Journal Pre-proof

Novel octapeptide-DTX prodrugs targeting MMP-7 as effective agents for the treatment of colorectal cancer with lower systemic toxicity

Zheng-Yu Liu, Mei-Lin Tang, Jinfeng Ning, Yun-Peng Hao, Lu Zhou, Xun Sun

PII: S0223-5234(20)30161-6

DOI: https://doi.org/10.1016/j.ejmech.2020.112194

Reference: EJMECH 112194

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 25 October 2019

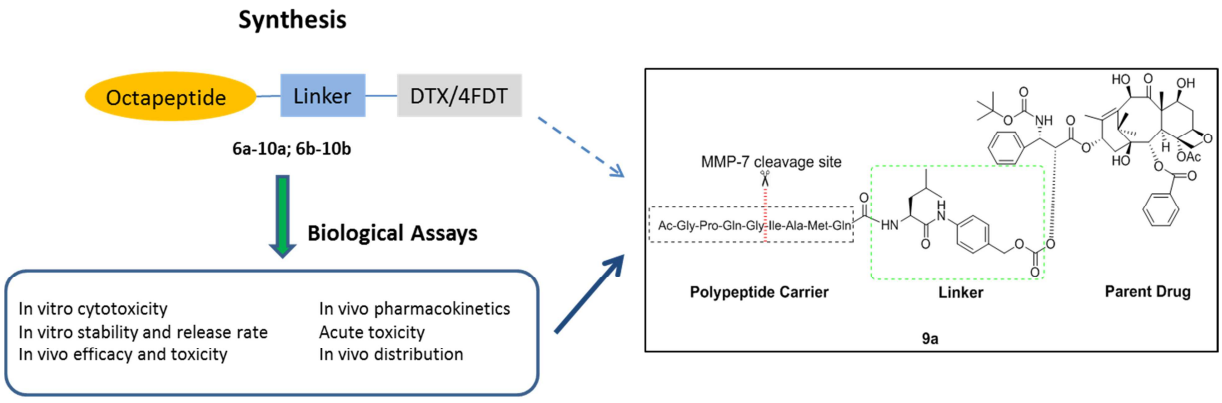
Revised Date: 25 February 2020

Accepted Date: 25 February 2020

Please cite this article as: Z.-Y. Liu, M.-L. Tang, J. Ning, Y.-P. Hao, L. Zhou, X. Sun, Novel octapeptide-DTX prodrugs targeting MMP-7 as effective agents for the treatment of colorectal cancer with lower systemic toxicity, *European Journal of Medicinal Chemistry* (2020), doi: https://doi.org/10.1016/j.ejmech.2020.112194.

Graphic Abstract:

Ten MMP-7 activated octapeptide-DTX/4FDT prodrugs were synthesized and evaluated. The preferred compound **9a** possessed lower systemic toxicity and similar anti-CRC activity to its parent drug DTX.



Novel Octapeptide-DTX Prodrugs targeting MMP-7 as Effective Agents for the Treatment of Colorectal Cancer with Lower Systemic Toxicity

Zheng-Yu Liu [a], Mei-Lin Tang [a,c], Jinfeng Ning [a], Yun-Peng Hao [a], Lu Zhou [d], Xun Sun [a,b,*]

[a] Department of Natural Medicine, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, China
[b] The Institutes of Integrative Medicine of Fudan University, 12 Wulumuqi Zhong Road, Shanghai 200040, China
[c] State Key Laboratory of Molecular Engineering and Institutes of Biomedical Sciences, Fudan University, 220 Handan Road, Shanghai 200433, China
[d] Department of Medicinal Chemistry, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, China

*Corresponding author. Tel./Fax: +86-21-51980101. E-mail address: sunxunf@shmu.edu.cn (X. Sun).

Abstract

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer death around the world. The current treatments of CRC exhibited high occurrence rate of side effects. Docetaxel (DTX), an important drug widely used in cancer chemotherapy, showed serious toxicity in CRC. Reducing toxicity of DTX could be a feasible and promising way to achieve the new indication of DTX for CRC. In this study, a series of MMP-7 activated octapeptide-DTX/4FDT prodrugs (**6a-10a** and **6b-10b**) were designed and synthesized based on the features of MMP-7 which is highly expressed in CRC and could specially recognize octapeptides with specific sequences. Among them, **9a** and **9b**, both possessing an octapeptide Gly-Pro-Gln-Gly-Ile-Ala-Met-Gln moiety, were the most potent prodrugs. Compounds **9a** and **9b** were also tested their release rate in HCT116 cell culture fluids and tumor homogenate along with in vivo anti-CRC activity and systemic toxicity. Since **9a** showed better anti-CRC activity and lower systemic toxicity than **9b** in CRC tumor bearing mice, it was further evaluated for its acute toxicity, pharmacokinetics and tissue distribution in comparison with its parent drug DTX. These results revealed that **9a** possessed good systemic stability, rapid release rate in CRC and reduced systemic toxicity, while retaining similar anti-CRC activity to its parent drug DTX. Thus, **9a**, an MMP-7 polypeptide prodrug of DTX, has been identified as a promising candidate for the treatment of CRC.

Keywords: CRC, prodrug, DTX, MMP-7, systemic toxicity.

1. Introduction

Colorectal cancer (CRC) is one of the most incident and fast-growing malignancies around the world, with an estimated 1.4 million new cases and 693,900 deaths in 2012 [1]. It will increase by 60% to more than 2.2 million new cases and 1.1 million deaths to 2030 [2]. Currently, the most commonly used drugs for CRC include fluoropyrimidines, irinotecan, oxaliplatin, angiogenesis inhibitors, and EGFR inhibitors [3-5]. However, the serious toxicity of these drugs, such as diarrhea, myelosuppression, neutropenia and alopecia, limited their clinical application and treatment efficacy.

Docetaxel (DTX, **Figure 1**), which induces cell-cycle arrest and apoptosis by stablishing microtubules through binding to beta tubulin, is one of most important and exciting drugs used in cancer chemotherapy. This agent has been approved to treat many types of cancer in clinical, including breast carcinoma, prostate cancer, non-small cell lung cancer, head & neck cancer and stomach cancer [6-8]. However, even DTX shows some efficacy with about 20% of response rate in advanced CRC [9], the high rate of serious toxicity hinders its application in CRC[10, 11].

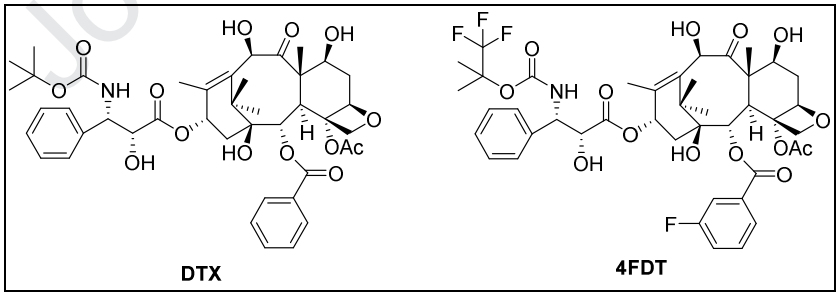
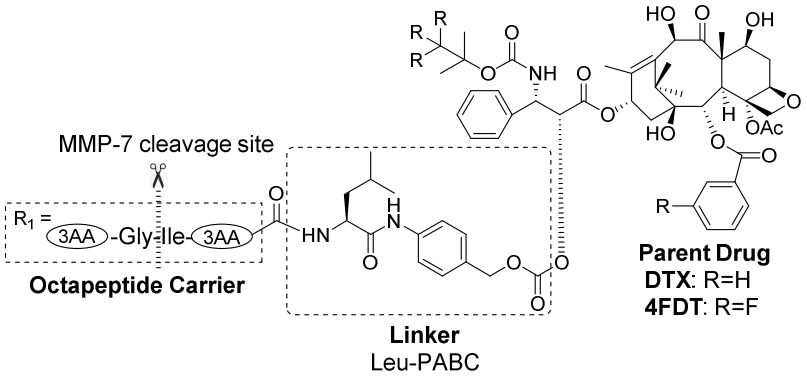


Figure 1. Structures of DTX and 4FDT

Interestingly, DTX exhibited high antiproliferative activity among 50 CRC cell lines in a high-throughput study [12-14]. In our previous work, we synthesized a series of fluorinated DTX derivatives [15] and found the compound 4FDT (**Figure 1**) also exhibited good antiproliferative activity in CRC with better metabolic stability and water solubility than DTX. Nevertheless, 4FDT still had the high systemic toxicity issue [16, 17]. Therefore, developing novel DTX and 4FDT analogs via

reducing systemic toxicity could be an achievable goal to identify promising candidates for the treatment of CRC.

For decades, the prodrug strategy has been sought as the “magic bullet” in drug discovery to increase the druggability by improving solubility, metabolic stability, target specificity and reducing systemic toxicity [18, 19]. Some prodrugs of DTX had been reported to reduce toxicity, such as chitosan-conjugated DTX prodrugs [20], ester-linked DTX prodrugs [21-24], albumin-hitchhiking DTX prodrugs [25] and lipophilic thioether-bridged oleate DTX prodrugs [26]. However, how to empower DTX to specially exert antiproliferation function in tumor is far from being well addressed. In an effort to solve this challenge, the strategy of prodrug that can be activated by particular biomarker of CRC was applied in the present work [18, 19]. Reports have shown that MMP-7 specifically expresses in primary and metastatic CRC, but not in adjacent normal tissues [27, 28]. As a zinc-containing endopeptidase, MMP-7 can promote tumor growth, microenvironment development, metastasis and invasion by degrading several components of the extracellular matrix (ECM) [29, 30]. More importantly, the key character of MMP-7 is that it can recognize octapeptides with specific sequences and hydrolyze them at P4-P5 peptide bond [31], including Gly-Pro-Met-Gly-Ile-Ala-Gly-Gln, Gly-Pro-Gln-Ala-Ile-Ala-Gly-Gln, Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln, Gly-Pro-Gln-Gly-Ile-Ala-Met-Gln, and Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln. This significant character of MMP-7 could be incorporated into prodrug strategy that polypeptides with specific sequences were used as carriers to confer special antiproliferative function on chemotherapy drugs in CRC [32]. The polypeptide-prodrug strategy has successfully been used before, such as on doxorubicin [33] and auristatin [34]. Furtherly, this tactics enables prodrugs to own the strengths of facile introduction of chemical modifications, low immunogenicity and high tumor affinity and specificity [32, 35].



6a: R_1 = Fmoc-Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln; R = H
7a: R_1 = Ac-Gly-Pro-Met-Gly-Ile-Ala-Gly-Gln; R = H
8a: R_1 = Ac-Gly-Pro-Gln-Ala-Ile-Ala-Gly-Gln; R = H
9a: R_1 = Ac-Gly-Pro-Gln-Gly-Ile-Ala-Met-Gln; R = H
10a: R_1 = Ac-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln; R = H
6b: R_1 = Fmoc-Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln; R = F
7b: R_1 = Ac-Gly-Pro-Met-Gly-Ile-Ala-Gly-Gln; R = F
8b: R_1 = Ac-Gly-Pro-Gln-Ala-Ile-Ala-Gly-Gln; R = F
9b: R_1 = Ac-Gly-Pro-Gln-Gly-Ile-Ala-Met-Gln; R = F
10b: R_1 = Ac-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln; R = F

Figure 2. Design strategy and structures of DTX and 4FDT prodrugs

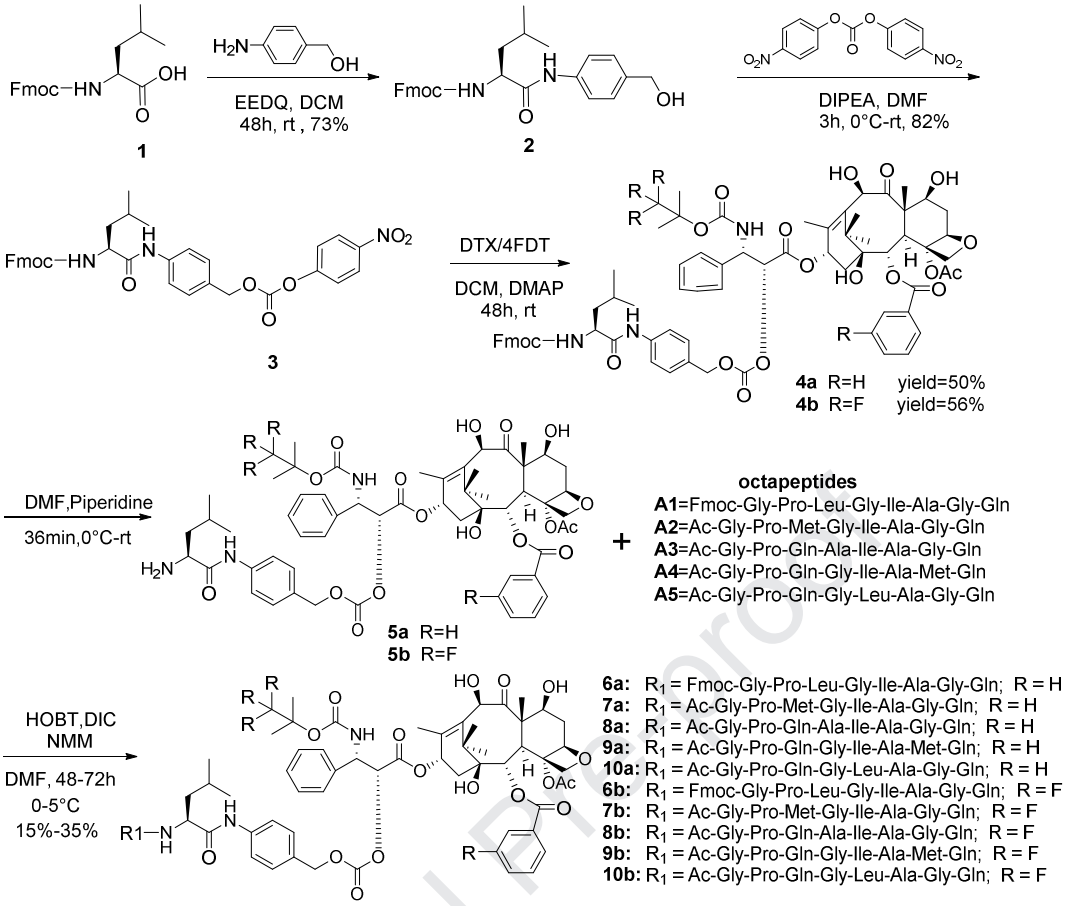
Herein, we designed a series of MMP-7 activated octapeptide prodrugs of DTX and 4FDT (**6a**-**10a** and **6b**-**10b**). These prodrugs consist of three subunits (**Figure 2**) with octapeptide carrier, linker and parent drug. The octapeptide carrier could be specifically recognized and hydrolyzed peptide bond between fourth and fifth peptide by MMP-7 [31]. The bridge chain was Leu-PABC which could possess both the function of self-degradation and linker of the octapeptide carrier with the chemotherapy drug [36]. DTX or 4FDT was chosen as the parent drug, since both already have demonstrated efficacy for anti-CRC activity. It was envisaged that promising analogs with good anti-CRC activity and reduced side effects could be achieved via transporting octapeptide-prodrug into CRC environment, being recognized and hydrolyzed by the high-expressing MMP-7 and releasing the parent drug by self-degradation of the linker.

