the cell cycle changes were evaluated on LNCaP-FGC (PSMA<sup>+</sup>) using flow cytometric methods. As shown in Figure 8, with the concentration of CPT-HT-J-ZL<sub>12</sub> increased from 0.63 to 1.25 to 2.50  $\mu$ M, the percentage of LNCaP-FGC cells in S phase increased dramatically (from 4.47% to 70.86% to 83.66%), while the cells in G<sub>0</sub>/G<sub>1</sub> phases decreased. This indicated that CPT-HT-J-ZL<sub>12</sub> was able to induce cell cycle arrest at the S phase.



**Figure 8.** Effect of CPT-HT-J-ZL<sub>12</sub> on cell cycle progression in LNCaP-FGC cells (high expression of PSMA): (a) control group; (b) 0.63  $\mu$ M; (c) 1.25  $\mu$ M; and (d) 2.5  $\mu$ M.

### 3. Discussion

In this article, four novel water-soluble PSMA-activated CPT prodrugs were successfully achieved by coupling pentapeptide HT-J, a PSMA hydrolyzing substrate, to 20-OH of CPT. Their selective cytotoxic activities were determined on several PSMA-positive and PSMA-negative cancer cell and normal cell lines by the standard MTT assay. The results indicated that the prodrug CPT-HT-J-ZL<sub>n</sub> showed significantly selective cytotoxicity against the PSMA-positive and PSMA-negative cell lines. CPT-HT-J-ZL<sub>12</sub>, the lead PSMA-activated prodrug identified herein, was ~40-fold more toxic in vitro to PSMA-positive cell LNCaP-FGC ( $IC_{50} = 1.00 \pm 0.20 \mu$ M) compared to PSMA-negative cells HepG2 ( $IC_{50} > 40.00 \mu$ M) and Hela ( $IC_{50} > 40.00 \mu$ M). Moreover, CPT-HT-J-ZL<sub>12</sub> exhibited low cytotoxicity ( $IC_{50} > 40 \mu$ M) towards MDCK and LO2 cells. The in vitro cellular uptake study and the cell apoptosis analyses demonstrated the PSMA-oriented targeting of CPT-HT-J-ZL<sub>12</sub>. The cell cycle analysis indicated that CPT-HT-J-ZL<sub>12</sub> could induce cell cycle arrest at the S phase. The PSMA present at the membrane of the PSMA-positive cells was able to hydrolyze the pentapeptide; then the subsequent active compound was able to enter cells and exert the toxic effect. The preclinical results presented here suggested that the attempt to discover tumor-targeting lead compounds by using this PSMA-activated prodrug strategy was viable. Consequently, our original design concept should be perfectly illustrated by the biological evaluations above. In addition, the compound CPT-HT-J-ZL<sub>12</sub> was selected for further pharmacodynamics and pharmacokinetic evaluation, including in vivo antitumor activity against LNCaP-FGC human prostate cancer mouse xenograft and MCF-7 human breast cancer xenograft, in vivo pharmacokinetics, in the hope of producing drug candidates for drug development.

### 4. Materials and Methods

#### 4.1. Chemistry

Materials were obtained from commercial suppliers and used without further purification unless otherwise mentioned. Reactions were monitored by TLC using silica gel-coated aluminum sheets (Qingdao Haiyang Chemical Co., Qingdao, China). All melting points were determined on a micro melting point apparatus and were uncorrected. NMR spectra were recorded on a BRUKER AVANCE

500 and 400 MHz spectrometer (Fällanden, Switzerland) with tetramethylsilane (TMS) as an internal standard; chemical shifts  $\delta$  were given in ppm and coupling constants  $J$  in Hz. HR-MS were acquired using a Thermo Scientific TM LTQ Orbitrap XL hybrid FTMS instrument (Thermo Technologies, New York, NY, USA).

#### 4.1.1. General Synthetic Procedure of Condensation Reaction (Method A)

The corresponding intermediate (1 equiv.) was dissolved in dry DCM (20 mL), EDCI (1.5 equiv.) and the corresponding protected amino acid (1.1 equiv.) were added. After addition of HOBT (1.5 equiv.) and DIPEA (2.5 equiv.), the mixture was stirred at 25 °C for 4 h. The reaction mixture was diluted with 50 mL CH<sub>2</sub>Cl<sub>2</sub>, then successively washed with water and brine (10 mL each), dried over sodium sulfate and filtered, and the solvent was evaporated. Then the crude product was purified by flash chromatography (silica gel, petroleum ether:ethyl acetate =10:1 to 3:1).

#### 4.1.2. General Synthetic Procedure of Deprotection (Method B)

To a solution of the Fmoc-protected compound (1 equiv.) in dry DMF (20 mL), piperidine (3 equiv.) was added. The mixture was allowed to stir at room temperature for 0.5 h. After completion of the reaction (as monitored by TLC), the solution was evaporated, and the residue was dissolved with ethyl acetate (50 mL). The organic extracts were washed with distilled water (3 × 25 mL) and brine (20 mL), dried over sodium sulfate, filtrated and evaporated. Purification was performed by flash chromatography (silica gel, petroleum ether:ethyl acetate = 3:1 to 0:1).

#### 4.1.3. General Synthetic Procedure of Deprotection (Method C)

Pd/C (10%; 80 mg) was added to a solution of the Cbz-protected compound in 30 mL MeOH. The mixture was stirred at room temperature for 12 h. After completion of the reaction (as monitored by TLC), the solvent was filtered to remove Pd/C. The filtrate was concentrated in vacuum and the residue was purified by flash chromatography (silica gel, dichloromethane:methanol = 40:1 to 10:1).

*Characterization of di-tert-butyl ((S)-2-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-(tert-butoxy)-4-oxobutanoyl)-L-glutamate (HT-A).* Obtained from Fmoc-L-aspartic acid  $\beta$ -tert-butyl ester by method A as a white powder; m.p. 116.6–116.9 °C; Yield: 85.8%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  1.44 (m, 27 H, 9 × -CH<sub>3</sub>), 1.90 (m, 1H), 2.16 (m, 1H), 2.31 (m, 2H), 2.60 (m, 1H), 2.99 (m, 1 H), 4.24 (t,  $J$  = 7.1 Hz, 1H), 4.42 (m, 2H), 4.45 (m, 1H), 4.56 (m, 1H), 5.99 (d,  $J$  = 8.5 Hz, 1H), 7.14 (d,  $J$  = 7.6 Hz, 1H), 7.32 (t,  $J$  = 7.5 Hz, 2H), 7.40 (t,  $J$  = 7.5 Hz, 2H), 7.60 (m, 2H), 7.76 (d,  $J$  = 7.6 Hz, 2H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>):  $\delta$  27.8, 28.1, 28.2, 31.4, 37.7, 47.2, 51.2, 52.6, 67.4, 80.7, 82.0, 82.4, 120.1, 125.2, 127.2, 127.9, 141.4, 144.0, 156.1, 170.4, 170.6, 171.5, 172.3; HRMS (ESI)  $m/z$ : [M + H]<sup>+</sup> 653.3429, calcd. for C<sub>36</sub>H<sub>48</sub>N<sub>2</sub>O<sub>9</sub> 652.33598.

*Characterization of di-tert-butyl ((S)-2-amino-4-(tert-butoxy)-4-oxobutanoyl)-L-glutamate (HT-B).* Obtained from HT-A by method B as a white oily liquid; Yield: 87.9%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  1.43 (s, 9H, 3 × -CH<sub>3</sub>), 1.44 (s, 9H, 3 × -CH<sub>3</sub>), 1.46 (s, 9H, 3 × -CH<sub>3</sub>), 1.89 (m, 2H), 2.29 (m, 2H), 2.58 (dd,  $J$  = 16.7, 8.0 Hz, 1H), 2.79 (dd,  $J$  = 16.7, 3.7 Hz, 1H), 3.66 (dd,  $J$  = 8.0, 3.7 Hz, 1H), 4.45 (td,  $J$  = 8.4, 4.9 Hz, 1H), 7.89 (d,  $J$  = 8.4 Hz, 1H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>):  $\delta$  28.1, 28.2, 28.3, 29.8, 31.6, 40.6, 52.1, 52.2, 80.7, 81.4, 82.3, 171.2, 171.3, 172.3, 173.5; HRMS (ESI)  $m/z$ : [M + H]<sup>+</sup> 431.2751, calcd. for C<sub>21</sub>H<sub>38</sub>N<sub>2</sub>O<sub>7</sub> 430.26790.

*Characterization of di-tert-butyl ((S)-2-((S)-4-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(tert-butoxy)-5-oxopentanamido)-4-(tert-butoxy)-4-oxobutanoyl)-L-glutamate (HT-C).* Obtained from HT-B by method A as a white powder; m.p. 106.3–107.3 °C; Yield: 88.1%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  1.41 (s, 9H, 3 × -CH<sub>3</sub>), 1.42 (s, 9H, 3 × -CH<sub>3</sub>), 1.43 (s, 9H, 3 × -CH<sub>3</sub>), 1.47 (s, 9H, 3 × -CH<sub>3</sub>), 1.91 (m, 3H), 2.12 (m, 1H), 2.28 (m, 5H), 2.60 (dd,  $J$  = 17.1, 6.3 Hz, 1H), 2.91 (dd,  $J$  = 17.2, 3.8 Hz, 1H), 4.22 (dd, 1H), 4.40 (m, 3H), 4.78 (d,  $J$  = 4.3 Hz, 1H), 5.63 (d,  $J$  = 7.7 Hz, 1H), 6.94 (d,  $J$  = 7.4 Hz, 1H), 7.17 (d,  $J$  = 7.7 Hz, 1H), 7.31 (t,  $J$  = 7.3 Hz, 2H), 7.39 (t,  $J$  = 7.3 Hz, 2H), 7.61 (d,  $J$  = 7.3 Hz, 2H), 7.75 (d,  $J$  = 7.4 Hz, 2H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>):  $\delta$  27.7, 28.1, 28.2, 28.8, 31.5, 32.4, 37.2, 47.3, 49.4, 52.5, 53.9, 67.1,

80.7, 81.9, 82.3, 82.6, 120.1, 125.3, 127.2, 127.8, 141.4, 143.9, 144.0, 156.3, 170.5, 171.2, 171.6, 171.9, 172.2. HRMS (ESI)  $m/z$ :  $[M + H]^+$  838.4416, calcd. for  $C_{45}H_{63}N_3O_{12}$  837.44117.

