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Synthesis and anticancer evaluation of acetylated-lysine conjugated gemcitabine prodrugs†

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Gemcitabine is an antimetabolite drug approved for the treatment of various cancers. However, its use is limited due to several issues such as stability, toxicity and drug resistance. Herein, we present the design and synthesis of a series of gemcitabine prodrugs with modifications on the 4-*N*-amino group by employing an acetylated L- or D-lysine moiety masked by different substitutions. Prodrugs **1–3** and **6–8** showed up to 2.4 times greater anticancer activity than gemcitabine in A549 lung cells, while they exhibited potent activity against BxPC-3 pancreatic cells with IC_{50} values in the range of 7–40 nM. Moreover, prodrugs **2–3** and **7–8** were found to be less potent against CTSL low expression Caco-2 cells and at least 69-fold less toxic towards human normal HEK-293T cells compared to gemcitabine, leading to improved selectivity and safety profiles. Further stability studies showed that representative prodrugs **2** and **7** exhibited enhanced metabolic stability in human plasma, human liver microsomes and cytidine deaminase. Prodrug **1** can be cleaved by tumor cell-enriched CTSL to release parent drug gemcitabine. Overall, these results demonstrated that acetylated lysine conjugated gemcitabine prodrugs could serve as promising leads for further evaluation as new anticancer drugs.

1. Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine, GEM, Fig. 1) is an antimetabolite drug approved by the FDA for the treatment of various cancers[1] and has been used as first-line therapy for pancreatic and non-small cell lung cancer.[2,3] GEM enters into cells by nucleoside transporters (CNTs) such as human equilibrative nucleoside transporter 1 (hENT1),[4,5] which is then phosphorylated by deoxycytidine kinase (dCK) with the formation of 5′-monophosphate (dFdCMP)[6,7] and undergoes further phosphorylation to form diphosphate (dFdCDP) and triphosphate (dFdCTP).[8] dFdCDP acts as an inhibitor of ribonucleotide reductase and results in the depletion of dNTPs which are required for DNA synthesis, while dFdCTP can incorporate into DNA strands and interrupt the DNA replication and repair processes.[9] The inhibition of the two processes eventually leads to cell apoptosis. The clinical potential of GEM is restricted by the drug resistance and high toxicity to normal cells, and the rapid deamination by cytidine deaminase (CDA) to its inactive metabolite 2′,2′-difluorodeoxyuridine (dFdU) as well.[10]

To overcome the issues of GEM, prodrug strategies have been widely used to improve metabolic stability while maintaining its high efficacy, which mainly focused on the modifications of the 4-*N*-amine and 5′-hydroxyl groups of GEM.[11,12] For instance, structural modification of the 4-*N*-amino group of GEM by linking valproic acid led to the discovery of an orally active prodrug LY2334737 with carboxylesterase activation and high stability (Fig. 1).[13,14] There were also GEM prodrugs such as GEMP-2 and Gem-Thr modified with amino acids or long alkyl chains that showed improved anticancer activity and stability.[15–17]

Upregulation of cysteine cathepsin L (CTSL), a lysosomal acid cysteine protease, has been recognized as a valid marker of cancer progression and multiple stages of metastasis.[18,19] The enhanced activity of CTSL in tumor cells could provide a specific environment for targeted cancer therapy, which has been successfully used for the development of anticancer prodrug strategies as well as selective metabolic labeling of RNA in cancer cells.[20–23]

Herein, we present the synthesis of gemcitabine prodrugs with modifications on the 4-*N*-amino group where an L- or

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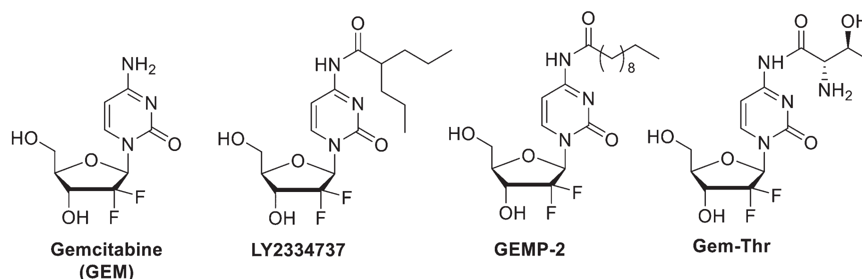


Fig. 1 Structures of GEM and its representative prodrugs with 4-*N*-amine modifications.