2. Results and Discussion

2.1. Synthesis

The synthesis of prodrugs **6a**-**10a** and **6b**-**10b** was outlined in **Scheme 1** by applying a five-step sequence. Followed a previously reported method [35],

Fmoc-L-Leu (**1**) was reacted with *p*-aminobenzyl alcohol (PABC) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) to give Fmoc-Leu-PABC (**2**) in 73% yield [36]. Compound **2** was then treated with bis(p-nitrophenyl) carbonate (bis-PNP) under Hünig's base to form an activated ester **3** in 82% yield by recrystallization. Intermediate **3** was coupled with DTX or 4FDT [14] catalyzed by DMAP to afford their desired corresponding **4a** or **4b** in 50-56% yield. Removal of Fmoc group of **4a** and **4b** to be **5a** and **5b** was problematic here mainly due to the unstable feature of the free amine. When exposed longer reaction time, multiple impurities were appeared which resulted in lower yield and tedious separation. After tried several conditions (*e.g.* HCl/Et_2O, Et_2NH/CH_3CN, *etc*.), it was found that the reaction was completed in 30 min under piperidine/DMF condition to give crude **5a** or **5b** in 80-90% yield. With **5a** and **5b** in hand, analogs of **6a-10a** and **6b-10b** were prepared in 15-35% yield (2 steps) by reacting with different octapeptides (**A1-A5**) in the presence of 1-hydroxybenzotriazole (HOBT), N,N'-diisopropylcarbodiimide (DIC) and N-methylmorpholine (NMM) in DMF.



Scheme 1. Synthesis of prodrugs **6a-10a** and **6b-10b**.

2.2. Biological Evaluation

2.2.1 In vitro cytotoxicity

All synthesized target compounds were evaluated for their in vitro cytotoxicity against colon cancer cell lines HCT116 and SW620, normal colon cell line CCD18Co, and normal kidney cell line HEK293 in comparison with their parent drugs (DTX or 4FDT) (**Table 1**). In this study, 5-FU was employed as a positive control. Results revealed that the antiproliferative activity of all prodrugs were much higher than the 5-FU. Except **6b**, most prodrugs showed good activity against HCT116 cell line. Among them, **9b** showed a slightly more potent than DTX (IC_{50} = 2.77 nM vs. 3.47 nM), though 4FDT was much more potent. For SW620 cell line, compound **9a** had the most potent activity in this series which was similar to that of DTX (IC_{50} = 30 nM vs. 20 nM) and 9-fold more potent than that of 4FDT (IC_{50} = 280 nM). Except **6b**, all prodrugs showed higher antiproliferative activity against HCT116 than that of

SW620.

All prodrugs (**6a-10a** and **6b-10b**) were significantly reduced toxicity compared to their corresponding parent drugs DTX or 4FDT against normal cell line CCD18Co. Most prodrugs also demonstrated weak toxicity against normal cell line HEK293. Compound **9a** and **9b**, contained a common octapeptide carrier (octapeptide A4), exhibited little or no toxicity effect against both normal cell line CCD18Co (IC_{50} > 200 μM) and HEK293 (IC_{50} > 86 μM). Particularly, **9a** retained strong antiproliferative activity against HCT116 and SW620 cell line (IC_{50} = 9.0 nM for HCT116; IC_{50} = 30 nM for SW620). These data initially validated the strategy that linking an octapeptide carrier to DTX or 4FDT could significantly reduce the toxicity of parent drugs against normal cell lines.

Table 1. Cytotoxic activity of prodrugs against CRC and normal cell lines.

compound	IC_{50} (μM)[a]			
	HCT116[b]	SW620[b]	CCD18Co[b]	HEK293[b]
5-FU	$26042.5 \pm 335.2 \times 10^{-3}$	6.96 ± 0.07	>200	79.32 ± 8.01
DTX	$3.5 \pm 0.05 \times 10^{-3}$	0.02 ± 0.001	116.54 ± 4.31	19.70 ± 1.31
4FDT	$0.1 \pm 0.01 \times 10^{-3}$	0.28 ± 0.013	17.13 ± 0.74	0.36 ± 0.02
6a	$9.8 \pm 0.2 \times 10^{-3}$	1.01 ± 0.03	> 200	15.24 ± 0.76
6b	$439.4 \pm 24.3 \times 10^{-3}$	0.21 ± 0.01	> 200	38.31 ± 1.71
7a	$7.2 \pm 0.1 \times 10^{-3}$	1.14 ± 0.03	> 200	11.02 ± 0.83
7b	$20.9 \pm 1.3 \times 10^{-3}$	> 200	> 200	21.31 ± 0.52
8a	$11.3 \pm 0.8 \times 10^{-3}$	> 200	> 200	106.00 ± 3.51
8b	$5.4 \pm 0.1 \times 10^{-3}$	0.65 ± 0.02	> 200	94.11 ± 2.73
9a	$9.1 \pm 0.1 \times 10^{-3}$	0.03 ± 0.001	> 200	136.83 ± 4.32
9b	$2.8 \pm 0.1 \times 10^{-3}$	6.59 ± 0.73	> 200	86.79 ± 2.71
10a	$5.2 \pm 0.2 \times 10^{-3}$	21.92 ± 1.02	> 200	165.34 ± 5.33
10b	$25.7 \pm 0.6 \times 10^{-3}$	> 200	> 200	6.17 ± 0.34

a: the concentration of drug to inhibit cell proliferation by 50% after 48 h or 72 h of treatment. *b*: cell lines were treated for 48 h (HCT116, HEK293) or 72 h (SW620, CCD18Co) with different concentrations of compound (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7},10^{-6}, 10^{-5}, and 10^{-4} mol/L).

2.2.2. In vitro stability and release rate

To investigate the stability of ten prodrugs, the half-life time in PBS and in plasma of SD rat were tested (**Table 2**). After dissolving prodrugs in media, the unhydrolyzed

prodrugs were measured at set time points by RP-HPLC. The time needed for prodrugs to reduce their concentration to half was calculated. Except **10a** ($t_{1/2}$ = 35.11 h) and **10b** ($t_{1/2}$ = 48.33 h), all other prodrugs showed a longer hydrolysis half-life time in PBS (> 72 h). The high stability of these prodrugs in plasma of SD rat was also observed. Except **8a** and **10a,** hydrolysis half-life time of all others reached to around or more than 24 h. Overall, these results of in vitro stability indicated that **6a**, **6b**, **9a** and **9b** exhibited higher stability in both PBS and rat plasma.

Taken the results of their cytotoxic activity and stability in vitro together, compounds **9a** and **9b**, both containing the carrier octapeptide A4, were chosen to further evaluate their release rate in cell culture fluids and tumor homogenate of HCT116. After incubating **9a** or **9b** in the culture mediate of HCT116 cells or in HCT116 tumor homogenate at 37 ^{0}C, the unhydrolyzed **9a** or **9b** and their corresponding released drug DTX (as DTX*) or 4FDT (as 4FDT*) were measured by RP-HPLC. Results showed that the percentage of release rate for **9a** and **9b** reached to 96.9% and 88.7% respectively during 4 h in HCT116 cell culture mediate; whilst in HCT116 tumor homogenate, their percentage release rate reached to 96.3% and 98.7% during 5 min respectively. These indicated that **9a** and **9b** can be rapidly released to be their parent drugs in CRC environment while keeping stable in mock systemic circulations of PBS and rat plasma.

As mentioned before, the hypothesis to design peptide-prodrugs was that these prodrugs could keep stable in circulating system and then release parent drug in CRC environment. The above results supported the feasibility of our rationale. The preferred compounds **9a** and **9b**, stable in PBS and rat plasma but rapidly released DTX* and 4FDT* in CRC circumstance, might have good antitumor activity to CRC with lower systemic toxicity compared to their parent DTX and 4FDT. These results inspired us to continue to explore their in vivo antitumor activity and systemic toxicity.

Table 2. Stability of prodrugs in phosphate saline buffer (PBS) and rat plasma (37 ^{0}C).

compound	$T_{1/2}$[a]	
	PBS	Plasma
6a	＞ 72 h	35.87 h
6b	＞ 7 2h	47.81 h
7a	＞ 72 h	23.93 h
7b	＞ 72 h	23.96 h
8a	＞ 72 h	11.97 h
8b	＞ 72 h	24.04 h
9a	＞ 72 h	24.18 h
9b	＞ 72 h	43.80 h
10a	35.11 h	14.01 h
10b	48.33 h	24.05 h

a: At fixed time points (*e.g.* 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 10 h, 24 h, 48 h, 72 h), equal samples were taken out and analyzed by RP-HPLC. See Experimental Section for details.

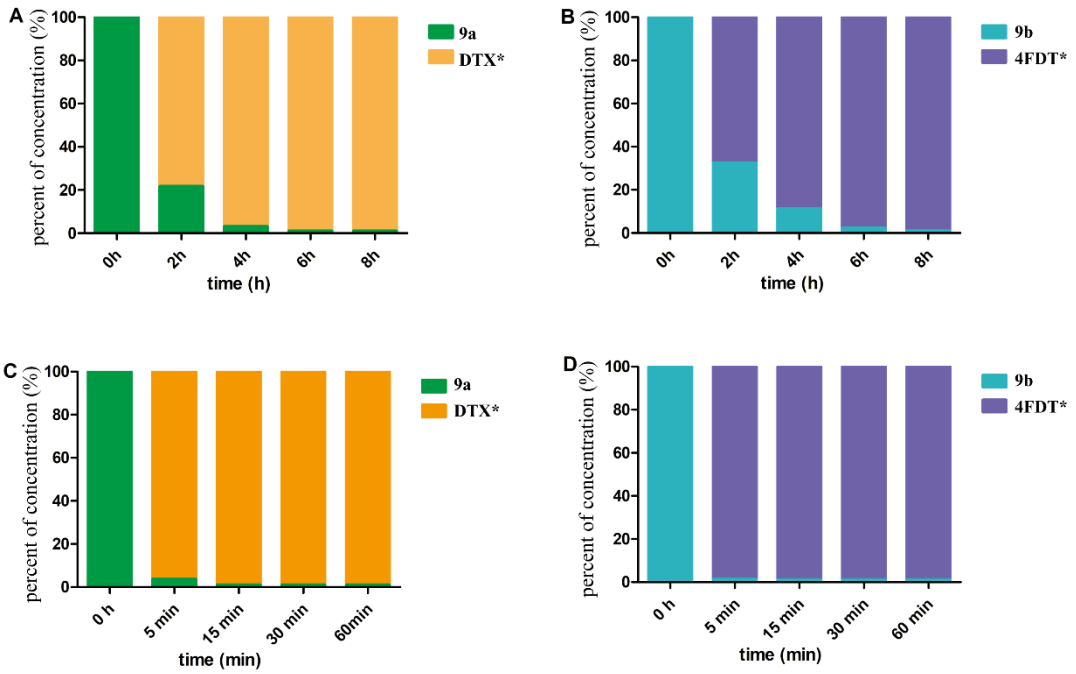


Figure 3. Release rate of **9a** and **9b** in the culture mediate of HCT116 cells or in HCT116 tumor homogenate. (A) Concentration percentage of **9a** and its released DTX (DTX*) in HCT116 cells culture fluids at different time points. (B) Concentration percentage of **9b** and its released 4FDT (4FDT*) in HCT116 cells culture fluids at different time points. (C) Concentration percentage of **9a** and DTX* in HCT116 tumor homogenate at different time points. (D) Concentration percentage of **9b**

and 4FDT* in HCT116 tumor homogenate at different time points.

2.2.3. In vivo efficacy and toxicity of compound 9a and 9b

To evaluate in vivo efficacy and toxicity of compound **9a** and **9b**, human CRC cancer xenograft mouse models were established by subcutaneous inoculation of HCT116 cells in the male BALB/C nude mice. After establishing solid tumor, the mice were randomized into ten groups and each group assigned six mice. The mice were injected intraperitoneally once every 7 days for two consecutive weeks and tested groups received a different dose with 23.4 mg/kg of **9a** (equivalent to DTX 10 mg/kg), or 11.7 mg/kg of **9a** (equivalent to DTX 5 mg/kg), or 5.85 mg/kg of **9a** (equivalent to DTX 2.5 mg/kg), or 22.2 mg/kg of **9b** (equivalent to 4FDT 10 mg/kg), or 11.1 mg/kg of **9b** (equivalent to 4FDT 5 mg/kg), or 5.55 mg/kg of **9b** (equivalent to 4FDT 2.5 mg/kg). The parent drug control groups received 5 mg/kg of DTX or 5 mg/kg of 4FDT and the positive control group received 5 mg/kg of 5-FU. The vehicle control group received the solvent formula only. Tumor progression and weight of mice were monitored once every other day. At the end of observation day, the mice were scarified and the tumors were stripped and weighed.