*Characterization of di-tert-butyl ((S)-2-((S)-4-amino-5-(tert-butoxy)-5-oxopentanamido)-4-(tert-butoxy)-4-oxobutanoyl)-L-glutamate (HT-D)*. Obtained from HT-C by method B as a white oily liquid; Yield: 90.4%;  $^1H$ -NMR (500 MHz,  $CDCl_3$ ):  $\delta$  1.43 (d, 36H,  $12 \times -CH_3$ ), 1.82 (m, 2H), 1.91 (m, 2H), 2.08 (m, 2H), 2.28 (m, 2H), 2.42 (m, 1H), 2.51 (m, 1H), 2.98 (m, 1H), 3.30 (m, 1H), 4.44 (d,  $J = 4.5$  Hz, 1H), 4.80 (d,  $J = 3.7$  Hz, 1H), 7.06 (d,  $J = 8.5$  Hz, 1H), 7.49 (d,  $J = 8.0$  Hz, 1H).  $^{13}C$ -NMR (126 MHz,  $CDCl_3$ ):  $\delta$  28.0, 28.1, 28.2, 29.7, 29.8, 31.5, 33.9, 37.1, 49.1, 52.4, 55.4, 80.6, 81.4, 81.8, 82.2, 170.7, 170.8, 171.8, 172.3, 172.7, 174.9. HRMS (ESI)  $m/z$ :  $[M + H]^+$  616.3830, calcd. for  $C_{30}H_{53}N_3O_{10}$  615.37309.

*Characterization of tetra-tert-butyl (5S,10S,15S,18S)-15-(2-(tert-butoxy)-2-oxoethyl)-1-(9H-fluoren-9-yl)-3,8,13,16-tetraoxo-2-oxa-4,9,14,17-tetrazaicosane-5,10,18,20-tetracarboxylate (HT-E)*. Obtained from HT-D by method B as a white powder; m.p. 91.6–92.2 °C; Yield: 86.7%;  $^1H$ -NMR (500 MHz,  $CDCl_3$ ):  $\delta$  1.40 (s, 9H,  $3 \times -CH_3$ ), 1.41 (s, 9H,  $3 \times -CH_3$ ), 1.43 (s, 9H,  $3 \times -CH_3$ ), 1.45 (s, 9H,  $3 \times -CH_3$ ), 1.46 (s, 9H,  $3 \times -CH_3$ ), 1.89 (m, 4H), 2.25 (m, 10H), 2.62 (dd,  $J = 17.2, 6.8$  Hz, 1H), 2.84 (dd,  $J = 17.2, 4.7$  Hz, 1H), 4.23 (m, 1H), 4.40 (m, 2H), 4.48 (m, 1H), 4.76 (m, 1H), 5.62 (d,  $J = 8.2$  Hz, 1H), 6.58 (d,  $J = 7.6$  Hz, 1H), 7.13 (d,  $J = 7.7$  Hz, 1H), 7.18 (d,  $J = 7.7$  Hz, 1H), 7.30 (m, 2H), 7.38 (m, 2H), 7.62 (d,  $J = 7.4$  Hz, 2H), 7.75 (d,  $J = 7.5$  Hz, 2H).  $^{13}C$ -NMR (126 MHz,  $CDCl_3$ ):  $\delta$  27.7, 28.0, 28.1, 28.2, 28.6, 28.8, 31.5, 32.1, 32.3, 37.2, 42.1, 47.3, 49.5, 52.3, 52.5, 53.9, 67.1, 80.7, 81.8, 82.2, 82.5, 82.6, 120.1, 125.3, 127.2, 127.8, 141.4, 141.5, 143.8, 144.1, 156.5, 170.5, 170.6, 171.2, 171.3, 171.3, 172.0, 172.2, 172.2. HRMS (ESI)  $m/z$ :  $[M + H]^+$  1023.5528, calcd. for  $C_{54}H_{78}N_4O_{15}$  1022.54637.

*Characterization of di-tert-butyl ((S)-2-((S)-4-((S)-4-amino-5-(tert-butoxy)-5-oxopentanamido)-5-(tert-butoxy)-5-oxopentanamido)-4-(tert-butoxy)-4-oxobutanoyl)-L-glutamate (HT-F)*. Obtained from HT-E by method B as a white oily liquid; Yield: 87.3%;  $^1H$ -NMR (500 MHz,  $CDCl_3$ ):  $\delta$  1.41 (s, 9H,  $3 \times -CH_3$ ), 1.42 (s, 9H,  $3 \times -CH_3$ ), 1.43 (s, 9H,  $3 \times -CH_3$ ), 1.44 (s, 9H,  $3 \times -CH_3$ ), 1.45 (s, 9H,  $3 \times -CH_3$ ), 1.89 (m, 4H), 2.09 (m, 2H), 2.22 (m, 2H), 2.31 (t,  $J = 7.3$  Hz, 2H), 2.38 (t,  $J = 7.0$  Hz, 2H), 2.61 (dd,  $J = 17.2, 6.6$  Hz, 1H), 2.86 (m, 1H), 3.36 (m,  $J = 8.4, 4.8$  Hz, 1H), 4.42 (m, 2H), 4.76 (m, 1H), 6.89 (d,  $J = 7.6$  Hz, 1H), 7.13 (d,  $J = 7.8$  Hz, 1H), 7.22 (d,  $J = 7.8$  Hz, 1H).  $^{13}C$ -NMR (126 MHz,  $CDCl_3$ ):  $\delta$  27.7, 28.1, 28.2, 28.5, 30.2, 31.5, 32.5, 32.9, 37.2, 49.5, 52.3, 52.5, 54.5, 80.7, 81.5, 81.8, 82.3, 82.4, 170.5, 170.6, 171.2, 171.4, 172.2, 172.3, 172.8, 174.8. HRMS (ESI)  $m/z$ :  $[M + H]^+$  801.4883, calcd. for  $C_{39}H_{68}N_4O_{13}$  800.47829.

*Characterization of 6-benzyl 11,16,24,26-tetra-tert-butyl (6S,11S,16S,21S,24S)-21-(2-(tert-butoxy)-2-oxoethyl)-2,2-dimethyl-4,9,14,19,22-pentaoxo-3-oxa-5,10,15,20,23-pentaazahexacosane-6,11,16,24,26-pentacarboxylate (HT-I)*. Obtained from HT-F by method A as a white powder; m.p. 107.0–107.7 °C; Yield: 83.6%;  $^1H$ -NMR (500 MHz,  $CDCl_3$ ):  $\delta$  1.39 (s, 27H,  $9 \times -CH_3$ ), 1.41 (s, 18H,  $6 \times -CH_3$ ), 1.42 (s, 9H,  $3 \times -CH_3$ ), 1.74 (m, 2H), 2.13 (s, 6H), 2.14 (s, 2H), 2.22 (m, 2H), 2.27 (m, 4H), 2.62 (dd,  $J = 17.1, 6.9$  Hz, 1H), 2.78 (m,  $J = 17.0, 4.8$  Hz, 1H), 4.41 (m, 3H), 4.75 (dd,  $J = 12.8, 6.8$  Hz, 1H), 5.14 (m, 2H), 5.57 (d,  $J = 8.3$  Hz, 1H), 6.52 (m, 1H), 6.86 (d,  $J = 7.5$  Hz, 1H), 7.21 (d,  $J = 7.8$  Hz, 1H), 7.32 (m, 5H).  $^{13}C$ -NMR (126 MHz,  $CDCl_3$ ):  $\delta$  27.7, 28.0, 28.1, 28.4, 31.0, 31.5, 31.6, 32.0, 32.2, 37.3, 49.5, 52.0, 52.2, 52.4, 53.1, 67.2, 80.1, 80.6, 81.6, 82.1, 82.4, 128.4, 128.5, 128.7, 135.5, 156.0, 170.6, 170.6, 171.0, 171.1, 171.3, 171.9, 172.1, 172.2, 172.3. HRMS (ESI)  $m/z$ :  $[M + H]^+$  1120.6292, calcd. for  $C_{56}H_{89}N_5O_{18}$  1119.62026.

*Characterization of (7S,10S,15S,20S,25S)-10-(2-(tert-butoxy)-2-oxoethyl)-7,15,20-tris(tert-butoxycarbonyl)-25-((tert-butoxycarbonyl)amino)-2,2-dimethyl-4,9,12,17,22-pentaoxo-3-oxa-8,11,16,21-tetraazahexacosan-2-6-ic acid (HT-J)*. Obtained from HT-I by method C as a white powder; m.p. 94.3–95.0 °C; Yield: 92.2%;  $^1H$ -NMR (500 MHz,  $CDCl_3$ ):  $\delta$  1.41 (s, 9H,  $3 \times -CH_3$ ), 1.42 (s, 27H,  $9 \times -CH_3$ ), 1.44 (s, 9H,  $3 \times -CH_3$ ), 1.45 (s, 9H,  $3 \times -CH_3$ ), 1.87 (m, 3H), 2.08 (m, 1H), 2.24 (m, 4H), 2.33 (m, 4H), 2.40 (m, 4H), 2.73 (m, 2H), 4.29 (m, 1H), 4.43 (m, 2H), 4.54 (t,  $J = 7.3$  Hz, 1H), 4.79 (dd,  $J = 12.7, 7.2$  Hz, 1H), 5.56 (d,  $J = 7.3$  Hz, 1H), 6.57 (s, 1H), 7.10 (d,  $J = 7.8$  Hz, 1H), 7.34 (d,  $J = 7.9$  Hz, 1H), 7.53 (d,  $J = 7.8$  Hz, 1H).  $^{13}C$ -NMR (126 MHz,  $CDCl_3$ ):  $\delta$  27.7, 28.1, 28.2, 28.3, 28.5, 31.2, 31.5, 32.0, 32.4, 37.3, 49.5, 51.7, 52.5, 52.6, 52.8, 80.3, 81.2, 81.8, 82.1, 82.3, 82.6, 156.2, 170.7, 170.8, 171.2, 171.4, 172.2, 172.3, 172.8, 173.0, 174.0. HRMS (ESI)  $m/z$ :  $[M + H]^+$  1130.5793, calcd. for  $C_{49}H_{83}N_5O_{18}$  1029.57331.