D-Lys(Ac) moiety is masked by different substitutions. The amide bonds in these prodrugs are theorized to be specifically cleaved by enriched CTSL inside cancer cells to finally release the parent compound gemcitabine. Additionally, these modifications would provide gemcitabine with enhanced metabolic stability in human plasma and tissues as well as improved safety profiles.

2. Results and discussion

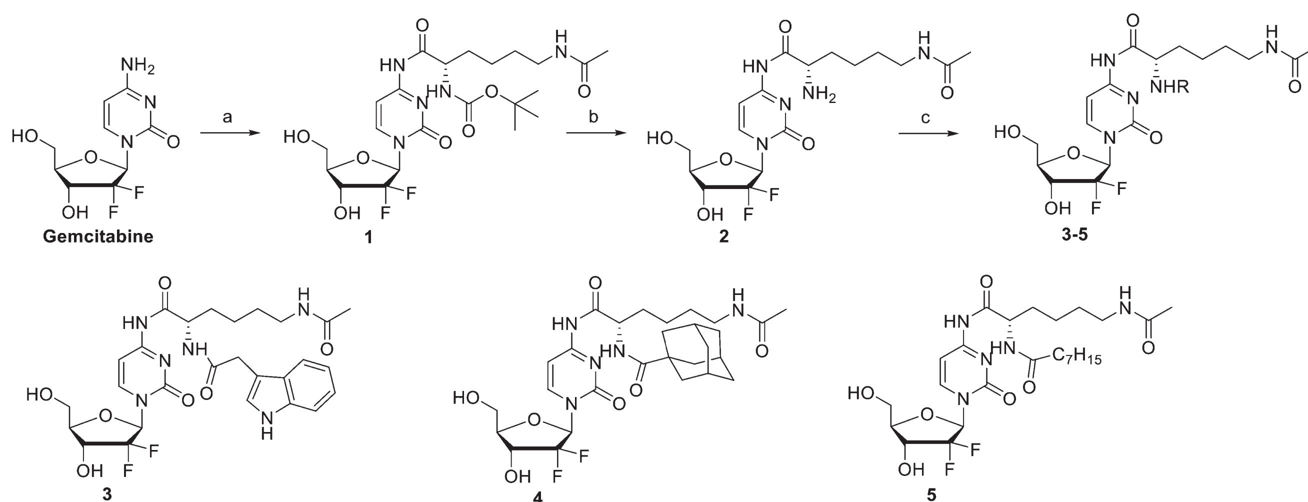
2.1. Synthesis of gemcitabine prodrugs

The synthesis of gemcitabine prodrugs with different substitutions on L-Lys(Ac) is shown in Scheme 1. Initially, prodrug **1** with a *tert*-butyloxycarbonyl (Boc) protected L-Lys(Ac) moiety was synthesized in 58% yield by a coupling reaction of gemcitabine and Boc–L-Lys(Ac)–OH in the presence of 1-hydroxy-1*H*-benzotrizole (HOBt), 4-methylmorpholine, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI),[17] followed by the cleavage of the Boc protecting group to yield prodrug **2** in 75% yield. Prodrug **3** was designed to act as a better substrate for CTSL as the enzyme has a preference for bulky aromatic residues.[21,24] The enhancement of lipophilicity may lead to an improvement of anticancer activity in the target cells.[15,22,25] Therefore, prodrugs **4** and **5**, with a fatty acyl moiety and an adamantyl moiety on the α-amino Lys(Ac) residue, respectively, were designed. Compounds **3–5** were prepared in 36–43% yield by coupling compound **2** with indole-3-acetic acid, 1-adamantane carboxylic acid and octanoic acid, respectively.