As shown in **Figure 4A**, at the end of observation period (14th day), both tested groups and the parent drug control groups exhibited statistically significant reduction in tumor progression compared with the vehicle. There was no notable difference between the positive control group of 5-FU (5 mg/kg) and vehicle control group in tumor weight. Moreover, both **9a** and **9b** inhibited tumor growth in a dose-dependent manner. At the same molar equivalent dose with 5 mg/kg DTX or 5 mg/kg 4FDT, the tumor growth inhibition (TGI) of **9a** (11.7 mg/kg), **9b** (11.1 mg/kg), DTX (5 mg/kg) and 4FDT (5 mg/kg) was 46.3%, 35.2%, 42.7% and 41.9% respectively ($p < 0.05$). The TGI of 5-FU (5 mg/kg) was 11.4% ($p > 0.05$). At the same molar equivalent dose with DTX (10 mg/kg) or 4FDT (10 mg/kg), groups of **9a (**23.4 mg/kg) and **9b** (22.2 mg/kg) exhibited enhanced inhibitory effect on the tumor growth with the TGI of 56.1% ($p < 0.01$) and 46.3% ($p < 0.05$) respectively. At the same molar equivalent dose with DTX (2.5 mg/kg) or 4FDT (2.5 mg/kg), tumor inhibition rate of groups **9a** (5.85

mg/kg) and **9b** (5.55 mg/kg) remained to reach 28.8% ($p < 0.05$) and 30.5% ($p < 0.05$) respectively. These results indicated that **9a** and **9b** demonstrated similar in vivo antitumor efficacy for CRC to their corresponding parent drugs DTX or 4FDT and much more efficacious than 5-FU. These results were consistent with the conclusion derived from in vitro cytotoxicity study.

At the end of observation period, animals treated with DTX (5 mg/kg), **9a (**23.4 mg/kg), **9a (**11.7 mg/kg), and **9a** (5.85 mg/kg) exhibited body weight loss of 13.0%, 12.2%, 8.5% and 4.9% respectively. Similarly, mice treated with 4FDT (5 mg/kg), **9b** (22.2 mg/kg), **9b** (11.1 mg/kg) and **9b** (5.55 mg/kg) exhibited body weight loss of 9.5%, 8.7%, 8.5% and 4.6% respectively (**Figure 4B** and **4C**). It was observed that **9a** and **9b** had less effect on body weight of mice than their corresponding parent drugs DTX or 4FDT at same molar dose (5 mg/kg) and even in high dose (10 mg/kg). This indicated that prodrugs **9a** or **9b** might have lower systemic toxicity than DTX or 4FDT in vivo.

To further assess the systemic toxicity of prodrugs **9a** and **9b**, the heart and liver tissues were collected from xenograft mice and stained by H&E at the end of observation time. The systemic toxicity was compared among vehicle control group, positive control group for 5-FU (5 mg/kg), groups for DTX (5 mg/kg) and 4FDT (5 mg/kg), **9a (**11.73 mg/kg) and **9b** (11.17 mg/kg). As shown in **Figure 4D**, DTX caused concentrated nucleus and 4FDT caused tissue necrosis in liver, both which did not appear on group of **9a** and **9b**. As shown in **Figure 4E**, disordered myocardial fibers and inflammatory cell invasion in heart were significantly observed on DTX and 4FDT, but not on **9a** and **9b**. This observation again indicated that prodrugs **9a** or **9b** may have lower systemic toxicity compared to DTX or 4FDT in vivo at the same molar dose.

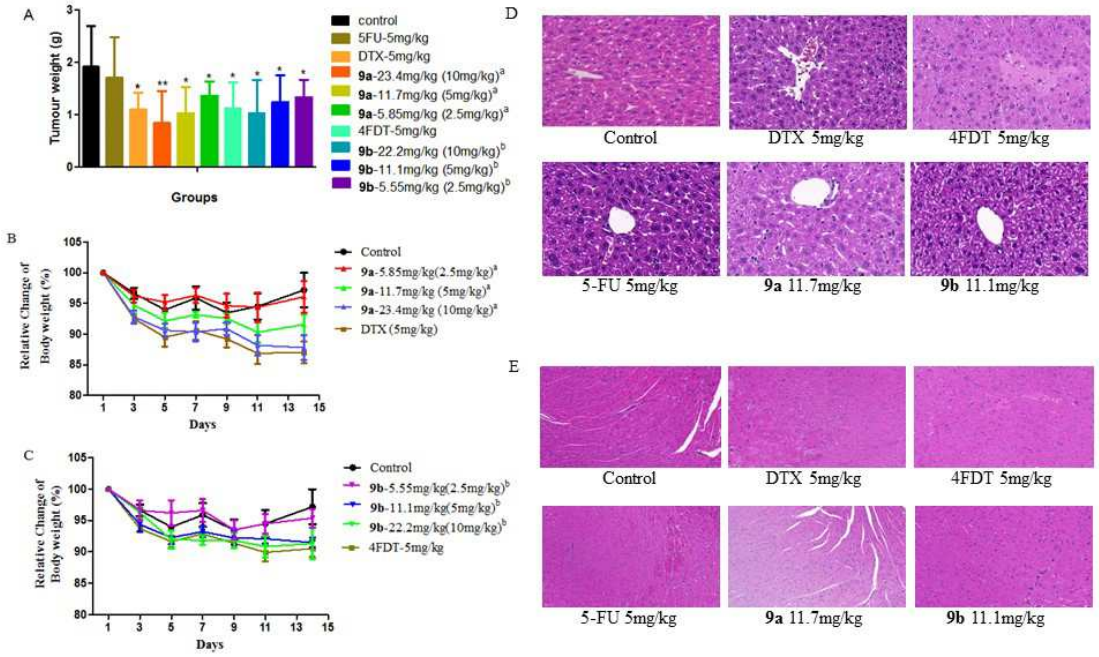


Figure 4. Inhibition of subcutaneous solid tumor growth in vivo by **9a**, **9b**, DTX, 4FDT and 5-FU. (A) The weight of the excised tumor at the end of the observation period. (B) The mouse body weights of groups **9a** and DTX recorded during the observation period. (C) The mouse body weights of groups **9b** and 4FDT recorded during the observation period. (D) Histological appearance of the liver (400×). (E) Histological appearance of the heart (100×). Data are expressed as the mean ± SD (n = 6): (∗) $p < 0.05$ and (∗∗) $p < 0.01$ vs control. a: The same molar equivalent dose of DTX; b: the same molar equivalent dose of 4FDT.

2.2.4. Acute toxicity in mice

Compound **9a** was chosen for further evaluation of its acute toxicity in mice, since it had better anti-CRC activity and lower systemic toxicity in vivo. Healthy Kunming mice (weighing 20 ± 2 g) were randomly divided into five groups with equal of both sexual and behavioral status. Death status of mice was continuously recorded for 14 days after intraperitoneally injected with different dose of tested compounds. Bliss method was applied to calculate the value of LD_{50}. Results showed that the LD_{50} of prodrug **9a** (245.25 mg/kg) was 3.3-fold higher than that of its parent drug DTX (74.1 mg/kg). When converted the LD50 value of **9a** to the same molar equivalent dose of DTX (104.3 mg/kg), it still showed 1.4-fold higher than that of parent drug DTX

(74.1 mg/kg). This confirmed that the safety margin of **9a** was larger than its parent drug DTX.

2.2.5. In vivo pharmacokinetics

Prodrug **9a** and its parent drug DTX were administrated intravenously to Sprague Dawley (SD) rats at a dose of 5 mg/kg respectively. The plasma concentrations of **9a**, DTX* (DTX released from **9a**) and DTX were determined by LC-MS/MS. The parameters of DTX group were calculated after converting DTX dose to the same molar equivalent of 9a. The exposure level of **9a** was ten-fold higher than that of DTX and volume of distribution of **9a** was two times lower than that of DTX (**Table 3**). Moreover, **9a** showed significantly larger mean residence time ($MRT_{0-\infty}$) and longer half-life time than that of DTX, whereas the concentration of DTX* was much less than that of the free parent drug DTX (**Table 3**, **Figure 5**). This meant that **9a** could keep primary type for long period in plasma and might contribute to decrease the systemic toxicity induced by DTX*.

Table 3. The pharmacokinetic parameters of **9a**, DTX*and **DTX**（iv, 5 mg/kg）

Parameters	Compound **9a**		DTX [i]
	9a	**DTX***	
AUC_{0-t} (μg/L*h)	9069 ± 1786	247 ± 124	930± 139
$AUC_{0-\infty}$(μg/L*h)	9244 ± 1800	299 ± 103	867 ± 146
$MRT_{0-\infty}$ (h)	11.57 ± 2.76	9.04 ± 2.53	3.76 ± 0.39
$t_{1/2}$ (h)	12.81 ± 2.32	7.03 ± 1.76	5.75 ± 0.88
Vz (L/kg)	10.39 ± 2.47	206.32 ± 124.39	22.6 ± 2.76
CLz (L/h/kg)	0.56 ± 0.12	18.98 ± 6.90	2.76 ± 0.43

Pharmacokinetic data were evaluated using a non-compartment model and showed as mean ± SD (n = 3). *i*: The parameters of DTX group were calculated after converting DTX dose to the same molar equivalent of **9a**.

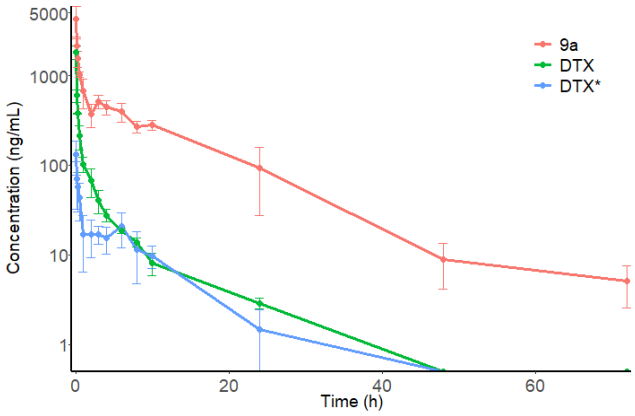


Figure 5. The concentration-time profile after intravenous administration of **9a** and DTX in SD rats (n = 3).

2.2.6. In vivo distribution

Tissue distribution was evaluated in mice subcutaneously implanted with HCT116 tumors following administration of **9a** and its parent drug DTX respectively. After intravenous injection, concentrations of **9a**, DTX* (DTX released from **9a**) and the free parent drug DTX in heart, liver, spleen, lung, kidney and tumor were measured time course by LC-MS/MS. As shown in **Figure 6**, the concentration of free DTX decreased rapidly in all tissues after 0.5 h, while the prodrug **9a** kept relatively high level for long time. Considering that **9a** shows long half-life time ($t_{1/2}$ for **9a** was 12.8 h) and great $AUC_{0-\infty}$ (9069 μg/L*h) in plasma of SD rats in pharmacokinetics study, **9a** was speculated more likely to be distributed in the blood of mice initially and slowly distributed to various tissues and organs over time. As shown in **Figure 6**, the concentration of DTX* was lower than that of **9a** in heart, liver, spleen, lung, and kidney at most time points, excepting in heart at 0.5 h and 4 h, and in lung and kidney at 24 h. Moreover, the concentration of DTX* and **9a** showed decreased trend in all normal tissues since 8 h. However, in tumor, the concentration of DTX* was higher than that of **9a** from 8 h to 24 h and continued to increase over time, while 9a showed decreased trend since 4 h. The different release rates of **9a** in tumor and other tissues indicated that the **9a** may possess relatively higher stability in plasma and normal tissues and more easily recognized and hydrolyzed in CRC tumor by MMP-7 to release DTX. Thus, **9a** may exhibit sustainable therapeutic efficacy for CRC and

meanwhile reduce systemic toxicity for other tissues.

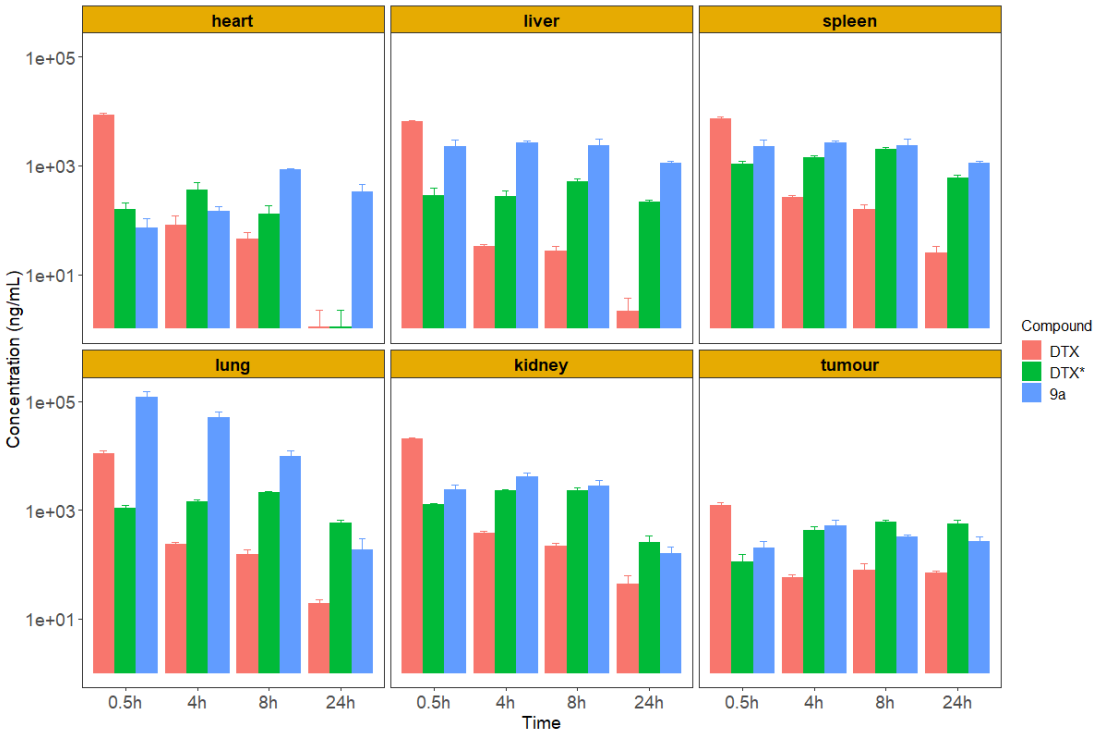


Figure 6. Tissue bio-distribution of **9a** and DTX in mice bearing HCT116 xenograft at 0.5, 4, 8 and 24 h after intravenous injection (n = 5 for each group at each time point).