#### 4.1.4. General Synthetic Procedure of the Preparation of PSMA-Activated Prodrug **CPT-HT-J-ZLn**

##### General Synthetic Procedure of the Intermediate **CPT-A-Ln** (Method D)

The compound camptothecin (1 equiv.) was dissolved in dry DCM (25 mL), DMAP (0.1 equiv.) and the corresponding *N*-*boc*-amino aliphatic acid (1.2 equiv.) were added. After addition of EDCI (1.5 equiv.), the mixture was stirred at 25 °C for 12 h. The reaction mixture was diluted with 50 mL CH<sub>2</sub>Cl<sub>2</sub>, then successively washed with water and brine (10 mL each), dried over sodium sulfate and filtered, and the solvent was evaporated. Then the crude product was purified by flash chromatography (silica gel, dichloromethane:methanol = 100:1 to 40:1).

##### General Synthetic Procedure of the Intermediate **CPT-B-Ln** (Method E)

To a solution of the *Boc*-protected compound **CPT-B-Ln** in dry DCM (30 mL), TFA (3 mL/10 mL DCM) was added. The mixture was stirred in an ice bath for 2 h. After completion of the reaction (as monitored by TLC), the solution was evaporated and the residue was dissolved with DCM (50 mL). The organic extracts were washed with distilled water (3 × 25 mL) and brine (20 mL), dried over sodium sulfate, filtrated and evaporated. Purification was performed by flash chromatography (silica gel, dichloromethane:methanol = 20:1 to 5:1).

##### General Synthetic Procedure of the Intermediate **CPT-HT-J-Ln** (Method F)

The corresponding intermediate **CPT-B-Ln** (1 equiv.) was dissolved in dry DCM (20 mL), EDCI (1.5 equiv.) and **HT-J** (1.1 equiv.) were added. After addition of HOBT (1.5 equiv.) and DIPEA (2.5 equiv.), the mixture was stirred at 25 °C for 4 h. The reaction mixture was diluted with 50 mL CH<sub>2</sub>Cl<sub>2</sub>, then successively washed with water and brine (10 mL each), dried over sodium sulfate and filtered, and the solvent was evaporated. Then the crude product was purified by flash chromatography (silica gel, dichloromethane:methanol = 1:0 to 50:1).

##### General Synthetic Procedure of the Prodrug **CPT-HT-J-ZLn** (Method G)

To a solution of the intermediate **CPT-HT-J-Ln** in 20 mL mixed solution (THF:Thioanisole:H<sub>2</sub>O = 95:2.5:2.5), the mixture was stirred at room temperature for 2.5 h. After completion of the reaction (as monitored by RP-HPLC), the filtrate was concentrated in vacuum and the residue was purified by PR-HPLC (A = H<sub>2</sub>O (0.2% TFA), and B = CH<sub>3</sub>CN (0.2% TFA); solvent gradient: 35–80% B in 25 min).

*Characterization of (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano [3',4':6,7]indolizino[1,2-b]quinolin-4-yl(tert-butoxycarbonyl)glycinate (CPT-A-L<sub>2</sub>)*. Obtained from **CPT** by method D as a faint yellow powder; mp > 220 °C; Yield: 65.0%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>): δ 0.98 (t, J = 7.4 Hz, 3H, -CH<sub>3</sub>), 1.42 (s, 9H, 3 × -CH<sub>3</sub>), 2.16 (m, 2H), 4.06 (dd, J = 18.0, 4.8 Hz, 1H), 4.21 (dd, J = 18.4, 6.1 Hz, 1H), 5.07 (s, 1H), 5.25 (m, 2H), 5.38 (d, J = 17.1 Hz, 1H), 5.67 (d, J = 17.1 Hz, 1H), 7.30 (s, 1H), 7.65 (t, J = 7.4 Hz, 1H), 7.81 (t, J = 7.5 Hz, 1H), 7.91 (d, J = 8.1 Hz, 1H), 8.23 (d, J = 8.4 Hz, 1H), 8.37 (s, 1H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>): δ 7.7, 28.4, 31.8, 42.5, 50.1, 67.2, 80.3, 96.5, 120.2, 128.2, 128.3, 128.5, 129.8, 130.8, 131.3, 145.7, 146.5, 148.9, 152.3, 155.7, 157.4, 167.3, 169.7. HRMS (ESI) *m/z*: [M + H]<sup>+</sup> 506.1917, calcd. for C<sub>27</sub>H<sub>27</sub>N<sub>3</sub>O<sub>7</sub> 505.18490.

*Characterization of (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl glycinate (CPT-B-L<sub>2</sub>)*. Obtained from **CPT-A-L<sub>2</sub>** by method E as a faint yellow powder; m.p. 194.8–195.2 °C; Yield: 61.2%; <sup>1</sup>H-NMR (500 MHz, CD<sub>3</sub>OD): δ 1.08 (t, J = 7.5 Hz, 3H, -CH<sub>3</sub>), 2.27 (m, 2H), 4.18 (m, 1H), 4.29 (m, 1H), 5.35 (m, 2H), 5.50 (d, J = 16.7 Hz, 1H), 5.64 (d, J = 16.7 Hz, 1H), 7.44 (s, 1H), 7.1 (t, J = 7.3 Hz, 1H), 7.88 (t, J = 7.5 Hz, 1H), 8.07 (d, J = 8.0 Hz, 1H), 8.15 (d, J = 8.0 Hz, 1H), 8.64 (s, 1H). <sup>13</sup>C-NMR (126 MHz, CD<sub>3</sub>OD): δ 8.0, 31.9, 41.0, 51.6, 67.7, 79.6, 97.7, 120.6, 129.3, 129.8, 130.0, 130.9, 132.1, 133.1, 133.5, 147.4, 147.9, 149.6, 153.4, 159.0, 168.1, 168.6. HRMS (ESI) *m/z*: [M + H]<sup>+</sup> 406.1397, calcd. for C<sub>22</sub>H<sub>19</sub>N<sub>3</sub>O<sub>5</sub> 405.13247.

*Characterization of 14,22,24,9-tetra-tert-butyl 1-((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano [3',4':6,7]indolizino[1,2-b]quinolin-4-yl) (4S,9S,14S,19S,22S)-19-(2-(tert-butoxy)-2-oxoethyl)-4-((tert-butoxyca*

*rbonyl*)amino)-3,7,12,17,20-pentaoxo-2,8,13,18,21-pentaazatetracosane-1,9,14,22,24-pentacarboxylate (**CPT-HT-J-L<sub>2</sub>**). Obtained from **CPT-B-L<sub>2</sub>** by method F as a faint yellow powder; m.p. 167.4–168.1 °C; Yield: 30.0%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>): δ 0.97 (t, *J* = 7.5 Hz, 3H, -CH<sub>3</sub>), 1.26 (s, 2H), 1.35 (s, 9H, 3 × -CH<sub>3</sub>), 1.37 (s, 9H, 3 × -CH<sub>3</sub>), 1.42 (s, 36H, 12 × -CH<sub>3</sub>), 1.90 (m, 2H), 2.19 (m, 14H), 2.64 (m, 1H), 2.83 (m, 1H), 4.16 (m, 2H), 4.42 (m, 4H), 4.18 (s, 1H), 5.29 (s, 2H), 5.38 (d, *J* = 17.4 Hz, 1H), 5.67 (d, *J* = 17.4 Hz, 1H), 6.67 (m, 1H), 7.11 (m, 1H), 7.33 (m, 4H), 7.67 (t, *J* = 6.9 Hz, 1H), 7.83 (t, *J* = 7.7 Hz, 1H), 7.94 (d, *J* = 7.9 Hz, 1H), 8.24 (d, *J* = 8.3 Hz, 1H), 8.40 (s, 1H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>): δ 7.7, 27.7, 28.0, 28.1, 28.2, 28.4, 29.4, 31.6, 31.9, 32.5, 37.4, 41.4, 49.4, 50.2, 51.8, 52.3, 52.5, 53.9, 67.2, 79.9, 80.7, 81.6, 82.0, 82.3, 82.5, 82.6, 96.5, 120.3, 128.2, 128.3, 128.6, 129.7, 130.8, 131.4, 145.6, 146.4, 148.8, 152.3, 156.0, 157.4, 167.2, 169.5, 170.9, 171.0, 171.7, 172.3, 172.6, 172.7, 172.9. HRMS (ESI) *m/z*: [M + H]<sup>+</sup> 1417.7008, calcd. for C<sub>71</sub>H<sub>100</sub>N<sub>8</sub>O<sub>22</sub> 1416.69522.

Characterization of ((*S*)-4-((*S*)-4-((*S*)-4-amino-5-((2-(((*S*)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3',4':6,7]indolizino[1,2-*b*]quinolin-4-yl)oxy)-2-oxoethyl)amino)-5-oxopentanamido)-4-carboxybutanamido)-4-carboxybutanoyl)-L-aspartyl-L-glutamic acid (**CPT-HT-J-ZL<sub>2</sub>**). Obtained from **CPT-HT-J-L<sub>2</sub>** by method G as a faint yellow powder; m.p. 179.0–179.5 °C; Yield: 19.9%; <sup>1</sup>H-NMR (500 MHz, DMSO): δ 0.93 (t, *J* = 7.4 Hz, 3H, -CH<sub>3</sub>), 1.52 (d, *J* = 6.6 Hz, 2H), 1.76 (s, 3H), 1.96 (s, 6H), 2.16 (s, 6H), 2.26 (m, 5H), 4.15 (m, 4H), 4.58 (m, 2H), 5.27 (s, 1H), 5.35 (s, 1H), 7.18 (s, 1H), 7.73 (t, *J* = 7.5 Hz, 1H), 7.88 (t, *J* = 7.5 Hz, 1H), 8.06 (d, *J* = 7.1 Hz, 1H), 8.16 (m, 6H), 8.27 (d, *J* = 6.6 Hz, 1H), 8.71 (s, 1H), 8.93 (s, 1H). <sup>13</sup>C-NMR (101 MHz, DMSO): δ 7.6, 26.2, 27.1, 29.9, 30.4, 31.5, 31.7, 36.2, 36.5, 49.3, 51.3, 51.5, 51.7, 66.3, 76.6, 95.1, 119.0, 127.8, 128.0, 128.7, 128.9, 129.8, 130.5, 131.7, 145.0, 146.1, 147.9, 152.4, 156.5, 167.0, 168.6, 169.0, 171.0, 171.2, 171.4, 171.7, 173.1, 173.3, 173.4, 173.8; HRMS (ESI) *m/z*: [M + H]<sup>+</sup> 1037.3386, calcd. for C<sub>46</sub>H<sub>52</sub>N<sub>8</sub>O<sub>20</sub> 1036.330.