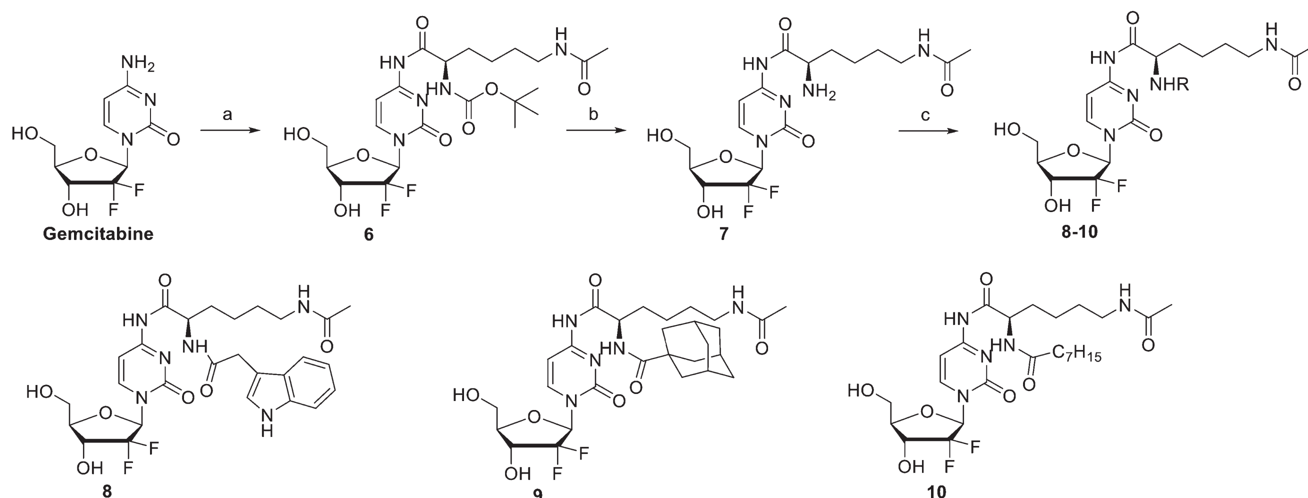
To investigate the influence of the configuration of Lys(Ac) on the anticancer activity and further enhance the metabolic stability, prodrugs with D-configuration Lys(Ac) were synthesized. Similarly, the synthesis of prodrugs **6–10** (Scheme 2) with D-configuration Lys(Ac) was accomplished by following similar procedures to those described for prodrugs **1–5**. The structures of all the synthesized prodrugs were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS analyses.

2.2. Anticancer and selectivity effect against cancer cells

All synthesized gemcitabine prodrugs with different substituents on Lys(Ac) were evaluated for their anticancer activity against CTSL overexpression A549 (non-small cell lung cancer) and BxPC-3 (pancreatic cancer) cell lines.[20,26] The evaluation was performed by the Cell Counting Kit-8



Scheme 1 Synthesis of gemcitabine prodrugs with different substitutions on L-Lys(Ac). Reagents and conditions: (a) Boc–L-Lys(Ac)–OH, 4-methylmorpholine, HOBt, EDCI, DMF, 55 °C, 17 h, 58%; (b) TFA, CH_2Cl_2, r.t, 2 h, 75%; (c) RCOOH, 4-methylmorpholine, HOBt, EDCI, DMF, r.t, 2 h, 36–43%.



Scheme 2 Synthesis of gemcitabine prodrugs with different substitutions on D-Lys(Ac). Reagents and conditions: (a) Boc–D-Lys(Ac)–OH, 4-methylmorpholine, HOBt, EDCI, DMF, 55 °C, 17 h, 86%; (b) TFA, CH_2Cl_2, r.t, 2 h, 67%; (c) RCOOH, 4-methylmorpholine, HOBt, EDCI, DMF, r.t, 2 h, 31–43%.

(CCK8) assay. The cells were incubated with different concentrations of tested compounds for 72 h before being assayed. Anticancer activity was expressed as IC_{50} and is summarized in Table 1. In A549 cells, prodrugs **1–3** and **6–8** with a Boc, an indole-3-acetyl or a free amino group exhibited potent anticancer activity with IC_{50} values in the range of 2.23–5.24 μM, which was more potent or comparable to that of gemcitabine (IC_{50} = 5.35 μM). However, the anticancer activity of compounds **4**, **5**, **9** and **10** that contain an adamantyl or an octanoyl moiety was less pronounced ($IC_{50} > 10.48$ μM). In BxPC-3 cells, similar trends were found for their anticancer activity compared to their activity in A549 cells. Prodrugs **1–3** and **6–8** showed nanomolar anticancer activity against BxPC-3 (IC_{50} = 7–40 nM), while prodrugs **4**, **5**, **9** and **10** exhibited less potent activity (IC_{50} = 0.14–94 μM). The less potent anticancer activity of compounds **4**, **5**, **9** and **10** in A549 and BxPC-3 cells compared to other prodrugs indicated that prodrugs with a 1-adamantane carbonyl or an octanoyl substituent may not be efficiently recognized by enzymes in cancer cells to release the parent compound gemcitabine. Moreover, no significant difference of anticancer activity was found between L- and D-configuration prodrugs.