3. Conclusion

In present study, with the aim of reducing systemic toxicity of DTX or 4FDT and developing their new indication for CRC, a series of MMP-7 activated octapeptide-DTX/4FDT prodrugs were designed, synthesized and biologically evaluated. Most of the synthesized prodrugs displayed comparable antitumor activity to CRC cells (HCT116, SW620) and less cytotoxicity to normal cells (CCD18Co, HEK293) when compared with their parent drugs. In addition, all prodrugs exhibited good stability in PBS and plasma of rats. Among them, compound **9a** and **9b**, both containing the same octapeptide carrier A4, were chosen to test their release rate in CRC circumstance of cell culture fluids and tumor homogenate of HCT116. The fast release rates of **9a** and **9b** in CRC circumstance and good stability in plasma encouraged us to further study their systemic toxicity and anti-CRC activity in vivo. At the same molar equivalent (5 mg/kg) as their parent drugs, **9a** and **9b** exhibited less

effect on body weight and lower toxicity injury on heart and liver of mice than DTX and 4FDT. Also, **9a** and **9b** showed similar antitumor activity to DTX and 4FDT in a subcutaneous solid tumor model of CRC. Prodrug **9a** was further tested for its acute toxicity, pharmacokinetics and bio-distribution. Acute toxicity experiment showed that LD_{50} of **9a** was increased 3.3-fold than its parent drug DTX which demonstrated that **9a** had a relatively larger safety margin than parent drug DTX. Pharmacokinetic study indicated that **9a** exhibits much higher distribution and longer half-life time in plasma than DTX. The release rate of **9a** in plasma was only 16.2% and the concentration of DTX* (**9a**-released DTX) was much lower than that of free parent drug DTX. In bio-distribution study, it was found the release rate of **9a** was high in tumor, but relatively low in other organs. In conclusion, the new prodrug **9a** possessed low systemic toxicity to normal tissues, while kept similar antitumor activity for CRC to its parent drug DTX. These results demonstrated that these novel prodrugs of DTX or 4FDT, conjugated an octapeptide by linker, could be feasible way to reduce systemic toxicity of DTX or 4FDT and maintain good antitumor activity for CRC.

4. EXPERIMENTAL SECTION

4.1. Materials and physical measurements

Compound **4FDT** was previously synthesized and published by our group [15]. Octapepetides were custom-made by Jie peptide Biological Technology Co., Ltd. (Shanghai, China) with a purity $\geqslant$ 95%. Commercially available chemicals were purchased from Aldrich and TCI Chemical companies. All solvents were purified and dried in accordance with standard procedures unless otherwise indicated. Reactions were monitored by TLC using Yantai (China) GF254 silica gel plates (5 $\times$ 10 cm) or by HPLC using Waters e2695 with X-Bridge C18-column (5 μm, 3.0 $\times$ 50 mm). Silica gel column chromatography was performed on silica gel (300-400 mesh) from Yantai (China). Semi-preparative reversed phase liquid chromatography was conducted on Combi Flash Rf from Teledyne Isco (USA). ^{1}H NMR spectra were

recorded at Varian Mercury 400 (400 MHz) or Bruker Avance DPX400, ^{13}C NMR spectra were recorded at Bruker (151 MHz). Low-resolution Mass spectra data were obtained using an Agilent 1946D LC/MSD (ESI) and high-resolution Mass spectra data were performed on AB SCIEX TripleTOF™ 5600^{+}.

4.2. Chemistry

4.2.1. Fmoc-Leu-PABC-PNP (**3**)

To a solution of N-ethoxycarbonyl-2-ethoxy-1,2- dihydroquinoline (EEDQ) (3.15 g, 2.59 mmol) and Fmoc-L-Leu (**1**, 4.15 g, 2.35 mmol) in anhydrous DCM (250 mL) was added 4-aminobenzyl alcohol (PAB-OH) (1.55 g, 2.59 mmol). The mixture was continuously stirred at room temperature for 24 h. The solvent was then evaporated under reduced pressure and the resulting residue was purified by silica gel chromatography (DCM/MeOH = 50 : 1 to 10 : 1) to afford **2** (1.00 g, 73%) as a colorless powder. ESI-MS: m/z 459.2 $[M+H]^{+}$, 481.2 $[M+Na]^{+}$, HPLC analysis (λ = 220 nm) > 95% of peak area.

To a solution of **2** (4.00 g, 8.73 mmol) and bis(p-nitrophenyl)carbonate (bis-PNP) (13.30 g, 43.65 mmol) in anhydrous N,N-dimethylformamide (DMF) (100 mL), N,N-diisopropylethylamine (DIPEA) (4.33 mL, 26.19 mmol) was added dropwise at 0 ^{0}C. After stirring at ice-bath for 5 minutes, the reaction mixture was continuously stirred at room temperature for approximately 6 h. Subsequently, water (300 mL) was added and the mixture was extracted with ethyl acetate (3 × 200 mL). The combined organic extracts were washed with brine (200 mL) and dried over Na_2SO_4. After filtered, the solvent was concentrated and the resulting residue was purified roughly by flash chromatography (petroleum ether/acetone = 7 : 1) to remove most of impurities. Then, the concentrated residue was further recrystallized with ethyl acetate/petroleum ether (V/ V = 1 : 1) to give compound **3** (4.46 g, 82%) as a colorless powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.27 (d, J = 8.0 Hz, 2H), 7.76 (d, J = 8.0 Hz, 2H), 7.61-7.49 (m, 4H), 7.49-7.44 (m, 1H), 7.41-7.35 (m, 6H), 7.30-7.28 (m, 1H), 5.24 (s, 2H), 4.51-4.45 (m, 2H), 4.29 (s, 1H), 4.21 (dd, J = 8.0 Hz, 12.0 Hz, 1H), 1.80-1.68 (m, 2H), 1.04 (d, J = 4.0 Hz, 1H), 0.98-0.95 (m, 6H); ^{13}C

NMR (100 MHz, $CDCl_3$) δ ppm: 170.34, 155.53, 152.44, 145.43, 143.56, 141.36, 129.73, 127.83, 126.60, 125.47, 125.35, 124.89, 122.14, 121.78, 120.08, 70.60, 67.24, 47.17, 24.76, 22.93. ESI-MS: m/z 623.6 $[M+H]^+$, 646.6 $[M+Na]^+$; $C_{35}H_{33}N_3O_8$: HRMS calcd. 646.2160 $[M+Na]^+$, found 646.2162.

4.2.2. General procedure for the synthesis of **4a** or **4b**.

Compound **3** (535.95 mg, 0.86 mmol), 4-(dimethylamino)pyridine (DMAP) (105.07 mg, 0.86 mmol) and appropriate DTX (or 4FDT) (0.86 mmol) were dissolved in anhydrous DCM (37 mL) under N_2. The reaction was stirred at room temperature for 48 h. Then, the organic solvent was concentrated under reduced pressure, and the crude residue was purified by flash chromatography column (DCM/MeOH = 100 : 1) to afford **4a** (550 mg, 50%) as a colorless powder or **4b** (550 mg, 54%) as a light yellow powder.

Fmoc-Leu-PABC-DTX (**4a**). Colorless powder. Yield: 50%. ^{1}H NMR (400 MHz, Acetone-d6) δ ppm: 9.43 (s, 1H), 8.12 (d, *J* = 7.6 Hz, 2H), 7.86 (d, *J* = 7.6 Hz, 2H), 7.76-7.64 (m, 5H), 7.58 (t, *J* = 7.6 Hz, 2H), 7.51 (d, *J* = 7.6 Hz, 2H), 7.46-7.35 (m, 6H), 7.34-7.25 (m, 3H), 7.04 (d, *J* = 9.6 Hz, 1H), 6.83 (d, *J* = 8.0 Hz, 1H), 6.11 (d, *J* = 8.4 Hz, 1H), 5.68 (d, *J* = 7.2 Hz, 1H), 5.40 (d, *J* = 9.2 Hz, 1H), 5.30 (d, *J* = 5.2 Hz, 1H), 5.25 (s, 1H), 5.19 (d, *J* = 11.80 Hz, 1H), 5.11 (d, *J* = 11.80 Hz, 1H), 4.97 (d, *J* = 8.8 Hz, 1H), 4.39-4.30 (m, 5H), 4.27-4.21 (m, 2H), 4.17 (s, 2H), 3.93 (d, *J* = 6.8 Hz, 1H), 3.68 (s, 1H), 2.83 (s, 1H), 2.45 (d, *J* = 17.6 Hz, 4H), 2.38-2.28 (m, 1H), 2.12 (d, *J* = 8.4 Hz, 1H), 1.93-1.77 (m, 5H), 1.75-1.65 (m, 5H), 1.45- 1.25 (m, 20H), 1.17 (d, *J* = 12.4 Hz, 6H), 0.96 (dd, *J* = 10.8, 6.6 Hz, 6H), 0.88 (t, *J* = 6.6 Hz, 1H). ^{13}C NMR (151 MHz, Acetone-d6) δ ppm: 209.96, 204.48 , 170.69, 169.18, 168.00, 165.15, 155.70, 154.72, 153.59, 143.60, 140.59, 138.83, 137.22, 137.10, 135.99, 132.56, 129.80, 129.74, 129.34, 128.72, 128.05, 127.88, 127.47, 127.01, 126.61, 126.42, 126.40, 124.62, 119.28, 118.86, 83.58, 80.24, 78.15, 77.27, 77.13, 75.30, 74.52, 73.67, 73.55, 71.43, 70.85, 70.74, 69.12, 65.66, 57.04, 54.34, 53.71, 46.55, 45.95, 42.63, 40.49, 36.10, 35.08, 31.10, 30.47, 30.16, 29.08, 28.8, 27.00, 25.50, 23.97, 21.94, 21.79, 21.62, 20.43, 19.88. ESI-MS: m/z 1292.4 $[M+H]^+$, 1314.2 $[M+Na]^+$;

$C_{72}H_{81}N_3O_{19}$: HRMS calcd. 1314.5356 $[M+Na]^+$, found 1314.5366.

Fmoc-Leu-PABC-4FDT (**4b**). Light yellow powder. Yield: 54%. ^{1}H NMR (400 MHz, Acetone-d6) δ ppm: 9.46 (s, 1H), 7.95 (d, *J* = 7.6 Hz, 1H), 7.86 (d, *J* = 7.6 Hz, 2H), 7.79 (d, *J* = 9.6 Hz, 1H), 7.75-7.67 (m, 5H), 7.67-7.61 (m, 1H), 7.56-7.47 (m, 3H), 7.47-7.35 (m, 7H), 7.34-7.25 (m, 3H), 6.84 (d, *J* = 8.0 Hz, 1H), 6.10 (t, *J* = 9.2 Hz, 1H), 5.66 (d, *J* = 6.8 Hz, 1H), 5.40-5.34 (m, 1H), 5.32 (d, *J* = 5.6 Hz, 1H), 5.24 (brs, 1H), 5.20 (d, *J* = 12.0 Hz, 1H), 5.12 (d, *J* = 12.0 Hz, 1H), 4.98 (d, *J* = 9.2 Hz, 1H), 4.41-4.28 (m, 7H), 4.24 (dd, *J* = 6.8 Hz, 8.0 Hz, 1H), 4.20-4.12 (m, 2H), 3.92 (d, *J* = 7.2 Hz, 1H), 3.79 (brs, 1H), 2.52-2.42 (m, 5H), 2.26 (dd, *J* = 15.6, 8.6 Hz, 2H), 2.02-1.95 (m, 1H), 1.90-1.75 (m, 7H), 1.73-1.63 (m, 6H), 1.62-1.50 (m, 7H), 1.44-1.34 (m, 1H), 1.34-1.24 (m, 5H), 1.17 (brs, 4H), 1.00-0.84 (m, 7H). ^{13}C NMR (151 MHz, Acetone-d6) δ ppm: 209.83, 170.74, 169.13, 167.96, 163.95, 161.90 (d, *J* = 245.4 Hz), 155.72, 153.52, 153.07, 143.60, 143.45, 140.59, 138.86, 137.00, 136.51, 135.99, 132.11 (d, *J* = 7.4 Hz), 130.07 (*J* = 7.7 Hz), 129.69, 128.77, 128.18, 127.76, 127.01, 126.68, 126.42, 126.40, 125.35, 124.63, 124.62 (q, *J* = 282.1 Hz), 119.56 (d, *J* = 21.4 Hz), 119.28, 118.89, 115.74 (d, *J* = 23.1 Hz), 83.57, 80.23, 77.13, 77.06, 75.19, 75.05, 73.64, 71.39, 70.81, 69.22, 65.68, 57.01, 54.81, 53.72, 46.55, 45.90, 42.60, 40.49, 36.05, 35.13, 25.46, 23.97, 21.94, 21.57, 20.44, 19.89, 18.30, 12.91, 8.82. ESI-MS: m/z 1364.2 $[M+H]^+$, 1386.2 $[M+Na]^+$; $C_{72}H_{77}F_4N_3O_{19}$: HRMS calcd. 1386.4980 $[M+Na]^+$, found 1386.5001.