Characterization of (*S*)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3',4':6,7]indolizino[1,2-*b*]quinolin-4-yl 4-((*tert*-butoxycarbonyl)amino)butanoate (**CPT-A-L<sub>4</sub>**). Obtained from **CPT** by method D as a faint yellow powder; m.p. 167.8–170.2 °C; Yield: 46.0%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>): δ 0.98 (t, *J* = 7.5 Hz, 3H, -CH<sub>3</sub>), 1.42 (s, 9H, 3 × -CH<sub>3</sub>), 1.86 (m, 2H), 2.14 (m, 2H), 2.54 (m, 2H), 3.14 (m, 2H), 5.28 (m, 1H), 5.38 (m, 1H), 5.66 (m, 1H), 7.23 (m, 1H), 7.66 (m, 1H), 7.82 (m, 1H), 7.93 (m, 1H), 8.25 (m, 1H), 8.40 (m, 1H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>): δ 7.7, 25.1, 28.5, 31.1, 31.8, 39.6, 50.1, 67.2, 76.0, 79.3, 96.1, 120.3, 128.2, 128.3, 128.6, 129.6, 130.9, 131.5, 146.1, 146.3, 148.8, 152.3, 156.2, 157.5, 167.7, 172.3; HRMS (ESI) *m/z*: [M + H]<sup>+</sup> 534.2231, calcd. for C<sub>29</sub>H<sub>31</sub>N<sub>3</sub>O<sub>7</sub> 533.21620.

Characterization of (*S*)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3',4':6,7]indolizino[1,2-*b*]quinolin-4-yl 4-aminobutanoate (**CPT-B-L<sub>4</sub>**). Obtained from **CPT-A-L<sub>4</sub>** by method E as a faint yellow powder; mp > 220 °C; Yield: 65.3%; <sup>1</sup>H-NMR (500 MHz, DMSO): δ 0.93 (t, *J* = 7.4 Hz, 3H, -CH<sub>3</sub>), 1.84 (m, 2H), 2.16 (m, 2H), 2.70 (m, 2H), 2.83 (m, 2H), 5.28 (s, 2H), 5.53 (m, 2H), 7.06 (s, 1H), 7.71 (t, *J* = 6.0 Hz, 1H), 7.86 (d, *J* = 6.7 Hz, 1H), 8.13 (d, *J* = 7.5 Hz, 1H), 8.16 (d, *J* = 7.5 Hz, 1H), 8.69 (s, 1H). <sup>13</sup>C-NMR (126 MHz, DMSO): δ 7.7, 22.5, 30.33, 38.0, 50.3, 66.4, 76.1, 94.8, 118.9, 127.8, 128.1, 128.7, 129.0, 129.9, 130.5, 131.7, 145.4, 146.2, 148.0, 152.4, 156.6, 167.4, 171.6; HRMS (ESI) *m/z*: [M + H]<sup>+</sup> 434.1715, calcd. for C<sub>24</sub>H<sub>23</sub>N<sub>3</sub>O<sub>5</sub> 433.16377.

Characterization of 1,11,16,3-tetra-*tert*-butyl 26-((*S*)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3',4':6,7]indolizino[1,2-*b*]quinolin-4-yl) (3*S*,6*S*,11*S*,16*S*,21*S*)-6-(2-(*tert*-butoxy)-2-oxoethyl)-21-((*tert*-butoxycarbonyl)amino)-5,8,13,18,22-pentaoxo-4,7,12,17,23-pentaazahexacosane-1,3,11,16,26-pentacarboxylate (**CPT-HT-J-L<sub>4</sub>**). Obtained from **CPT-B-L<sub>4</sub>** by method F as a faint yellow powder; m.p. 129.0–129.6 °C; Yield: 49.3%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>): δ 0.95 (t, *J* = 7.4 Hz, 3H, -CH<sub>3</sub>), 1.39 (s, 9H, 3 × -CH<sub>3</sub>), 1.40 (s, 18H, 6 × -CH<sub>3</sub>), 1.41 (s, 18H, 6 × -CH<sub>3</sub>), 1.44 (s, 9H, 3 × -CH<sub>3</sub>), 1.72 (m, 2H), 1.84 (m, 4H), 2.15 (m, 2H), 2.26 (m, 4H), 2.37 (m, 8H), 2.56 (m, 2H), 2.83 (m, 1H), 3.30 (m, 2H), 3.64 (m, 1H), 4.11 (m, 1H), 4.43 (m, 4H), 4.79 (m, 1H), 5.29 (s, 2H), 5.39 (d, *J* = 17.2 Hz, 1H), 5.65 (d, *J* = 17.2 Hz, 1H), 5.79 (m, 1H), 6.38 (d, *J* = 8.4 Hz, 1H), 7.20 (d, *J* = 7.7 Hz, 1H), 7.33 (m, 2H), 7.53 (s, 1H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.83 (t, *J* = 7.4 Hz, 1H), 7.93 (d, *J* = 8.0 Hz, 1H), 8.23 (d, *J* = 8.0 Hz, 1H), 8.40 (s, 1H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>): δ 7.7, 24.5, 24.8, 27.6, 28.1, 28.2, 28.4, 28.5, 29.0, 31.3, 31.4, 31.6, 31.9, 32.5, 37.4, 38.7, 49.4, 50.1, 51.7, 52.3, 52.5, 53.2, 53.4, 67.2, 76.0, 79.9, 80.6, 81.6, 81.9, 82.2, 82.4, 96.4, 120.5, 128.2, 128.3, 128.7, 129.7,

130.9, 131.5, 145.9, 146.2, 148.8, 152.4, 156.2, 157.5, 167.7, 170.9, 171.0, 171.1, 171.5, 172.0, 172.1, 172.2, 172.5, 172.6, 172.7; HRMS (ESI)  $m/z$ :  $[M + H]^+$  1445.7339, calcd. for  $C_{73}H_{104}N_8O_{22}$  1444.72652.

*Characterization of (3S,6S,11S,16S,21S)-21-amino-6-(carboxymethyl)-27-(((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyranol[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-5,8,13,18,22,27-hexaoxo-4,7,12,17,23-pentaazaheptacosane-1,3,11,16-tetracarboxylic acid (CPT-HT-J-ZL<sub>4</sub>)*. Obtained from **CPT-HT-J-L<sub>4</sub>** by method G as a faint yellow powder; m.p. 189.0–189.5 °C; Yield: 19.9%; <sup>1</sup>H-NMR (500 MHz, DMSO):  $\delta$  0.92 (t,  $J = 7.4$  Hz, 3H, -CH<sub>3</sub>), 1.73 (m, 4H), 1.92 (s, 6H), 2.16 (s, 6H), 2.22 (m, 12H), 2.63 (m, 2H), 4.11 (m, 3H), 4.55 (s, 1H), 5.31 (s, 2H), 5.48 (d,  $J = 16.9$  Hz, 1H), 5.52 (d,  $J = 17.1$  Hz, 1H), 7.07 (s, 1H), 7.72 (t,  $J = 7.4$  Hz, 1H), 7.87 (t,  $J = 7.6$  Hz, 1H), 7.93 (s, 1H), 8.09 (s, 1H), 8.15 (m, 4H), 8.24 (s, 2H), 8.48 (s, 1H), 8.70 (s, 1H). <sup>13</sup>C-NMR (126 MHz, DMSO):  $\delta$  7.6, 24.3, 27.1, 27.2, 27.6, 29.6, 30.3, 30.6, 30.8, 31.5, 31.7, 36.3, 38.1, 50.3, 51.3, 51.4, 51.7, 51.9, 52.1, 66.4, 75.9, 94.7, 118.9, 127.8, 128.0, 128.6, 129.0, 129.9, 130.5, 131.7, 145.4, 146.1, 147.9, 152.4, 156.6, 167.4, 168.5, 170.7, 171.1, 171.5, 171.8, 172.4, 172.9, 173.6. HRMS (ESI)  $m/z$ :  $[M + H]^+$  1065.3690, calcd. for  $C_{48}H_{56}N_8O_{20}$  1064.36109.

*Characterization of (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyranol[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 6-((tert-butoxycarbonyl)amino)hexanoate (CPT-A-L<sub>6</sub>)*. Obtained from **CPT** by method D as a faint yellow powder; m.p. 152.0–152.5 °C; Yield: 55.0%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  0.97 (t,  $J = 7.5$  Hz, 3H, -CH<sub>3</sub>), 1.39 (s, 9H, 3  $\times$  -CH<sub>3</sub>), 1.50 (m, 2H), 1.66 (m, 4H), 2.14 (m, 2H), 2.49 (m, 2H), 3.07 (m, 2H), 5.29 (s, 2H), 5.39 (d,  $J = 17.0$  Hz, 1H), 5.67 (d,  $J = 17.0$  Hz, 1H), 7.23 (s, 1H), 7.67 (m, 1H), 7.83 (m, 1H), 7.94 (d,  $J = 8.0$  Hz, 1H), 8.22 (d,  $J = 8.0$  Hz, 1H), 8.40 (s, 1H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>):  $\delta$  7.7, 24.4, 26.2, 28.5, 29.9, 32.0, 33.8, 40.4, 50.1, 67.2, 75.9, 96.3, 120.5, 128.2, 128.3, 128.4, 128.6, 129.6, 130.9, 131.5, 146.2, 146.2, 148.9, 152.4, 157.5, 167.7, 172.7; HRMS (ESI)  $m/z$ :  $[M + H]^+$  562.2548, calcd. for  $C_{31}H_{35}N_3O_7$  561.24750.