4.2.3. General procedure for the synthesis of **5a** and **5b**.

To solution of compound **4a** or **4b** (0.5 mmol) in anhydrous DMF (1 mL) was added piperidine (5.00 equiv) at 0 ^{0}C. The mixture reaction was stirred at room temperature for half an hour. After removed DMF, the residue was diluted with DCM (20 mL) and washed with brine (20 mL). The organic layer was concentrated next and dried in vacuo to give crude **5a** or **5b** in 80%-90% purity which was used directly for the step without further purification.

4.2.4. General procedure for the synthesis of peptide-prodrugs (**6a-10a**, **6b-10b**).

To a solution of octapeptide (A1-A5) (0.04 mmol), **5a** or **5b** (0.04 mmol) and 1-hydroxybenzotriazole (HOBt) (0.12 mmol) in anhydrous DMF (4 mL), 4-methylmorpholine (0.16 mmol) was added dropwise under N_2 at 0 ^{0}C. After the reaction mixture stirred at 0 ^{0}C for 15 min, N,N'-diisopropylcarbodiimide (DIC) (0.24 mmol) was added and the mixture was stirred at 5 ^{0}C for about 72 h to fully generated peptide-prodrugs (**6a-10a**, **6b-10b**). Subsequently, DMF was removed by vacuum pump and the crude product was purified by reverse-phase preparative HPLC chromatography with acetonitrile/water (V/V = 0% ~ 100% within 50 min, 1 mL/min) as mobile phase to afford **6a-10a** and **6b-10b**. Purity analysis and reaction monitoring of peptide-prodrugs were performed using HPLC with the UV detector set at 254 nm, acetonitrile and water (V/V = 0 ~ 100% within 25 min) were used as mobile phase at a flow-rate of 0.5 mL/min.

DTX-PABC-Leu-A1 (**6a**)**.** Colorless powder. Yield: 17%. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 9.95 (s, 1H), 8.16-8.05 (m, 4H), 7.99 (d, J = 8 Hz, 1H), 7.94 (d, J = 6.4 Hz, 3H), 7.90 (d, J = 8 Hz, 2H), 7.85 (d, J = 7.6 Hz, 2H), 7.71 (d, J = 7.6 Hz, 3H), 7.67-7.63 (m, 3H), 7.43-7.39 (m, 6H), 7.37-7.28 (m, 7H), 7.16 (t, J = 7.2 Hz, 1H), 6.82 (brs, 1H), 5.77 (dd, J = 8.0 Hz, 12.0 Hz, 1H), 5.39 (d, J = 7.2 Hz, 1H), 5.14 (brs, 2H), 5.11-5.06 (m, 3H), 5.03 (d, J = 7.2 Hz, 2H), 4.97 (brs, 1H), 4.90 (d, J = 10.4 Hz, 1H), 4.46-4.39 (m, 3H), 4.31 (d, J = 8.0 Hz, 1H), 4.26-4.19 (m, 7H), 4.03-3.97 (m, 3H), 3.80-3.70 (m, 5H), 3.64 (d, J = 6.8 Hz, 2H), 3.51-3.43 (m, 1H), 2.27-2.21 (m, 5H), 2.15-2.08 (m, 2H), 1.93-1.83 (m, 4H), 1.72 (brs, 3H), 1.65-1.59 (m, 4H), 1.51 (brs, 6H), 1.33 (s, 9H), 1.26-1.19 (m, 6H), 0.96 (brs, 6H), 0.91 (d, J = 6.0 Hz, 3H), 0.88-0.84 (m, 6H), 0.83- 0.74 (m, 12H). ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm: 209.14, 173.76, 172.48, 172.22, 171.46, 171.32, 171.11, 171.00, 170.69, 169.42, 168.81, 168.73, 168.61, 167.47, 165.13, 156.35, 156.24, 154.94, 153.62, 143.69, 140.54, 139.08, 137.04, 136.81, 135.64, 133.24, 129.88, 129.54, 129.40, 129.03, 128.49, 127.97, 127.45, 127.20, 126.91, 125.09, 119.93, 83.61, 80.14, 78.36, 77.51, 76.61, 75.23, 74.63, 73.57, 71.36, 70.57, 69.39, 65.60, 65.54, 59.52, 58.37, 56.83, 56.60, 56.47, 54.99, 52.21, 51.89, 50.99, 50.88, 48.35, 48.30, 46.45, 45.81, 45.74,

42.71, 42.62, 42.08, 41.89, 40.58, 40.39, 40.34, 36.59, 36.48, 36.31, 34.50, 31.71, 31.27, 28.87, 27.93, 27.76, 26.28, 24.16, 24.10, 24.04, 23.99 22.90, 22.83, 22.29,21.89, 21.34, 21.04, 20.57, 17.54, 17.50, 15.09, 13.49, 10.86, 9.61. ESI-MS: m/z 1015.4 $[M/2+Na]^+$; $C_{103}H_{132}N_{12}O_{28}$: HRMS calcd. 2007.9166 $[M+Na]^+$, found 2007.9246.

DTX-PABC-Leu-A1 (**6b**). Colorless powder. Yield: 22%. ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 10.00 (s, 1H), 8.51 (d, *J* = 8.0 Hz, 1H), 8.28–8.07 (m, 4H), 8.05–7.93 (m, 3H), 7.90 (d, *J* = 8.0 Hz, 2H), 7.82 (d, *J* = 8.0 Hz, 2H), 7.75-7.69 (m, 3H), 7.67-7.60 (m, 2H), 7.49-7.37 (m, 7H), 7.35-7.27 (m, 5H), 7.19 (t, *J* = 8.0, 4.0 Hz, 1H), 6.82 (brs, 1H), 5.78 (dd, *J* = 8.0 Hz, 12.0 Hz, 1H), 5.38 (d, *J* = 4.0 Hz, 1H), 5.22-5.08 (m, 4H), 5.06-4.96 (m, 3H), 4.91 (d, *J* = 8.0 Hz, 1H), 4.54 (brs, 1H), 4.41 (brs, 1H), 4.34-4.17 (m, 7H), 4.16-4.08 (m, 6H), 4.07-3.95 (m, 3H), 3.90-3.49 (m, 5H), 3.19-3.15 (m, 14H), 2.89 (brs, 3H), 2.73 (brs, 3H), 2.24 (brs, 4H), 1.72 (brs, 5H), 1.57-1.49 (m, 10H), 1.23 (s, 5H), 0.98 (s, 5H), 0.91 (d, J = 8.0 Hz , 3H), 0.87 (brs, 5H), 0.80 (d, *J* = 8.0 Hz, 6H). ^{13}C NMR (151 MHz, DMSO-d6) δ ppm: 209.01, 173.77, 172.48, 172.23, 171.47, 171.11, 171.01, 170.70, 169.32, 168.80, 168.61, 167.47, 167.20, 163.95, 162.15, 160.99, 156.35, 153.56, 153.18, 143.69, 140.54, 139.09, 136.82, 136.36, 135.53, 132.16, 132.12, 130.89, 129.49, 129.02, 128.55, 128.22, 127.45, 127.20, 126.91, 125.60, 125.09, 120.29, 119.93, 119.06, 115.84, 83.57, 80.14, 78.52, 78.33, 77.26, 76.59, 75.15, 73.54, 71.34, 70.55, 69.48, 65.60, 65.54, 59.51, 56.80, 56.60, 56.46, 55.33, 52.20, 51.88, 50.98, 50.88, 48.43, 46.45, 45.76, 42.68, 42.62, 42.07, 40.58, 40.40, 40.33, 36.48, 36.27, 35.61, 34.49, 31.26, 30.62, 28.85, 27.75, 26.28, 24.15, 23.98, 22.89, 22.82, 22.24, 21.88, 21.33, 21.03, 20.56, 19.16, 19.11, 17.53, 17.49, 15.08, 13.46, 10.85, 9.58. ESI-MS: m/z 1029.4 $[M/2+H]^+$; $C_{103}H_{128}F4N_{12}O_{28}$: HRMS calcd. 2079.8789 $[M+Na]^+$, found 2079.8879.

DTX-PABC-Leu-A2 (**7a**). Colorless powder. Yield: 21%. ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 9.99 (s, 1H), 8.19 (d, *J* = 7.9 Hz, 1H), 8.13-7.09 (m, 3H), 8.05-7.92 (m, 6H), 7.86-7.79 (m, 1H), 7.75-7.69 (m, 1H), 7.65 (d, *J* = 7.2 Hz, 4H), 7.41 (t, *J* = 7.2 Hz, 2H), 7.38-7.31 (m, 4H), 7.29 (brs, 1H), 7.17 (t, *J* = 7.2 Hz 1H),

6.82 (brs, 1H), 5.78 (dd, *J* = 8.0 Hz, 12.0 Hz, 1H), 5.40 (d, *J* = 6.9 Hz, 1H), 5.15 (brs, 2H), 5.08 (d, *J* = 8.3 Hz, 2H), 5.03 (d, *J* = 7.3 Hz, 2H), 4.96 (s, 1H), 4.90 (d, *J* = 9.2 Hz, 1H), 4.5-4.37 (m, 3H), 4.33-4.17 (m, 5H), 4.12-4.96 (m, 4H), 3.95-3.89 (m, 1H), 3.87-3.78 (m, 2H), 3.78-3.67 (m, 4H), 3.64 (d, *J* = 6.4 Hz, 1H), 3.53 (brs, 1H), 2.24 (brs, 5H), 2.15-2.08 (m, 2H), 2.03 (brs, 4H), 1.86 (brs, 5H), 1.84 (brs, 2H), 1.72 (brs, 4H), 1.51 (brs, 6H), 1.34 (brs, 10H), 1.23 (d, *J* = 6.0 Hz, 6H), 0.98 (brs, 7H), 0.93-0.85 (m, 7H), 0.85-0.76 (m, 7H). ^{13}C NMR (151 MHz, DMSO-d6) δ ppm: 209.13 , 173.76, 172.47, 171.60, 171.26, 171.11, 170.70, 169.42, 169.37, 169.17, 168.81, 168.72, 168.54, 168.44, 167.42, 167.15, 165.14, 154.94, 153.62, 139.07, 137.04, 136.81, 135.64, 133.25, 129.88, 129.55,129.40, 129.04, 128.49, 127.97, 127.20, 119.06, 83.61, 80.14, 78.36, 77.51, 76.62, 75.23, 74.63, 73.57, 71.36, 70.57, 69.40, 59.59, 58.54, 56.83, 56.61, 54.99, 52.21, 51.88, 51.76, 48.35, 46.58, 45.88, 45.81, 42.72, 42.07, 41.22, 40.40, 36.49, 31.43, 31.27, 29.47, 28.92, 27.76, 26.29, 24.22, 24.17, 24.06, 22.84, 22.30, 22.18, 21.19, 21.35, 20.57, 17.52, 15.10, 14.42, 13.49, 10.88, 9.61. ESI-MS: m/z 1843.8 $[M+Na]^+$; $C_{89}H_{122}N_{12}O_{27}S$: HRMS calcd. 1845.8155 $[M+Na]^+$, found 1845.8219.

DTX-PABC-Leu-A2 (**7b**). Colorless powder. Yield: 23%. ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 10.00 (s, 1H), 8.52 (d, *J* = 8.0Hz, 1H), 8.11 (d, *J* = 8.0 Hz, 2H), 8.06-7.93 (m, 3H), 7.82 (dd, *J* = 8.0, 4.0 Hz, 2H), 7.78-7.61 (m, 5H), 7.47-7.39 (m, 3H), 7.37-7.29 (m, 4H), 7.18 (t, *J* = 7.0 Hz, 1H), 6.82 (brs, 1H), 5.78 (dd, *J* = 8.0 Hz, 12.0 Hz, 1H), 5.38 (d, *J* = 8.0 Hz, 1H), 5.35-5.29 (m, 1H), 5.21-5.07 (m, 4H), 5.05 (d, *J* = 8.0Hz, 1H), 5.01 (brs, 2H), 4.92 (d, *J* = 8.0 Hz, 1H), 4.54 (brs, 1H), 4.51-3.66 (m, 13H), 3.66-3.38 (m, 1H), 3.29-2.96 (m, 4H), 2.89 (brs, 1H), 2.73 (brs, 1H), 2.24 (brs, 3H), 2.15-2.08 (m, 2H), 2.03 (brs, 4H), 1.89-1.82 (m, 5H), 1.79-1.62 (m, 9H), 1.55 (brs, 3H), 1.54-1.48 (m, 7H), 1.23 (brs, 9H), 0.98 (brs, 6H), 0.91 (d, *J* = 8.0 Hz, 3H), 0.89-0.85 (m, 4H), 0.83-0.75 (m, 7H). ^{13}C NMR (151 MHz, DMSO-d6) δ ppm: 209.10, 174.14, 173.77, 172.48, 171.61, 171.52, 171.23, 170.71, 170.65, 169.39, 169.33, 169.18, 168.81, 168.61, 168.55, 167.42, 167.16, 163.96, 162.61, 160.99, 153.56, 153.18, 139.09, 136.82, 136.36, 135.53, 132.16, 130.89, 129.49, 129.03,

128.55, 127.21, 125.61, 120.43, 120.29, 119.07, 115.84, 115.69, 83.53, 80.14, 77.26, 76.59, 75.15, 73.55, 71.34, 70.55, 69.48, 59.59, 56.80, 56.61, 52.20, 51.87, 51.75, 48.35, 45.88, 45.77, 42.68, 41.86, 31.42, 31.26, 29.46, 28.91, 28.84, 28.65, 27.75, 26.29, 24.21, 24.15, 24.05, 22.82, 22.24, 22.17, 21.34, 20.56, 19.16, 19.12, 17.50, 15.09, 14.41, 13.46, 10.86, 9.58. ESI-MS: m/z 948.6 $[M/2+H]^+$; $C_{89}H_{118}F_4N_{12}O_{27}S$: HRMS calcd. 1917.7778 $[M+Na]^+$, found 1917.7831.