*Characterization of (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyranol[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 6-aminohexanoate (CPT-B-L<sub>6</sub>)*. Obtained from **CPT-A-L<sub>6</sub>** by method E as a faint yellow powder; m.p. 207.5–208.0 °C; Yield: 65.0%; <sup>1</sup>H-NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  1.05 (t,  $J = 7.4$  Hz, 3H, -CH<sub>3</sub>), 1.48 (m, 2H), 1.70 (m, 4H), 2.23 (m, 2H), 2.62 (m, 2H), 2.91 (m, 2H), 5.27 (s, 2H), 5.47 (d,  $J = 16.7$  Hz, 1H), 5.61 (d,  $J = 16.7$  Hz, 1H), 7.35 (s, 1H), 7.70 (m, 1H), 7.87 (t,  $J = 7.0$  Hz, 1H), 8.04 (d,  $J = 8.0$  Hz, 1H), 8.15 (d,  $J = 8.0$  Hz, 1H), 8.60 (s, 1H). <sup>13</sup>C-NMR (126 MHz, CD<sub>3</sub>OD):  $\delta$  8.1, 25.1, 26.6, 28.2, 32.1, 34.2, 40.5, 51.5, 67.7, 77.5, 97.8, 120.6, 129.2, 129.7, 129.7, 130.0, 130.9, 132.0, 133.4, 147.5, 148.4, 149.5, 153.4, 159.0, 169.6, 173.9. HRMS (ESI)  $m/z$ :  $[M + H]^+$  462.2030, calcd. for  $C_{26}H_{27}N_3O_5$  461.19507.

*Characterization of 1,11,16,3-tetra-tert-butyl 28-((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyranol[3',4':6,7]indolizino[1,2-b]quinolin-4-yl) (3S,6S,11S,16S,21S)-6-(2-(tert-butoxy)-2-oxoethyl)-21-((tert-butoxycarbonyl)amino)-5,8,13,18,22-pentaoxo-4,7,12,17,23-pentaazaocotacosane-1,3,11,16,28-pentacarboxylate (CPT-HT-J-L<sub>6</sub>)*. Obtained from **CPT-B-L<sub>6</sub>** by method F as a faint yellow powder; m.p. 138.5–139.0 °C; Yield: 45.2%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  0.96 (t,  $J = 7.5$  Hz, 3H, -CH<sub>3</sub>), 1.41 (s, 27H, 9  $\times$  -CH<sub>3</sub>), 1.42 (s, 9H, 3  $\times$  -CH<sub>3</sub>), 1.43 (s, 9H, 3  $\times$  -CH<sub>3</sub>), 1.45 (s, 9H, 3  $\times$  -CH<sub>3</sub>), 1.52 (m, 2H), 1.76 (m, 6H), 2.12 (m, 4H), 2.32 (m, 12H), 2.48 (m, 2H), 2.61 (m, 1H), 2.86 (m, 1H), 3.21 (m, 2H), 4.11 (s, 1H), 4.45 (m, 3H), 4.80 (m, 1H), 5.30 (s, 2H), 5.40 (d,  $J = 17.2$  Hz, 1H), 5.66 (d,  $J = 17.2$  Hz, 1H), 5.84 (d,  $J = 7.0$  Hz, 1H), 6.34 (d,  $J = 8.3$  Hz, 1H), 7.23 (d,  $J = 7.4$  Hz, 1H), 7.27 (s, 1H), 7.34 (t,  $J = 8.3$  Hz, 1H), 7.67 (t,  $J = 7.4$  Hz, 1H), 7.84 (t,  $J = 7.2$  Hz, 1H), 7.94 (d,  $J = 8.0$  Hz, 1H), 8.25 (d,  $J = 8.5$  Hz, 1H), 8.41 (s, 1H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>):  $\delta$  7.7, 24.4, 26.3, 27.6, 27.9, 28.1, 28.2, 28.6, 29.0, 29.2, 31.6, 32.0, 32.1, 32.5, 33.7, 37.4, 39.4, 49.4, 50.1, 51.7, 52.3, 52.6, 53.3, 67.2, 75.8, 79.9, 80.6, 81.6, 81.9, 82.2, 82.5, 96.5, 120.6, 128.3, 128.4, 128.7, 129.5, 131.0, 131.7, 146.1, 148.6, 152.3, 156.2, 157.5, 167.7, 170.9, 171.1, 171.5, 171.7, 172.2, 172.5, 172.7, 172.8. HRMS (ESI)  $m/z$ :  $[M + H]^+$  1473.7646, calcd. for  $C_{75}H_{108}N_8O_{22}$  1472.75782.

*Characterization of (3S,6S,11S,16S,21S)-21-amino-6-(carboxymethyl)-29-(((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyranol[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-5,8,13,18,22,29-hexaoxo-4,7,12,17,23-pentaazanonacosane-1,3,11,16-tetracarboxylic acid (CPT-HT-J-ZL<sub>6</sub>)*. Obtained from **CPT-HT-J-L<sub>6</sub>** by method G as a faint yellow powder; m.p. 190.0–190.4 °C; Yield: 25.0%; <sup>1</sup>H-NMR (500 MHz, DMSO):  $\delta$  0.93 (t,  $J = 7.5$  Hz, 3H, -CH<sub>3</sub>), 1.36 (m, 4H), 1.48 (m, 2H), 1.60 (m, 2H), 1.79 (m, 3H), 1.94 (m, 5H), 2.21 (m, 9H), 2.36 (m, 1H), 2.66 (m, 1H), 3.11 (m, 2H), 3.62 (m, 1H), 3.72 (s, 1H), 4.20 (m, 3H), 4.60 (m, 1H), 5.31 (s, 2H), 5.49 (m, 2H), 7.06 (s, 1H), 7.72 (t,  $J = 7.1$  Hz, 1H), 7.87 (t,  $J = 7.7$  Hz, 1H), 8.07 (d,  $J = 6.8$  Hz, 1H),

8.15 (m, 5H), 8.33 (m, 2H), 8.70 (s, 1H).  $^{13}\text{C-NMR}$  (126 MHz, DMSO):  $\delta$  7.6, 24.0, 25.7, 27.0, 27.7, 28.5, 29.7, 30.0, 30.3, 30.4, 31.6, 31.7, 33.1, 36.2, 38.6, 49.3, 50.3, 51.3, 51.5, 51.7, 51.8, 54.9, 66.4, 75.7, 94.7, 118.9, 127.8, 128.1, 128.6, 129.0, 129.9, 130.5, 131.7, 145.5, 146.0, 147.9, 152.3, 156.6, 163.1, 167.4, 167.9, 170.9, 171.0, 171.2, 171.5, 171.7, 172.1, 172.8, 172.9, 173.1, 173.4, 173.8. HRMS (ESI)  $m/z$ :  $[\text{M} + \text{H}]^+$  1093.4007, calcd. for  $\text{C}_{50}\text{H}_{60}\text{N}_8\text{O}_{20}$  1092.39239.

*Characterization of (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 12-((tert-butoxycarbonyl)amino)dodecanoate (CPT-A-L<sub>12</sub>)*. Obtained from CPT by method D as a faint yellow powder; m.p. 134.6–140.0 °C; Yield: 67.0%;  $^1\text{H-NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  0.96 (t,  $J = 7.4$  Hz, 3H,  $-\text{CH}_3$ ), 1.15 (m, 8H), 1.26 (m, 6H), 1.42 (s, 9H,  $3 \times -\text{CH}_3$ ), 1.62 (m, 2H), 2.16 (m, 1H), 2.27 (m, 1H), 2.47 (m, 2H), 3.06 (s, 2H), 5.26 (m, 2H), 5.40 (d,  $J = 17.1$  Hz, 1H), 5.66 (d,  $J = 17.1$  Hz, 1H), 7.22 (s, 1H), 7.66 (t,  $J = 7.3$  Hz, 1H), 7.82 (t,  $J = 7.3$  Hz, 1H), 7.93 (d,  $J = 8.1$  Hz, 1H), 8.20 (d,  $J = 8.5$  Hz, 1H), 8.39 (s, 1H).  $^{13}\text{C-NMR}$  (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.7, 24.7, 26.9, 28.5, 29.1, 29.3, 29.5, 29.5, 30.1, 32.0, 33.9, 40.7, 50.0, 67.2, 75.7, 79.1, 96.2, 120.5, 128.2, 128.3, 128.3, 128.6, 129.6, 130.8, 131.4, 146.1, 146.2, 148.9, 152.4, 156.1, 157.5, 167.7, 172.9. HRMS (ESI)  $m/z$ :  $[\text{M} + \text{H}]^+$  646.3484, calcd. for  $\text{C}_{37}\text{H}_{47}\text{N}_3\text{O}_7$  645.34140.

*Characterization of (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 12-aminododecanoate (CPT-B-L<sub>12</sub>)*. Obtained from CPT-A-L<sub>12</sub> by method E as a faint yellow powder; m.p. 215.5–216.0 °C; Yield: 72.0%;  $^1\text{H-NMR}$  (500 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  1.04 (t,  $J = 7.4$  Hz, 3H,  $-\text{CH}_3$ ), 1.23 (m, 8H), 1.33 (m, 6H), 1.63 (m, 4H), 2.23 (m, 2H), 2.54 (m, 2H), 2.89 (m, 2H), 5.27 (s, 2H), 5.47 (d,  $J = 16.8$  Hz, 1H), 5.60 (d,  $J = 16.8$  Hz, 1H), 7.35 (s, 1H), 7.69 (dd,  $J = 8.0, 7.1$  Hz, 1H), 7.86 (t,  $J = 7.7$  Hz, 1H), 8.04 (d,  $J = 8.2$  Hz, 1H), 8.14 (d,  $J = 8.5$  Hz, 1H), 8.58 (s, 1H).  $^{13}\text{C-NMR}$  (126 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  8.0, 25.8, 27.4, 28.6, 30.0, 30.1, 30.3, 30.4, 30.5, 32.2, 34.6, 40.8, 51.5, 67.7, 77.3, 97.9, 120.7, 129.2, 129.7, 129.8, 130.0, 130.8, 131.9, 133.3, 147.5, 148.4, 149.6, 153.4, 159.0, 169.6, 174.1. HRMS (ESI)  $m/z$ :  $[\text{M} + \text{H}]^+$  546.2972, calcd. for  $\text{C}_{32}\text{H}_{39}\text{N}_3\text{O}_5$  545.28897.