DTX-PABC-Leu-A3 (**8a**). Colorless powder. Yield: 18%. ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 9.97 (s, 1H), 8.22-8.10 (m, 4H), 8.07-7.95 (m, 6H), 7.79-7.61 (m, 7H), 7.47-7.27 (m, 8H), 6.81 (brs, 2H), 5.86-5.72 (m, 1H), 5.45-5.36 (m, 1H), 5.17-5.01 (m, 6H), 5.00-4.88 (m, 2H), 4.44 (brs, 2H), 4.26 (s, 4H), 4.18-4.11 (m, 2H), 4.07-3.98 (m, 3H), 3.90 (brs, 2H), 3.74 (brs, 3H), 3.58-3.47 (m,2H), 2.29-2.20(m, 4H), 2.17-2.06 (m, 5H), 1.91-1.79 (m, 8H), 1.76-1.68 (m, 6H), 1.67-1.61 (m, 3H), 1.52 (s, 5H), 1.33 (brs, 9H), 1.24 (brs, 9H), 1.01-0.95 (m, 6H), 0.92-0.87 (m, 6H), 0.83-0.77 (m, 6H). ^{13}C NMR (151 MHz, DMSO-d6) δ ppm: 209.13, 173.89, 173.75，173.53, 173.49, 172.56, 172.41, 172.07, 171.90, 171.71, 171.37, 171.13, 170.99, 170.66, 170.62, 170.76, 169.40, 168.88, 168.73, 167.68, 167.03, 165.13, 154.94, 153.62, 139.07, 137.04, 136.81, 135.64, 133.24, 129.88, 129.55, 129.40, 129.03, 128.49, 127.97, 127.20, 119.06, 83.61, 80.14, 78.36, 77.51, 76.62, 75.23, 74.63, 73.57, 71.36, 70.57, 69.40, 59.86, 58.53, 56.77, 54.99, 52.44, 52.31, 51.90, 48.46, 48.29, 47.90, 45.89, 45.81, 42.72, 42.12, 41.26, 40.38, 40.29, 36.31, 36.25, 34.50, 31.29, 28.88, 27.94, 27.68, 27.02, 26.29, 24.23, 24.15, 24.10, 22.85, 22.30, 22.19, 21.33, 20.57, 17.89, 17.69, 17.54, 15.13, 13.49, 10.85, 9.61. ESI-MS: m/z 940.2 [M/2+Na]+; $C_{90}H_{123}N_{13}O_{28}$: HRMS calcd. 1856.8493 [M+Na]+, found 1856.8486.

DTX-PABC-Leu-A3 (**8b**). Colorless powder. Yield: 21%. ^{1}H NMR (400 MHz, DMSO-d6, rotamers) δ ppm: 9.97 (d, J = 12.0 Hz, 1H), 8.52 (d, J = 12.0 Hz, 1H), 8.23-7.97 (m, 12.1 Hz, 7H), 7.82 (d, J = 8.0 Hz, 2H), 7.76 (d, J = 8.0 Hz, 1H), 7.74-7.68 (m, 2H), 7.65 (d, J = 8.0 Hz, 2H), 7.45-7.38 (m, 3H), 7.37-7.25 (m, 5H), 7.18 (d, J = 4.0 Hz, 1H), 6.81 (d, J = 8.0 Hz, 2H), 5.77 (dd, J = 8.0, 12.0 Hz, 1H), 5.38 (d, J = 8.0 Hz, 1H), 5.22-4.95 (m, 7H), 4.91 (d, J = 8.0 Hz, 1H), 4.54 (brs, 1H),

4.40 (brs, 1H), 4.34-4.18 (m, 4H), 4.12 (brs, 7H), 4.01 (brs, 2H), 3.90 (brs, 1H), 3.73 (brs, 1H), 3.66-3.46 (m, 1H), 3.19-3.16 (m, 5H), 2.88 (brs, 1H), 2.72 (brs, 1H), 2.34-2.17 (m, 5H), 2.16-2.05 (m, 5H), 1.91-1.82 (m, 6H), 1.76-1.69 (m, 6H), 1.56-1.48 (m, 10H), 1.28-1.15 (m, 7H), 1.01-0.95 (m, 5H), 0.93-0.84 (m, 6H), 0.84-0.73 (m, 7H). ^{13}C NMR (151 MHz, DMSO-d6) δ ppm: 209.02, 173.91, 173.77, 173.46, 173.07, 172.57, 172.08, 171.91, 171.72, 171.39, 171.14, 171.00, 170.93, 170.77, 169.42, 169.33, 168.88, 168.61, 167.69, 163.96, 162.62, 160.99, 153.56, 153.18, 139.08, 136.82, 136.36, 135.53, 132.16, 130,85, 129.49, 129.02, 128.55, 128.22, 127.21, 125.60, 120.43, 119.07, 83.58, 80.14, 77.26, 76.59, 75.12, 73.55, 71.34, 70.55, 69.48, 59.86, 56.80, 55.33, 52.44, 52.30, 51.89, 48.43, 48.29, 45.89, 45.77, 42.68, 42.11, 41.25, 36.24, 31.34, 31.28, 28.87, 27.67, 27.01, 26.28, 24.22, 24.14, 24.09, 22.83, 22.24, 22.17, 21.31, 20.65, 19.16, 19.12, 17.67, 17.52, 15.12, 13.46, 10.83, 9.58. ESI-MS: m/z 954.0 $[M/2+H]^+$; $C_{90}H_{119}F_4N_{13}O_{28}$: HRMS calcd. 1928.8116$[M+Na]^+$, found 1928.8183.

DTX-PABC-Leu-A4 (**9a**). Colorless powder. Yield: 24%. ^{1}H NMR (400 MHz, DMSO-d_6, rotamers) δ ppm: 10.03 (s, 1H), 8.29-8.15 (m, 3H), 8.12-7.91 (m, 8H), 7.84-7.61 (m, 6H), 7.47-7.16 (m, 8H), 6.80 (s, 2H), 5.90-5.68 (m, 1H), 5.40 (d, J = 4.0 Hz, 1H), 5.21-4.84 (m, 8H), 4.55-4.37 (m, 3H), 4.35-4.14 (m, 6H), 4.07-3.96 (m, 3H), 3.89-3.54 (m, 8H), 2.29-2.19 (m, 4H), 2.18-2.07 (m, 5H), 2.06-2.00 (m, 3H), 1.95-1.82 (m, 9H), 1.77-1.63 (m, 9H), 1.56-1.45 (m, 6H), 1.34 (brs, 9H), 1.27-1.16 (m, 6H), 1.03-0.95 (m, 6H), 0.93-0.85(m, 6H), 0.83-0.75 (m, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm: 209.13, 173.84, 173.64, 173.45, 172.28, 172.10, 171.66, 171.51, 171.44, 171.32, 171.08, 171.02, 170.75, 170.57, 169.51, 169.42, 168.73, 168.62, 168.33, 167.66, 167.04, 165.13, 154.94, 153.62, 139.09, 137.04, 136.81, 135.64, 133.24, 129.88, 129.55，129.39, 129.08, 128.49, 129.04, 128.49, 127.97, 127.20, 119.05, 83.61, 80.14, 78.36, 77.51, 76.62, 75.23, 74.63, 73.57, 71.36, 70.57, 69.40, 59.82, 58.56, 56.83, 56.70, 54.99, 52.52, 52.30, 52.03, 51.82, 48.33, 46.56, 45.88, 45.81, 42.72, 42.00, 41.31, 40.53, 36.49, 36.31, 34.50, 31.69, 31.29, 29.20, 28.81, 27.94, 27.48, 27.16, 26.29, 24.26, 24.17, 24.10, 22.88, 22.30, 22.19, 21.90, 21.27,

20.57, 17.42, 17.31, 15.05, 14.40, 13.49, 10.86, 9.61. ESI-MS: m/z 969.8 $[M/2+Na]^+$; $C_{92}H_{127}N_{13}O_{28}S$: HRMS calcd. 1916.8526 $[M+Na]^+$, found 1916.8535.

DTX-PABC-Leu-A4 (**9b**). Colorless powder. Yield: 25%. ^{1}H NMR (400 MHz, DMSO-d_6, rotamers) δ ppm: 10.01 (d, J = 20.8 Hz, 1H), 8.47 (d, J = 8.4 Hz, 1H), 8.14 (d, J =6.4 Hz, 2H), 8.08-7.98 (m, 3H), 7.95-7.91 (m, 2H), 7.78 (q, J = 16.4, 12.4 Hz, 2H), 7.71-7.66 (m, 1H), 7.66-7.57 (m, 4H), 7.37 (t, J = 7.6 Hz, 2H), 7.32 (d, J = 7.6 Hz, 2H), 7.29 (d, J = 8.6 Hz, 2H), 7.24 (s, 2H), 7.14 (t, J = 8.0 Hz, 1H), 6.76 (brs, 2H), 5.74 (dd, J = 8.0 Hz, 12.0 Hz, 1H), 5.34 (d, J = 8.0 Hz, 1H), 5.17-5.07 (m, 4H), 5.04 (d, J = 8.0 Hz, 1H), 5.01-4.96 (m, 2H), 4.87 (d, J = 8.0 Hz, 1H), 4.43-4.34 (m, 1H), 4.39 (s, 1H), 4.32-4.11 (m, 6H), 4.07-3.97 (m, 3H), 3.86 (dd, J = 16.0, 8.0 Hz, 1H), 3.80-3.64 (m, 2H), 3.62-3.42 (m, 2H), 3.34 (brs, 1H), 2.85 (brs, 3H), 2.69 (brs, 3H), 2.43 (brs, 2H), 2.20 (brs, 3H), 2.16-2.03 (m, 4H), 1.98 (s, 3H), 1.95-1.88 (m, 3H), 1.87-1.84 (m, 4H), 1.76-1.69 (m, 4H), 1.68 (brs, 3H), 1.65-1.57 (m, 4H), 1.55 (brs, 3H), 1.52-1.48 (m, 6H), 1.21-1.14 (m, 6H), 0.94 (s, 6H), 0.87 (d, J = 6.2 Hz, 3H), 0.83 (d, J = 6.2 Hz, 3H), 0.83-0.76 (m, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ ppm: 209.00, 173.84, 173.65, 173.46, 172.28, 171.66, 171.51, 171.07, 171.01, 170.74, 169.52, 169.32, 168.60, 167.66, 163.94, 162.15, 160.98, 153.55, 139.09, 136.81, 136.36, 135.52, 132.16, 132.11, 130.89, 130.83, 129.48, 129.02, 128.54, 128.21, 127.20, 125.60, 120.42, 120.28, 119.05, 115.69, 83.58, 80.14, 77.26, 76.59, 75.15, 73.54, 71.34, 70.55, 69.48, 59.82, 58.55, 56.80, 55.33, 52.51, 52.35, 52.28, 52.02, 51.81, 48.32, 45.88, 45.77, 42.68, 41.99, 41.30, 40.53, 36.49, 36.28, 35.61, 34.50, 31.69, 31.64, 31.28, 30.61, 29.19, 28.80, 28.65, 28.51, 28.40, 27.58, 27.54, 27.47, 27.15, 26.38, 26.29, 24.26, 24.17, 24.09, 22.87, 22.24, 22.18, 21.89, 21.26, 20.56, 19.17, 19.12, 17.41, 17.30, 15.04, 14.39, 13.47, 10.84, 9.58. ESI-MS: m/z 984.2 $[M/2+H]^+$; $C_{92}H_{123}F_4N_{13}O_{28}S$: HRMS calcd. 1988.8150 $[M+Na]^+$, found 1988.8169.

DTX-PABC-Leu-A5 (**10a**). Colorless powder. Yield: 24%. ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 9.96 (s, 1H), 8.14 (d, J = 28.0Hz, 5), 8.03 (s, 1H), 7.95 (d, J = 21.6 Hz, 5H), 7.68 (dd, J =24.4, 6.4 Hz, 5H), 7.41 (d, J =6.0 Hz, 2H), 7.31 (d, J = 26.4 Hz, 6H), 7.17 (brs, 1H), 6.81 (d, J = 14.8 Hz, 2H), 5.78 (dd, J = 8.0 Hz, 12.0 Hz, 1H),

5.39 (d, *J* = 6.0 Hz, 1H), 5.15 (brs, 3H), 5.09-4.98 (m, 3H), 4.96 (brs, 1H), 4.90 (d, *J* = 9.2 Hz, 1H), 4.47-4.37 (m, 2H), 4.35-4.12 (m, 5H), 4.10-3.89 (m, 4H), 3.88-3.77 (m, 1H), 3.72 (brs, 3H), 3.67-3.60 (m, 1H), 3.59-3.46 (m, 2H), 3.28 (brs, 1H), 2.24 (brs, 3H), 2.15-2.02 (m, 5H), 1.94-1.88 (m, 2H), 1.86 (brs, 3H), 1.85-1.73 (m, 3H), 1.68 -1.53 (m, 6H), 1.51 (brs, 3H), 1.47-1.42 (m, 2H), 1.33 (brs, 9H), 1.25-1.19 (m, 4H), 0.98 (brs, 6H), 0.92-0.82 (m, 13H). ^{13}C NMR (151 MHz, DMSO-d6) δ ppm: 209.14 , 173.89, 173.82, 173.79, 173.52, 172.63, 171.97, 171.89, 171.67, 171.51, 171.44, 171.35, 171.14, 171.01, 169.52, 169.44, 168.92, 168.74, 168.64, 168.48, 167.69, 167.06, 165.15, 154.95, 153.63, 139.07, 137.04, 136.81, 135.6, 133.26, 129.88, 129.55, 129.40, 129.04, 128.50, 127.98, 127.20, 119.07, 83.62, 80.14, 78.38, 77.52, 76.62, 75.23, 74.63, 73.58, 71.37, 70.58, 69.41, 59.79, 58.58, 56.83, 54.99, 54.48, 52.27, 51.09, 50.79, 48.49, 46.56, 45.89, 45.81, 42.72, 42.13, 41.92, 41.30, 40.38, 36.31, 34.50, 31.29, 28.79, 27.94, 27.68, 27.54, 27.13, 26.29, 24.25, 24.16, 23.90, 22.92, 22.85, 22.30, 22.18, 21.90, 21.33, 20.57,17.50, 17.45, 13.49, 9.61. ESI-MS: m/z 1842.8 $[M+Na]^+$; $C_{89}H_{121}N_{13}O_{28}$: HRMS calcd. 1842.8336 $[M+Na]^+$, found 1842.8347.