*Characterization of 1,11,16,3-tetra-tert-butyl 34-((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl) (3S,6S,11S,16S,21S)-6-(2-(tert-butoxy)-2-oxoethyl)-21-((tert-butoxycarbonyl)amino)-5,8,13,18,22-pentaoxo-4,7,12,17,23-pentaazatetatriacontane-1,3,11,16,34-pentacarboxylate (CPT-HT-J-L<sub>12</sub>)*. Obtained from CPT-B-L<sub>12</sub> by method F as a faint yellow powder; m.p. 121.5–121.9 °C; Yield: 42.0%;  $^1\text{H-NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  0.96 (t,  $J = 7.4$  Hz, 3H,  $-\text{CH}_3$ ), 1.14 (m, 10H), 1.24 (s, 4H), 1.30 (m, 4H), 1.41 (s, 27H,  $9 \times -\text{CH}_3$ ), 1.42 (s, 15H,  $5 \times -\text{CH}_3$ ), 1.44 (s, 12H,  $4 \times -\text{CH}_3$ ), 1.62 (m, 4H), 1.86 (m, 2H), 2.07 (m, 4H), 2.26 (m, 6H), 2.37 (m, 4H), 2.48 (m, 4H), 2.60 (m, 1H), 2.87 (m, 1H), 3.10 (m, 1H), 3.22 (m, 1H), 4.13 (s, 1H), 4.42 (m, 2H), 4.52 (m, 1H), 4.80 (s, 1H), 5.28 (m, 2H), 5.40 (d,  $J = 17.0$  Hz, 1H), 5.66 (d,  $J = 17.0$  Hz, 1H), 5.87 (d,  $J = 7.1$  Hz, 1H), 6.31 (d,  $J = 8.1$  Hz, 1H), 7.21 (s, 1H), 7.28 (d,  $J = 7.5$  Hz, 1H), 7.37 (t,  $J = 8.6$  Hz, 1H), 7.44 (s, 1H), 7.66 (t,  $J = 7.4$  Hz, 1H), 7.82 (t,  $J = 7.6$  Hz, 1H), 7.94 (d,  $J = 8.1$  Hz, 1H), 8.20 (d,  $J = 8.5$  Hz, 1H), 8.40 (s, 1H).  $^{13}\text{C-NMR}$  (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.7, 24.8, 27.0, 27.6, 27.8, 28.1, 28.2, 28.5, 29.0, 29.2, 29.4, 29.5, 29.6, 29.6, 31.5, 32.0, 32.1, 32.5, 33.9, 37.4, 39.8, 49.3, 50.1, 51.7, 52.2, 52.5, 53.3, 67.2, 75.7, 80.0, 80.6, 81.6, 81.9, 82.2, 82.5, 96.2, 120.5, 128.2, 128.3, 128.4, 128.6, 129.7, 130.8, 131.4, 146.1, 146.3, 149.0, 152.5, 156.3, 157.5, 167.7, 170.9, 171.1, 171.5, 171.7, 172.2, 172.5, 172.9, 172.9. HRMS (ESI)  $m/z$ :  $[\text{M} + \text{H}]^+$  1557.8584, calcd. for  $\text{C}_{81}\text{H}_{120}\text{N}_8\text{O}_{22}$  1556.85172.

*Characterization of (3S,6S,11S,16S,21S)-21-amino-6-(carboxymethyl)-35-(((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-5,8,13,18,22,35-hexaoxo-4,7,12,17,23-pentaazapentatriacontane-1,3,11,16-tetracarboxylic acid (CPT-HT-J-ZL<sub>12</sub>)*. Obtained from CPT-HT-J-L<sub>12</sub> by method G as a faint yellow powder; m.p. 183.9–184.3 °C; Yield: 24.8%;  $^1\text{H-NMR}$  (500 MHz, DMSO):  $\delta$  0.93 (t,  $J = 7.4$  Hz, 3H,  $-\text{CH}_3$ ), 1.12 (m, 10H), 1.26 (m, 5H), 1.37 (m, 4H), 1.45 (m, 1H), 1.54 (m, 3H), 1.80 (m, 2H), 1.93 (m, 4H), 2.22 (m, 7H), 2.35 (m, 2H), 3.10 (m, 2H), 3.30 (m, 1H), 3.40 (m, 1H), 3.59 (s, 1H), 3.62 (s, 1H), 3.72 (s, 1H), 4.17 (m, 3H), 4.57 (m, 1H), 5.31 (s, 2H), 5.50 (m, 2H), 7.05 (s, 1H), 7.73 (t,  $J = 7.5$  Hz, 1H), 7.87 (t,  $J = 7.5$  Hz, 1H), 8.15 (m, 6H), 8.32 (m, 2H), 8.71 (s, 1H).  $^{13}\text{C-NMR}$  (126 MHz, DMSO):  $\delta$  7.6, 24.0, 24.5, 25.3, 26.0, 26.4, 26.7, 27.0, 27.6, 28.4, 28.7, 28.8, 28.9, 29.7, 30.3, 30.4, 33.2, 38.8, 42.0, 45.8, 50.3, 51.4, 51.6, 52.0, 66.3, 75.6, 94.7, 119.0, 127.8, 128.1, 128.6, 129.0, 129.8, 130.4, 131.6, 145.4, 146.0, 147.9, 152.3, 156.6, 163.1, 167.3, 167.9, 169.3, 171.5, 171.7, 172.1, 172.9, 172.8, 173.4, 173.6, 173.8. HRMS (ESI)  $m/z$ :  $[\text{M} + \text{H}]^+$  1177.4963, calcd. for  $\text{C}_{81}\text{H}_{120}\text{N}_8\text{O}_{22}$  1176.48629.

#### 4.1.5. General Synthetic Procedure of the Preparation of PSMA Hydrolysate CPT-D-GLn

##### General Synthetic Procedure of the Intermediate CPT-C-GLn (Method H)

The corresponding intermediate **CPT-B-Ln** (1 equiv.) was dissolved in dry DCM (20 mL), EDCI (1.5 equiv.) and *N*-Cbz-L-glutamic acid 5-benzyl ester (1.1 equiv.) were added. After addition of HOBT (1.5 equiv.) and DIPEA (2.5 equiv.), the mixture was stirred at 25 °C for 4 h. The reaction mixture was diluted with 50 mL CH<sub>2</sub>Cl<sub>2</sub>, then successively washed with water and brine (10 mL each), dried over sodium sulfate and filtered, and the solvent was evaporated. Then the crude product was purified by flash chromatography (silica gel, dichloromethane:methanol = 1:0 to 50:1).

##### General Synthetic Procedure of PSMA Hydrolysate CPT-D-GLn (Method I)

The intermediate **CPT-C-GLn** was dissolved in 20 mL MeOH, Pd/C (10%, 80 mg) was added. The reaction mixture was stirred at room temperature for 12 h and filtered to remove Pd/C. The filtrate was concentrated in vacuum. Purification was performed by flash chromatography (silica gel, dichloromethane:methanol = 20:1 to 5:1).

*Characterization of benzyl (S)-4-(((benzyloxy)carbonyl)amino)-5-((2-(((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-2-oxoethyl)amino)-5-oxopentanoate (CPT-C-GL<sub>2</sub>)*. Obtained from **CPT-B-L<sub>2</sub>** by method H as a faint yellow powder; m.p. 213.9–214.2 °C; Yield: 50.4%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>): δ 0.97 (t, J = 7.4 Hz, 3H, -CH<sub>3</sub>), 1.97 (dd, J = 14.3, 7.4 Hz, 1H), 2.14 (m, 2H), 2.26 (dd, J = 14.3, 7.4 Hz, 1H), 2.48 (m, 2H), 4.12 (m, 1H), 4.31 (m, 1H), 4.37 (dd, J = 18.2, 6.1 Hz, 1H), 4.93 (t, J = 14.5 Hz, 2H), 5.02 (t, J = 14.5 Hz, 2H), 5.13 (d, J = 18.9 Hz, 1H), 5.22 (d, J = 18.9 Hz, 1H), 5.37 (d, J = 17.1 Hz, 1H), 5.66 (d, J = 17.1 Hz, 1H), 6.99 (s, 1H), 7.18 (m, 2H), 7.24 (m, 2H), 7.28 (m, 6H), 7.63 (t, J = 7.5 Hz, 1H), 7.78 (t, J = 7.6 Hz, 1H), 7.89 (d, J = 8.2 Hz, 1H), 8.22 (d, J = 8.5 Hz, 1H), 8.30 (d, J = 8.5 Hz, 1H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>): 7.7, 28.1, 30.5, 31.8, 41.4, 50.1, 54.1, 66.7, 67.1, 67.2, 96.4, 120.2, 128.1, 128.2, 128.3, 128.4, 128.6, 128.7, 129.7, 130.8, 131.4, 135.7, 136.1, 145.6, 146.4, 148.8, 152.3, 156.4, 157.4, 167.2, 168.9, 171.7, 173.2. HRMS (ESI) *m/z*: [M + H]<sup>+</sup> 759.2653, calcd. for C<sub>42</sub>H<sub>38</sub>N<sub>4</sub>O<sub>10</sub> 758.25879.

*Characterization of (S)-4-amino-5-((2-(((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-2-oxoethyl)amino)-5-oxopentanoic acid (CPT-D-GL<sub>2</sub>)*. Obtained from **CPT-C-GL<sub>2</sub>** by method I as a faint yellow powder; Yield: 39.5%; <sup>1</sup>H-NMR (500 MHz, DMSO): δ 0.92 (s, 3H), 2.16 (d, J = 6.3 Hz, 2H), 2.42 (dd, J = 16.1, 7.8 Hz, 1H), 2.82 (d, J = 16.1 Hz, 1H), 3.47 (s, 1H), 4.08 (d, J = 17.6 Hz, 1H), 4.19 (d, J = 16.9 Hz, 1H), 5.27 (s, 2H), 5.50 (s, 2H), 7.19 (s, 1H), 7.71 (t, J = 6.5 Hz, 1H), 7.85 (d, J = 6.7 Hz, 1H), 8.11 (d, J = 7.5 Hz, 1H), 8.20 (d, J = 8.1 Hz, 1H), 8.67 (s, 1H), 9.02 (s, 1H). <sup>13</sup>C-NMR (126 MHz, DMSO): δ 8.0, 28.0, 30.8, 36.4, 40.9, 50.7, 51.2, 66.8, 76.7, 95.9, 119.4, 128.2, 128.4, 129.0, 129.5, 130.2, 130.9, 132.1, 145.6, 146.4, 148.4, 152.8, 157.0, 167.6, 169.4, 171.7. HRMS (ESI) *m/z*: [M + H]<sup>+</sup> 535.1800, calcd. for C<sub>27</sub>H<sub>26</sub>N<sub>4</sub>O<sub>8</sub> 534.17506.