DTX-PABC-Leu-A5 (**10b**). Colorless powder. Yield: 22%. ^{1}H NMR (400 MHz, DMSO-d6) δ ppm: 9.97 (s, 1H), 8.52 (d, *J* = 8 Hz, 1H), 8.17 (d, *J* = 8.0 Hz, 2H), 8.11 (dd, *J* = 6.0, 4.0 Hz, 3H), 8.07-8.01 (m, 1H), 7.97 (d, *J* = 8.0 Hz, 1H), 7.91 (dd, *J* = 8.0, 4.0 Hz, 1H), 7.82 (d, *J* = 8.0 Hz, 1H), 7.78-7.58 (m, 5H), 7.43-7.28 (m, 9H), 7.18 (t, *J* = 8.0 Hz, 1H), 6.81 (d, *J* = 16 Hz, 2H), 5.78 (dd, *J* = 8.0 Hz, 12.0 Hz, 1H), 5.38 (d, *J* = 8.0 Hz, 1H), 5.21-5.08 (m, 4H), 5.07-4.96 (m, 3H), 4.92 (d, *J* = 8.0 Hz, 1H), 4.54 (s, 1H), 4.51-3.85 (m, 10H), 3.84-3.68 (m, 4H), 3.64 (d, *J* = 8.0 Hz, 1H), 3.60-3.35 (m, 2H), 3.34 (brs,1H), 2.36-2.19 (m, 4H), 2.17-1.99 (m, 5H), 1.98-1.82 (m, 7H), 1.80-1.57 (m, 10H), 1.55 (brs, 3H), 1.54-1.48 (m, 7H), 1.48-1.31 (m, 4H), 1.26-1.18 (m, 3H), 0.98 (brs, 5H), 0.93-0.89 (m, 3H), 0.89-0.85(m, 6H), 0.94-0.77 (m, 4H). ^{13}C NMR (151 MHz, DMSO-d6) δ ppm: 209.01, 173.87, 173.70, 173.49, 172.60, 171.87, 171.64, 171.49, 171.12, 171.05, 170.96, 169.48, 169.33, 168.89, 168.61, 167.67, 167.05, 163.95, 162.61, 160.99, 153.56, 153.18, 139.09, 136.82, 136.37,

135.53, 132.17, 130.89, 129.48, 129.03, 128.55, 128.22, 127.21, 125.61, 124.02, 120.43, 120.29, 119.06, 115.84, 115.69, 83.58, 80.14, 78.52, 78.33, 77.26, 76.59, 75.15, 73.55, 71.34, 70.55, 69.48, 59.78, 56.80, 55.33, 52.47, 51.88, 48.47, 45.88, 45.77, 42.68, 41.29, 36.28, 34.50, 31.63, 31.28, 28,78, 27.69, 27.54, 27.13, 26.29, 24.25, 24.15, 23.89, 22.92, 22.83, 22.24, 22.18, 21.89, 21.33, 20.56, 19.16, 19.12, 17.46, 13.47, 9.58. ESI-MS: m/z 947.2 $[M/2+H]^+$; $C_{89}H_{117}F_4N_{13}O_{28}$: HRMS calcd. 1914.7959 $[M+Na]^+$, found 1914.7963.

4.3. Biological evaluation procedures

4.3.1. Cell lines and cultures

HCT116 (human colon carcinoma cell line), SW620 (human colon carcinoma cell line), CCD18Co (human colon cell line) and HEK293 (human embryonic kidney 293) were purchased from American Type Culture Collection (ATCC) by commercial suppliers. DMEM and McCoy's 5A were obtained from NanJing KeyGen Biotech Co., Ltd. Fetal bovine serum (FBS) and Trypsine-EDTA (0.25%) were purchased from Gibco (Invitrogen, USA). All these cell lines were cultured as recommended: SW620 and HEK293 were cultured in DMEM with 10% fetal bovine serum, CCD18Co was cultured in DMEM with 15% FBS, HCT116 was cultured in McCoy's 5A with 10% FBS. All cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2.

4.3.2. Cell proliferation assay

Cell proliferation assays were conducted to measure and compare the cytotoxicity and selectivity of these prodrugs and their parent drug DTX or 4FDT. Cells were seeded onto a 96-well plate at a density of 5000 cells per well and precultured for 1 day. Stock solutions (0.1 mol/L) of test compounds were prepared freshly by dissolving in DMSO. An increased concentration (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7},10^{-6}, 10^{-5} and 10^{-4} mol/L) and a control (blank) were applied to the cells in sixplicate for 48h (HCT116 and HEK293) or 72 h (SW620 and CCD18Co) at 37 ^{o}C in 5% CO_2 atmosphere. Cell cytotoxicity was evaluated by MTT assay. MTT solution (25 μL, 5 mg/mL in PBS) was added to the culture media. After incubating at 37 ^{o}C in the dark

for 4 h, and the absorbance (OD value) of each well at 570 nm was measured by a microplate reader (Thermo Scientific, Multiskan GO). The half maximal inhibitory concentration (IC_{50}) for each compound was estimated by SPSS, version 19.0.

4.3.3. Stability study in PBS and rat plasma

The stability of prodrugs was measured in PBS or rat plasma. Compound (50 μg/mL) was incubated in phosphate buffer (12.5 mM, pH 7.4) or freshly rat plasma at 37 ^{o}C for 72 h. At fixed time points (0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 10 h, 24 h, 48 h, 72 h), 200 μL of aliquot sample were taken. Then plasma sample was mixed with 600 μL cold acetonitrile and centrifuged at 12000 rpm for 4 min. The supernatant or the PBS sample was evaporated on a nitrogen stream and reconstituted with 100 μL acetonitrile. After filtered by a 0.22 μm filter, the clear fraction was analyzed by RP-HPLC (A = 0.1% TFA in water, B = acetonitrile; isocratic: 60% B; λ = 254 nm; flow rate = 0.6 mL/min; injection volume = 10μL). $T_{1/2}$ was calculated according $t_{1/2} = \ln(2) / K_{obs}$. Based on pseudo-first order behavior, the values of K_{obs} was obtained from the plots of logarithms of the concentration versus time using the expression: $\ln (C_t) = -K_{obs} * t + \ln(C_0)$.

4.3.4. Assay for release rate in vitro

Release rate in HCT116 cell cultured medium. HCT116 cells (1×10^5 cells/well) were seeded onto 96-well plate for 24 h before drug treatment. Fresh cell culture medium with or without prodrug (50 μg/mL) was added into well (100 μL per well) and incubated at 37 ^{o}C for 0, 2, 4, 6 and 8 h. Subsequently, the cell culture medium was collected and extracted with 10 mL of dichloromethane three times. The organic extracts were evaporated by vacuum evaporator and the dry extracts were redissolved into 100 μL of acetonitrile. After filtered by the 0.22 μm filter, the clear fraction was analyzed by RP-HPLC (method was same with the stability test) to test the concentration of prodrug and released DTX (or 4FDT).

Release rate in HCT116 tumor homogenate. Adult male BALB/c nude mice (20 ± 2 g) were purchased from the BK Lab Animal Ltd. (Shanghai, China) and fed under

standard housing conditions (Humidity: 40-70%, temperature: 20-24 °C, ventilation time: 10-20 times per hour, 12h light/dark cycle) with food and water ad libitum. 0.2 mL of PBS with HCT116 cells (1×10^7) was subcutaneously injected in the axilla of the BALB/c nude mice. The inoculated mice were maintained in SPF-grade animal experimental facility for about 2 weeks. Then fresh HCT116 tumor tissue was homogenized with saline and diluted to approximately 250 mg/mL. After centrifuged at 5000 r/min for 10 min at 5 ^{o}C, 2 mL of the upper homogenate solution was extracted and used to incubate prodrug (100 μg) at 37 ^{o}C. 200 μL of aliquot sample was taken at fixed time points (0 min, 5 min, 15 min, 30 min and 1 h). Acetonitrile (600 μL) was added to each sample to precipitate the protein and centrifuged at 12000 r/min for 10 min at 5 ^{o}C. The supernatant was evaporated on a nitrogen stream and redissolved with acetonitrile (100 μL). After filtered by the 0.22 μm filter, the concentrations of the prodrug and the released parent drug in the clear fraction were measured by HPLC. The RP-HPLC conditions were the same as stability assays. The animal experimental protocols were approved by the Animal Ethics Committee of School of Pharmacy, Fudan University (Shanghai, China).

4.3.5. In vivo efficacy and toxicity study

Adult male BALB/c nude mice (20 ± 2 g) were purchased from the BK Lab Animal Ltd. (Shanghai, China) and housed individually in a specific pathogen free facility. The mice were inoculated subcutaneously with HCT116 cells (2×10^6 suspended in 0.2 mL of PBS for each mouse). After reaching an average tumor volume of 100-200 mm^3, the animals were randomized into ten groups with six mice per group. 5-FU (5 mg/kg), DTX (5 mg/kg), 4FDT (5 mg/kg), **9a** high dose group (equivalent to DTX 10 mg/kg), **9a** medium dose group (equivalent to DTX 5 mg/kg), **9a** low dose group (equivalent to DTX 2.5 mg/kg), **9b** high dose group (equivalent to 4FDT 10 mg/kg), **9b** medium dose group (equivalent to 4FDT 5 mg/kg) and **9b** low dose group (equivalent to 4FDT 2.5 mg/kg) were freshly prepared using the formulation solvent containing DMSO (0.5%), castor oil (5%), ethanol (5%), and physiological saline (89.5%). All the solutions were injected intraperitoneally in a volume of 0.1 mL per

10 g of body weight and the vehicle control was injected with formulation solvent. Compound or vehicle were administrated once every 7 days. Tumor progression and weight of mice were monitored every other day or three days. The tumor volumes were estimated by measuring the two dimensions of the tumors using a caliper and calculated by the formula $V = L \times W^2 \times 0.52$, with V being volume, L being length, and W being width of the tumor nodules. On the 14th day of dosing, the mice were weighed and sacrificed, and their tumors were dissected out and weighed. The animal experimental protocols were approved by the Animal Ethics Committee of School of Pharmacy, Fudan University (Shanghai, China).

Histopathological study. At the end of the in vivo experiment, heart and liver tissues of vehicle and the same equivalent groups (5 mg/kg for the parent drugs and 5-FU, 11.73 mg/kg for **9a**, and 11.17 mg/kg for **9b**) were harvested and fixed in a 10% formalin solution in PBS. The process of paraffin embedding and H&E staining were commissioned to Shanghai Ricci biological science and Technology Co. Lt. Then, the gross morphological or cellular abnormalities of tissues were examined by two pathologists in a blinded fashion to analyze the damage of each compound. The animal experimental protocols were approved by the Animal Ethics Committee of School of Pharmacy, Fudan University (Shanghai, China).

4.3.6. Acute toxicity study

Healthy Kunming mice of both sexes, weighing 20 ± 2 g, were randomly divided into 5 groups of 10 animals matched for weight and size. Prodrug **9a** was freshly prepared and intraperitoneally administered at the dose of 400, 300, 250, 150 mg/kg body weight. All the solutions were injected in a volume of 0.1 mL per 10 g of body weight and the vehicle control was injected with formulation solvent. The death of mice was monitored daily and recorded up to 14 days after injection. The LD_{50} values were calculated using Bliss method. All procedures used in this experiment were compliant with the regulations of the Animal Ethics Committee of School of Pharmacy, Fudan University (Shanghai, China).

4.3.7. Pharmacokinetic study

Male Sprague Dawley (SD) rats, weighing 180-200 g, were purchased from BK Lab Animal Ltd. (Shanghai, China) and fed under standard housing conditions. The rats were starved with freely drank for 12 h prior to divide into two groups (n = 3). Tested compounds DTX (5 mg/kg) and **9a** (5 mg/kg) were freshly prepared with formulation solvent (0.5% DMSO, 5% castor oil, 5% ethanol, and 89.5% physiological saline) and intravenously administrated via the tail vein in a volume of 1 mL per 200 g of body weight to the SD mice respectively. Blood samples were drawn from the retroorbital venous at 0.083, 0.133, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48 and 72 h post-injection with 1% sodium heparin as anticoagulant. Subsequently, the plasma fractions were collected in eppendorf tubes to centrifuge at 5000 rpm for 10 min at 4 ^{o}C and stored at -20 ^{o}C for analysis. Then, mice were sacrificed under CO_2.

To prepare samples for analysis, methanol (80 μL) containing 100 ng/mL internal standard (paclitaxel) was added into plasma sample (20 μL) to denature the proteins. The mixture was vortexed for 10 s to fully extract the test compound and centrifuged at 12000 rpm for 10 min at 4 ^{o}C. Subsequently, the supernatant was mixed with an equal volume of deionized water and filtered through the 0.22 μm microporous membrane. The filtrate was analyzed on liquid chromatography-tandem mass spectrometry (AB Sciex API4000 Q-Trap LC-MS-MS system). Liquid chromatography use acetonitrile/water (V/V = 65:35) as mobile phase at a flow rate of 0.35 mL/min. Detection of the ions was conducted in the multiple reaction monitoring (MRM) mode, monitoring the transition of the m/z 830.3 / 549.1 for DTX $[M + Na]^+$, 1917.7 / 970.5 for **9a** $[M + Na]^+$ and 876.6 /308.0 for PTX $[M + Na]^+$ respectively. DAS software (version 2.0) was used to determine the pharmacokinetic parameters with non-compartmental model. The used animal protocol was reviewed and approved by the Animal Ethics Committee of School of Pharmacy, Fudan University (Shanghai, China).