*Characterization of benzyl (S)-4-(((benzyloxy)carbonyl)amino)-5-((4-(((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-4-oxobutyl)amino)-5-oxopentanoate (CPT-C-GL<sub>4</sub>)*. Obtained from **CPT-B-L<sub>4</sub>** by method H as a faint yellow powder; m.p. 118.0–118.7 °C; Yield: 60.0%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>): δ 0.96 (t, J = 7.4 Hz, 3H, -CH<sub>3</sub>), 1.82 (m, 2H), 1.93 (m, 1H), 2.13 (m, 2H), 2.25 (m, 1H), 2.47 (m, 4H), 3.28 (m, 2H), 4.22 (d, J = 4.8 Hz, 1H), 5.04 (s, 2H), 5.09 (s, 2H), 5.22 (d, J = 18.9 Hz, 1H), 5.27 (d, J = 18.9 Hz, 1H), 5.38 (d, J = 17.1 Hz, 1H), 5.68 (d, J = 17.1 Hz, 1H), 5.76 (d, J = 7.5 Hz, 1H), 6.58 (s, 1H), 7.23 (s, 1H), 7.30 (m, 10H), 7.65 (d, J = 7.5 Hz, 1H), 7.81 (dd, J = 11.3, 4.1 Hz, 1H), 7.92 (d, J = 8.1 Hz, 1H), 8.22 (d, J = 8.5 Hz, 1H), 8.36 (s, 1H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>): δ 7.7, 24.2, 28.2, 30.6, 31.2, 31.9, 38.7, 50.1, 54.4, 66.6, 67.1, 67.3, 76.2, 96.2, 120.2, 128.1, 128.2, 128.3, 128.4, 128.6, 128.7, 129.6, 130.9, 131.5, 135.8, 136.3, 146.0, 146.3, 148.8, 152.4, 156.4, 157.4, 168.0, 171.5, 172.3, 173.2. HRMS (ESI) *m/z*: [2M + H]<sup>+</sup> 1573.7668, calcd. for C<sub>44</sub>H<sub>42</sub>N<sub>4</sub>O<sub>10</sub> 786.29009.

*Characterization of (S)-4-amino-5-((4-(((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-4-oxobutyl)amino)-5-oxopentanoic acid (CPT-D-GL<sub>4</sub>)*. Obtained from **CPT-C-GL<sub>4</sub>** by method I as a faint yellow powder; m.p. 127.5–128.1 °C; Yield: 42.6%; <sup>1</sup>H-NMR

(500 MHz, CDCl<sub>3</sub>):  $\delta$  0.93 (t,  $J = 7.4$  Hz, 3H, -CH<sub>3</sub>), 1.69 (m, 2H), 1.74 (m, 1H), 1.88 (m, 1H), 2.16 (m, 2H), 2.23 (t,  $J = 7.7$  Hz, 2H), 2.24 (m, 2H), 3.12 (d,  $J = 7.1$  Hz, 2H), 3.33 (s, 1H), 3.95 (dd,  $J = 13.9, 8.3$  Hz, 1H), 5.02 (s, 1H), 5.03 (s, 1H), 5.31 (m, 2H), 5.48 (d,  $J = 16.8$  Hz, 1H), 5.52 (d,  $J = 16.8$  Hz, 1H), 7.09 (s, 1H), 7.31 (dd,  $J = 8.5, 4.2$  Hz, 1H), 7.36 (s, 1H), 7.73 (t,  $J = 7.1$  Hz, 1H), 7.87 (t,  $J = 7.1$  Hz, 1H), 7.98 (t,  $J = 5.6$  Hz, 1H), 8.16 (dd,  $J = 18.4, 8.2$  Hz, 2H), 8.70 (s, 1H). <sup>13</sup>C-NMR (126 MHz, DMSO):  $\delta$  7.6, 24.4, 27.2, 30.2, 30.3, 30.7, 37.8, 50.3, 54.1, 65.4, 75.8, 94.8, 118.9, 127.7, 128.0, 128.3, 128.6, 129.0, 129.8, 130.4, 131.6, 145.4, 146.1, 147.9, 152.4, 156.6, 167.3, 171.4, 171.9, 173.9. HRMS (ESI)  $m/z$ : [M + H]<sup>+</sup> 563.2149, calcd. for C<sub>29</sub>H<sub>30</sub>N<sub>4</sub>O<sub>8</sub> 562.20636.

Characterization of (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 6-((S)-5-(benzyloxy)-2-(((benzyloxy)carbonyl)amino)-5-oxopentanamido)hexanoate (**CPT-CPT-C-GL<sub>6</sub>**). Obtained from **CPT-B-L<sub>6</sub>** by method H as a faint yellow powder; m.p. 106.3–107.3 °C; Yield: 62.6%; <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  0.90 (t,  $J = 7.4$  Hz, 3H, -CH<sub>3</sub>), 1.27 (m, 2H), 1.42 (m, 2H), 1.50 (m, 2H), 1.83 (m, 2H), 2.14 (m, 6H), 3.04 (m, 2H), 3.63 (s, 1H), 3.67 (s, 1H), 4.19 (m, 1H), 4.33 (m, 2H), 5.30 (d,  $J = 17.5$  Hz, 1H), 5.49 (d,  $J = 17.5$  Hz, 1H), 7.32 (dd,  $J = 17.0, 9.6$  Hz, 1H), 7.48 (d,  $J = 7.5$  Hz, 1H), 7.71 (m, 1H), 7.88 (dd,  $J = 17.0, 9.6$  Hz, 1H), 8.15 (m, 1H), 8.69 (s, 1H). <sup>13</sup>C-NMR (101 MHz, CDCl<sub>3</sub>): 7.7, 24.3, 25.9, 27.8, 28.9, 30.6, 31.9, 33.7, 39.2, 50.1, 52.7, 54.3, 66.6, 67.1, 67.3, 75.9, 96.3, 120.4, 128.1, 128.3, 128.4, 128.7, 129.4, 131.0, 131.6, 135.9, 136.4, 146.1, 146.2, 148.7, 152.4, 157.4, 168.0, 171.1, 172.7, 173.2. HRMS (ESI)  $m/z$ : [M + H]<sup>+</sup> 815.3279, calcd. for C<sub>46</sub>H<sub>46</sub>N<sub>4</sub>O<sub>10</sub> 814.32139.

Characterization of (S)-4-amino-5-((6-(((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-6-oxohexyl)amino)-5-oxopentanoic acid (**CPT-D-GL<sub>6</sub>**). Obtained from **CPT-C-GL<sub>6</sub>** by method I as a faint yellow powder; m.p. 114.3–114.9 °C; Yield: 35.9%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  0.93 (t,  $J = 7.4$  Hz, 3H, -CH<sub>3</sub>), 1.69 (m, 2H), 1.74 (m, 1H), 1.88 (m, 1H), 2.16 (m, 2H), 2.23 (t,  $J = 7.7$  Hz, 2H), 2.24 (m, 2H), 3.12 (d,  $J = 7.1$  Hz, 2H), 3.33 (s, 1H), 3.95 (dd,  $J = 13.9, 8.3$  Hz, 1H), 5.02 (s, 1H), 5.03 (s, 1H), 5.31 (m, 2H), 5.48 (d,  $J = 16.8$  Hz, 1H), 5.52 (d,  $J = 16.8$  Hz, 1H), 7.09 (s, 1H), 7.31 (dd,  $J = 8.5, 4.2$  Hz, 1H), 7.36 (s, 1H), 7.73 (t,  $J = 7.1$  Hz, 1H), 7.87 (t,  $J = 7.1$  Hz, 1H), 7.98 (t,  $J = 5.6$  Hz, 1H), 8.16 (dd,  $J = 18.4, 8.2$  Hz, 2H), 8.70 (s, 1H). <sup>13</sup>C-NMR (101 MHz, DMSO): 7.6, 24.5, 25.4, 25.6, 28.7, 29.3, 30.3, 33.1, 38.3, 53.1, 54.7, 55.9, 65.5, 75.7, 94.8, 118.9, 127.3, 127.8, 128.1, 129.4, 129.9, 130.5, 131.7, 145.5, 146.0, 147.9, 152.3, 156.6, 167.3, 172.1, 173.4, 177.1, 177.4. HRMS (ESI)  $m/z$ : [M + H]<sup>+</sup> 591.2467, calcd. for C<sub>31</sub>H<sub>34</sub>N<sub>4</sub>O<sub>8</sub> 590.23766.

Characterization of (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 12-((S)-5-(benzyloxy)-2-(((benzyloxy)carbonyl)amino)-5-oxopentanamido)dodecanoate (**CPT-C-GL<sub>12</sub>**). Obtained from **CPT-B-L<sub>12</sub>** by method H as a faint yellow powder; m.p. 90.7–91.5 °C; Yield: 48.6%; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  0.97 (t,  $J = 7.5$  Hz, 3H, -CH<sub>3</sub>), 1.16 (s, 10H), 1.25 (s, 2H), 1.30 (m, 2H), 1.40 (m, 2H), 1.63 (m, 2H), 1.95 (m, 1H), 2.14 (m, 1H), 2.28 (m, 1H), 2.46 (m, 4H), 3.17 (m, 2H), 4.20 (m, 1H), 5.07 (m, 2H), 5.11 (m, 2H), 5.24 (d,  $J = 19.0$  Hz, 1H), 5.29 (d,  $J = 19.0$  Hz, 1H), 5.40 (d,  $J = 17.0$  Hz, 1H), 5.66 (d,  $J = 17.0$  Hz, 1H), 5.71 (s, 1H), 6.24 (s, 1H), 7.23 (s, 1H), 7.32 (s, 6H), 7.32 (s, 4H), 7.66 (t,  $J = 7.4$  Hz, 1H), 7.82 (t,  $J = 7.4$  Hz, 1H), 7.93 (d,  $J = 8.1$  Hz, 1H), 8.21 (d,  $J = 8.5$  Hz, 1H), 8.38 (s, 1H). <sup>13</sup>C-NMR (126 MHz, CDCl<sub>3</sub>):  $\delta$  7.7, 24.7, 26.9, 28.4, 29.1, 29.3, 29.3, 29.4, 29.5, 29.5, 30.5, 32.0, 33.9, 39.7, 50.0, 54.3, 66.7, 67.1, 67.2, 75.7, 96.2, 120.5, 128.1, 128.2, 128.3, 128.3, 128.4, 128.6, 128.6, 128.7, 129.6, 130.8, 131.4, 135.8, 136.3, 146.1, 146.2, 148.9, 152.4, 156.4, 157.5, 167.7, 171.0, 172.9, 173.3. HRMS (ESI)  $m/z$ : [M + H]<sup>+</sup> 899.4223, calcd. for C<sub>52</sub>H<sub>58</sub>N<sub>4</sub>O<sub>10</sub> 898.41529.

Characterization of (S)-4-amino-5-((12-(((S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-12-oxododecyl)amino)-5-oxopentanoic acid (**CPT-D-GL<sub>12</sub>**). Obtained from **CPT-C-GL<sub>12</sub>** by method I as a faint yellow powder; m.p. 180.1–180.9 °C; Yield: 24.2%; <sup>1</sup>H-NMR (500 MHz, DMSO):  $\delta$  0.91 (t,  $J = 7.4$  Hz, 3H, -CH<sub>3</sub>), 1.26 (m, 16H), 1.54 (m, 2H), 1.65 (m, 2H), 1.78 (m, 2H), 2.13 (m, 4H), 2.24 (m, 2H), 3.03 (m, 2H), 3.31 (s, 1H), 5.26 (d, 2H), 5.44 (m, 1H), 5.49 (s, 1H), 6.82 (s, 1H), 7.04 (s, 1H), 7.31 (dd,  $J = 14.7, 7.1$  Hz, 1H), 7.46 (d,  $J = 7.1$  Hz, 1H), 7.71 (t,  $J = 7.5$  Hz, 1H), 7.86 (t,  $J = 7.6$  Hz, 1H), 8.04 (s, 1H), 8.13 (m, 2H), 8.69 (s, 1H). <sup>13</sup>C-NMR (126 MHz, DMSO):  $\delta$  7.5, 24.5, 26.3, 26.4, 28.4, 28.7, 28.7, 28.8, 28.9, 29.0, 29.4, 30.3, 32.3, 33.2, 38.4, 39.0, 39.2, 39.3, 39.5, 39.7, 39.8, 40.0, 50.2,

53.6, 66.3, 75.6, 94.7, 119.0, 127.7, 128.0, 128.6, 128.9, 129.8, 130.4, 131.6, 145.4, 146.0, 147.9, 152.3, 156.6, 165.1, 167.3, 172.1, 172.4, 174.7. HRMS (ESI)  $m/z$ :  $[M + H]^+$  675.3390, calcd. for  $C_{37}H_{46}N_4O_8$  674.33156.

#### 4.2. Bio-Evaluation Methods

##### 4.2.1. Cell Culture

The human prostate cancer cell line (LNCaP-FGC), human prostate cancer cell line (PC-3), human prostate cancer cell line (DU-145), human breast cancer cell line (MCF-7), human hepatocellular carcinoma cell line (HepG2), human cervical cancer cell line (Hela), were obtained from the Chinese Academy of Medical Sciences and Peking Union Medical College. The cultures of the cells were maintained as a monolayer in RPMI 1640 or DMEM supplemented with 10% (*v/v*) heat inactivated fetal bovine serum and 1% (*v/v*) enicillin/streptomycin (Corning, New York, NY, USA) and incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. The tested compounds were dissolved in DMSO (Sigma, St. Louis, MO, USA) and added at required concentrations to the cell culture.

##### 4.2.2. Cytotoxicity Assay

The cytotoxicity of all the tested compounds was evaluated *in vitro* via the MTT method against PSMA-expressing LNCaP-FGC cells and non-PSMA-expressing cells HepG2, Hela, MCF-7, DU145, PC-3 cells using CPT as the positive control. Tumor cells growing in the logarithmic phase were seeded in 96-well plates at a density  $3 \times 10^3$  cells/well and incubated overnight. The following day, cells were then treated with serial dilutions of the tested compounds for 72 h. At the end of this incubation, 20  $\mu$ L of 5 mg/mL methylthiazol tetrazolium (MTT) was added to each well and incubation proceeded at 37 °C for another 4 h. After the supernatant medium was thrown away, 150  $\mu$ L dimethylsulphoxide (DMSO) were added to each well and absorbance was measured at 490 nm using a plate reader (BIORAD 550 spectrophotometer, Bio-rad Life Science Development Ltd., Berkeley, CA, USA). Experiments were performed in triplicate and the values were the average of three ( $n = 3$ ) independent experiments. The concentration of the compound that results in 50% growth inhibition corresponds to the IC<sub>50</sub>. Tumor cell growth inhibitory rate was calculated using Equation (1):

$$\% \text{ inhibition} = (1 - \text{Sample group OD} / \text{Control group OD}) \times 100\% \quad (1)$$

##### 4.2.3. Aqueous Solubility Study

The equilibrium solubility of CPT and CPT-HT-J-ZL<sub>12</sub> was determined in triplicate in PBS, according to the reference method with some modifications. An excess amount (~5 mg) of the tested compounds were placed into tubes, and 1 mL PBS was added to the tube. Then the tubes were incubated at 37 °C for 24 h in an ermostated oscillator (HZQ-QX, Harbin Donglian Instrument Co., Ltd., Harbin, China). After incubation, samples (0.1 mL) were removed and centrifuged at  $10,000 \times g$  for 10 min with high speed centrifuge (Sorvall™ Legend™ Micro 21, Thermo Fisher Scientific, Waltham, MA, USA). A 500  $\mu$ L sample of the supernatant was diluted, and the concentration was measured with HPLC according the method below. CPT and CPT-HT-J-ZL<sub>12</sub> were measured by HPLC systems configured with a Waters 2695 separation module and a Waters 2695 ultraviolet (UV) detector with the following conditions: Xbridge C18 column (250 mm  $\times$  4.6 mm, 5 mm); temperature, 25 °C; elution flow rate, 1.0 mL/min; detection wavelength, 365 nm. mobile phase, 0.2% b in water (A) and 0.2% formic acid in acetonitrile (B) using a gradient elution of 10–60% B at 0–5.0 min, 60–80% B at 5.0–15.0 min, 80% B at 15.0–17.0 min, and 10% B at 17.1–23.0 min.

##### 4.2.4. In Vitro Cellular Uptake

To evaluate the cellular uptake of the CPT-HT-J-ZL<sub>12</sub>, LNCaP-FGC (PSMA<sup>+</sup>) and HepG2 (PSMA<sup>-</sup>) cells were seeded at a density of  $1 \times 10^4$  cells/well in 35 mm glass bottom dishes with 1 mL complete 1640 medium [28]. After incubation for 24 h, the culture medium was replaced with 2 mL of

fresh medium containing 10  $\mu\text{M}$  CPT-HT-J-ZL<sub>12</sub>. Then the cells were incubated further for different incubation time periods (1 and 4 h). Following the removal of the culture media, cells were washed with cold PBS, fixed with 70% ethanol for 30 min at 4 °C. Subsequently, the cells were stained with 5  $\mu\text{g}/\text{mL}$  propidium iodide (excitation wavelength was 561 nm) for 5 min to visualize nucleus. The cells were observed by a UltraView RS confocal laser scanning microscopy (PerkinElmer, Waltham, MA, USA).

#### 4.2.5. Detection of Apoptosis Using Annexin V-FITC/PI Staining

To determine early apoptosis and secondary necrosis, LNCaP-FGC (PSMA<sup>+</sup>) and HepG2 (PSMA<sup>-</sup>) cells were stained with annexin-V FITC apoptosis detection kit (Beijing BioDee Biotech. Co., Ltd., Beijing, China) as per the manufacturer's instructions. After exposure to different concentrations of CPT-HT-J-ZL<sub>12</sub> (0.63, 1.25, 2.50  $\mu\text{M}$  for LNCaP-FGC; 20.00, 40.00, 80.00  $\mu\text{M}$  for HepG2) for 72 h, the cells were collected, washed twice with cold PBS, and centrifuged at 3000  $\times g$  for 5 min. The resulting pellet was mixed with 200  $\mu\text{L}$  binding buffer of the Annexin V-FITC kit, then 5  $\mu\text{L}$  FITC labeled annexin V was added and mixed gently. After incubation at 4 °C for 10 min in the dark, 5  $\mu\text{L}$  PI was added and mixed gently. Then the cells were immediately analyzed with a flow cytometry.

#### 4.2.6. Detection of Cell Cycle Using PI Staining

The cell cycle was measured using PI staining and flow cytometry analysis according to the instructions of the manufacturer (Cell Cycle and Apoptosis Analysis Kit, Beyotime Biotechnology, Jiangsu, China). LNCaP-FGC cells ( $2 \times 10^4$  cells/mL) in logarithmic growth phase were seeded in 6-well culture plate and incubated for 24 h at 37 °C in incubator with 5% CO<sub>2</sub>. After exposure to different concentrations of CPT-HT-J-ZL<sub>12</sub> (0.63, 1.25, 2.50  $\mu\text{M}$ ) for 72 h, cells were harvested and washed twice with cold PBS. Then the cells were centrifuged at 2400  $\times g$  for 10 min, resuspended in 1 mL 70% cold ethanol and fixed for 12 h at -20 °C. After being washed twice with cold PBS, the cells were incubated with 0.5 mL propidium iodide for 30 min at 37 °C. Detection of the cell cycle was carried out by the Flow Cytometer at 488 nm excitation wavelength

#### 4.2.7. Statistical Analysis

Each experimental value is expressed as the mean  $\pm$  SD (standard deviation). One-way analysis of variance (ANOVA) was performed to determine the significance between groups;  $p < 0.05$  was considered to be statistically significant.

**Supplementary Materials:** Supplementary materials can be found at <http://www.mdpi.com/1422-0067/19/10/3251/s1>.

**Author Contributions:** Ideas and experiment design: B.X., T.M., P.-L.W., and H.-M.L.; Chemistry and Biology: B.X., F.Z., M.-M.Y., D.-S.C.; Analysis and interpretation of data: W.-B.G., Y.-Q.Y., X.-H.J.; Writing and review of the manuscript: All the authors; Study supervision: H.-M.L., P.-L.W., and T.M.

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