4.3.8. In vivo distribution study

The human CRC cancer xenograft mouse models were established by the same method described in section 4.3.5. After reaching an average tumor volume of 200-300 mm^3, the animals were randomized into two groups (20 mice for each group). DTX (5 mg/kg) and **9a** (5 mg/kg) were freshly prepared and administrated via the tail vein in a volume of 0.1 mL per 10 g of body weight respectively. At 0.5, 4, 8 and 24 h of post-injection, five mice of each group were sacrificed and heart perfused with PBS. Subsequently, heart, liver, spleen, lung, kidney and tumor tissues were collected and weighted. The tissues were stored at -80 ^oC until analysis. After the organ samples were homogenized in a volume of 4 μL normal saline per 1 mg of tissue, the homogenate was added with 4 fold volumes of methanol containing paclitaxel (100 ng/mL) as internal standard to precipitated proteins and the supernatant following centrifugation was subjected to LC-MS/MS analysis as described in pharmacokinetics study.

Acknowledgments：

This work was supported by the National Natural Science Foundation of China (No: 81673297), Shanghai Municipal Committee of Science and Technology (No: 17431902500), National Postdoctoral Program for Innovative Talents (BX20180065), and China Postdoctoral Science Foundation (2019M651384).

References

[1] L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal, Global cancer statistics, 2012, CA Cancer J Clin, 65 (2015) 87-108.
[2] M. Arnold, M.S. Sierra, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global patterns and trends in colorectal cancer incidence and mortality, Gut, 66 (2017) 683-691.
[3] B.M. Wolpin, R.J. Mayer, Systemic treatment of colorectal cancer, Gastroenterology, 134 (2008) 1296-1310.
[4] T. Watanabe, M. Itabashi, Y. Shimada, S. Tanaka, Y. Ito, Y. Ajioka, T. Hamaguchi, I. Hyodo, M. Igarashi, H. Ishida, S. Ishihara, M. Ishiguro, Y. Kanemitsu, N. Kokudo, K. Muro, A. Ochiai, M. Oguchi, Y. Ohkura, Y. Saito, Y. Sakai, H. Ueno, T. Yoshino, N. Boku, T. Fujimori, N. Koinuma, T. Morita, G. Nishimura, Y. Sakata, K. Takahashi, O. Tsuruta, T. Yamaguchi, M. Yoshida, N. Yamaguchi, K. Kotake, K. Sugihara, Japanese Society for Cancer of the Colon and Rectum (JSCCR) Guidelines 2014 for treatment of colorectal cancer, International journal of clinical oncology, 20 (2015) 207-239.
[5] J.A. Meyerhardt, R.J. Mayer, Systemic therapy for colorectal cancer, The New England journal of medicine, 352 (2005) 476-487.
[6] A. Montero, F. Fossella, G. Hortobagyi, V. Valero, Docetaxel for treatment of solid tumours: a systematic review of clinical data, The Lancet. Oncology, 6 (2005) 229-239.
[7] V.A. de Weger, J.H. Beijnen, J.H. Schellens, Cellular and clinical pharmacology of the taxanes docetaxel and paclitaxel--a review, Anti-cancer drugs, 25 (2014) 488-494.
[8] M.C. Bissery, D. Guenard, F. Gueritte-Voegelein, F. Lavelle, Experimental antitumor activity of taxotere (RP 56976, NSC 628503), a taxol analogue, Cancer research, 51 (1991) 4845-4852.
[9] H.J. Wilke, E. Van Cutsem, Current treatments and future perspectives in colorectal and gastric cancer, Annals of oncology : official journal of the European Society for Medical Oncology, 14 Suppl 2 (2003) ii49-ii55.
[10] R. Pazdur, Y. Lassere, L.T. Soh, J.A. Ajani, B. Bready, E. Soo, S. Sugarman, Y. Patt, J.L. Abbruzzese, B. Levin, Phase II trial of docetaxel (Taxotere) in metastatic colorectal carcinoma, Annals of oncology : official journal of the European Society for Medical Oncology, 5 (1994) 468-470.
[11] T.B. Clark, N.E. Kemeny, J.A. Conti, Y. Huang, A.M. Andre, J. Stockman, Phase II trial of docetaxel (Taxotere) for untreated advanced colorectal carcinoma, Cancer investigation, 16 (1998) 314-318.
[12] W. Yang, J. Soares, P. Greninger, E.J. Edelman, H. Lightfoot, S. Forbes, N. Bindal, D. Beare, J.A. Smith, I.R. Thompson, S. Ramaswamy, P.A. Futreal, D.A. Haber, M.R. Stratton, C. Benes, U. McDermott, M.J. Garnett, Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells, Nucleic Acids Res, 41 (2013) D955-961.
[13] M.J. Garnett, E.J. Edelman, S.J. Heidorn, C.D. Greenman, A. Dastur, K.W. Lau, P. Greninger, I.R. Thompson, X. Luo, J. Soares, Q. Liu, F. Iorio, D. Surdez, L. Chen, R.J. Milano, G.R. Bignell, A.T. Tam, H. Davies, J.A. Stevenson, S. Barthorpe, S.R.

Lutz, F. Kogera, K. Lawrence, A. McLaren-Douglas, X. Mitropoulos, T. Mironenko, H. Thi, L. Richardson, W. Zhou, F. Jewitt, T. Zhang, P. O'Brien, J.L. Boisvert, S. Price, W. Hur, W. Yang, X. Deng, A. Butler, H.G. Choi, J.W. Chang, J. Baselga, I. Stamenkovic, J.A. Engelman, S.V. Sharma, O. Delattre, J. Saez-Rodriguez, N.S. Gray, J. Settleman, P.A. Futreal, D.A. Haber, M.R. Stratton, S. Ramaswamy, U. McDermott, C.H. Benes, Systematic identification of genomic markers of drug sensitivity in cancer cells, Nature, 483 (2012) 570-575.

[14] F. Iorio, T.A. Knijnenburg, D.J. Vis, G.R. Bignell, M.P. Menden, M. Schubert, N. Aben, E. Goncalves, S. Barthorpe, H. Lightfoot, T. Cokelaer, P. Greninger, E. van Dyk, H. Chang, H. de Silva, H. Heyn, X. Deng, R.K. Egan, Q. Liu, T. Mironenko, X. Mitropoulos, L. Richardson, J. Wang, T. Zhang, S. Moran, S. Sayols, M. Soleimani, D. Tamborero, N. Lopez-Bigas, P. Ross-Macdonald, M. Esteller, N.S. Gray, D.A. Haber, M.R. Stratton, C.H. Benes, L.F. Wessels, J. Saez-Rodriguez, U. McDermott, M.J. Garnett, A Landscape of Pharmacogenomic Interactions in Cancer, Cell, 166 (2016) 740-754.

[15] H.F. Lu, X. Sun, L. Xu, L.G. Lou, G.Q. Lin, Design, synthesis and biological evaluation of novel fluorinated docetaxel analogues, European journal of medicinal chemistry, 44 (2009) 482-491.

[16] H.F. Lu, C. Xie, J. Chang, G.Q. Lin, X. Sun, Synthesis, cytotoxicity, metabolic stability and pharmacokinetic evaluation of fluorinated docetaxel analogs, European journal of medicinal chemistry, 46 (2011) 1743-1748.

[17] Y.P. Hao, Z.Y. Liu, C. Xie, L. Zhou, X. Sun, Novel fluorinated docetaxel analog for anti-hepatoma: Molecular docking and biological evaluation, European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 88 (2016) 274-281.

[18] J. Rautio, H. Kumpulainen, T. Heimbach, R. Oliyai, D. Oh, T. Jarvinen, J. Savolainen, Prodrugs: design and clinical applications, Nature reviews. Drug discovery, 7 (2008) 255-270.

[19] J. Rautio, N.A. Meanwell, L. Di, M.J. Hageman, The expanding role of prodrugs in contemporary drug design and development, Nature reviews. Drug discovery, (2018).

[20] E. Lee, H. Kim, I.H. Lee, S. Jon, In vivo antitumor effects of chitosan-conjugated docetaxel after oral administration, Journal of controlled release : official journal of the Controlled Release Society, 140 (2009) 79-85.

[21] K.S. Chu, M.C. Finniss, A.N. Schorzman, J.L. Kuijer, J.C. Luft, C.J. Bowerman, M.E. Napier, Z.A. Haroon, W.C. Zamboni, J.M. DeSimone, Particle replication in nonwetting templates nanoparticles with tumor selective alkyl silyl ether docetaxel prodrug reduces toxicity, Nano letters, 14 (2014) 1472-1476.

[22] Y. Fan, Q. Wang, G. Lin, Y. Shi, Z. Gu, T. Ding, Combination of using prodrug-modified cationic liposome nanocomplexes and a potentiating strategy via targeted co-delivery of gemcitabine and docetaxel for CD44-overexpressed triple negative breast cancer therapy, Acta biomaterialia, 62 (2017) 257-272.

[23] I.V. Zhigaltsev, G. Winters, M. Srinivasulu, J. Crawford, M. Wong, L. Amankwa, D. Waterhouse, D. Masin, M. Webb, N. Harasym, L. Heller, M.B. Bally, M.A.

Ciufolini, P.R. Cullis, N. Maurer, Development of a weak-base docetaxel derivative that can be loaded into lipid nanoparticles, Journal of controlled release : official journal of the Controlled Release Society, 144 (2010) 332-340.
[24] A.R. Wohl, A.R. Michel, S. Kalscheuer, C.W. Macosko, J. Panyam, T.R. Hoye, Silicate esters of paclitaxel and docetaxel: synthesis, hydrophobicity, hydrolytic stability, cytotoxicity, and prodrug potential, Journal of medicinal chemistry, 57 (2014) 2368-2379.
[25] W. Wei, C. Luo, J. Yang, B. Sun, D. Zhao, Y. Liu, Y. Wang, W. Yang, Q. Kan, J. Sun, Z. He, Precisely albumin-hitchhiking tumor cell-activated reduction/oxidation-responsive docetaxel prodrugs for the hyperselective treatment of breast cancer, Journal of controlled release : official journal of the Controlled Release Society, 285 (2018) 187-199.
[26] W. Cui, S. Zhang, H. Zhao, C. Luo, B. Sun, Z. Li, M. Sun, Q. Ye, J. Sun, Z. He, Formulating a single thioether-bridged oleate prodrug into a self-nanoemulsifying drug delivery system to facilitate oral absorption of docetaxel, Biomaterials science, (2019).
[27] M. Yoshimoto, F. Itoh, H. Yamamoto, Y. Hinoda, K. Imai, A. Yachi, Expression of MMP-7(PUMP-1) mRNA in human colorectal cancers, International journal of cancer, 54 (1993) 614-618.
[28] Y. Adachi, H. Yamamoto, F. Itoh, Y. Hinoda, Y. Okada, K. Imai, Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers, Gut, 45 (1999) 252-258.
[29] C. Streuli, Extracellular matrix remodelling and cellular differentiation, Current Opinion in Cell Biology, 11 (1999) 634-640.
[30] S. Banskota, S.C. Regmi, J.A. Kim, NOX1 to NOX2 switch deactivates AMPK and induces invasive phenotype in colon cancer cells through overexpression of MMP-7, Molecular cancer, 14 (2015) 123.
[31] S. Netzel-Arnett, Q.X. Sang, W.G. Moore, M. Navre, H. Birkedal-Hansen, H.E. Van Wart, Comparative sequence specificities of human 72- and 92-kDa gelatinases (type IV collagenases) and PUMP (matrilysin), Biochemistry, 32 (1993) 6427-6432.
[32] D.G. Vartak, R.A. Gemeinhart, Matrix metalloproteases: underutilized targets for drug delivery, Journal of drug targeting, 15 (2007) 1-20.
[33] A.M. Mansour, J. Drevs, N. Esser, F.M. Hamada, O.A. Badary, C. Unger, I. Fichtner, F. Kratz, A new approach for the treatment of malignant melanoma: enhanced antitumor efficacy of an albumin-binding doxorubicin prodrug that is cleaved by matrix metalloproteinase 2, Cancer research, 63 (2003) 4062-4066.
[34] T. Kline, M.Y. Torgov, B.A. Mendelsohn, C.G. Cerveny, P.D. Senter, Novel Antitumor Prodrugs Designed for Activation by Matrix Metalloproteinases-2 and -9, Molecular pharmaceutics, 1 (2004) 9-22.
[35] D. Bohme, A.G. Beck-Sickinger, Controlling toxicity of Peptide-drug conjugates by different chemical linker structures, ChemMedChem, 10 (2015) 804-814.
[36] B. Elsadek, R. Graeser, N. Esser, C. Schafer-Obodozie, K. Abu Ajaj, C. Unger, A. Warnecke, T. Saleem, N. El-Melegy, H. Madkor, F. Kratz, Development of a novel prodrug of paclitaxel that is cleaved by prostate-specific antigen: an in vitro and in

vivo evaluation study, European journal of cancer (Oxford, England : 1990), 46 (2010) 3434-3444.

Highlights:

- A series of MMP-7 activated octapeptide-DTX/4FDT prodrugs were synthesized and evaluated both in vitro and in vivo.
- **9a** had similar anti-CRC activity and lower systemic toxicity compared to its parent drug DTX.
- **9a** possessed good stability in plasma and rapid release rate in CRC tumor.

Declaration of interest

All authors declare that they have no conflict of interest with respect to authorship, and/or publication of this study.