To further investigate their anticancer selectivity, Caco-2 selected as CTSL was less expressed in this cancer cell line.[20] All the prodrugs displayed weak or loss of activity regardless of different substitutions and lipophilicity profiles, leading to a boost in anticancer selectivity. It is worth noting that prodrugs **1** and **6** that contain a Boc protecting group were slightly toxic to Caco-2 cells with IC_{50} values of 8.02 μM and 3.17 μM, respectively. The results verified that the designed gemcitabine prodrugs can be utilized as a selective modality to target tumors.

2.3. Toxicity effect against a normal cell line

Safety plays a crucial role in the discovery of anticancer agents. We next sought to investigate the safety profile of selected prodrugs **1–3** and **6–8** with promising anticancer activity by utilizing immortalized noncancerous cell line

Table 1 *In vitro* IC_{50} values of gemcitabine prodrugs in this study (μM)[a]

Compound	A549	BxPC-3	Caco-2	HEK-293T
1	2.23 ± 0.53	0.012 ± 0.001	8.02 ± 1.80	0.12 ± 0.005
2	5.24 ± 1.19	0.022 ± 0.0015	77.44 ± 17.19	0.69 ± 0.09
3	2.81 ± 0.43	0.04 ± 0.002	>100	2.08 ± 0.07
4	10.48 ± 1.04	5.49 ± 0.66	78.59 ± 15.73	ND[b]
5	49.20 ± 7.35	22.2 ± 2.47	29.58 ± 4.38	ND
6	3.26 ± 0.55	0.01 ± 0.0008	3.17 ± 0.62	0.03 ± 0.001
7	2.40 ± 0.36	0.025 ± 0.002	100.6 ± 28.85	0.80 ± 0.04
8	3.16 ± 0.46	0.007 ± 0.001	100.1 ± 33.25	1.62 ± 0.15
9	17.63 ± 2.30	93.96 ± 6.84	>100	ND
10	>100	0.14 ± 0.01	81.16 ± 23.89	ND
Gemcitabine	5.35 ± 0.94	0.0081 ± 0.0004	1.33 ± 0.3	0.01 ± 0.0007

[a] Cell growth inhibition activity was assayed by incubation with different compounds for 72 h and expressed as the concentration required to inhibit tumor cell growth by 50% (IC_{50}). The IC_{50} values are shown as mean ± SEM of three independent experiments. [b] Not determined.

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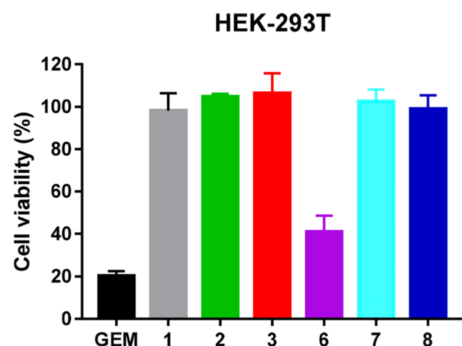

Fig. 2 Cell viability in HEK-293T cells treated with 0.032 μM concentration of compounds for 72 h. Data are presented as mean ± SD of three independent experiments.

HEK-293T (human embryonic kidney cells).[23] As shown in Fig. 2, when treated with 0.032 μM concentration of gemcitabine for 72 h, it induced 80% cell death with an IC_{50} value of 0.01 μM. Generally, prodrugs **1–3** and **6–8** were less toxic to HEK-293 cells compared to gemcitabine. An obvious safety profile was obtained for prodrugs **2–3** and **7–8** (Table 1), with IC_{50} values in the range of 0.69–2.08 μM, which were at least 69-fold less toxic than gemcitabine. These results demonstrated that the conjugation of gemcitabine with acetylated lysine could reduce its toxicity towards normal cells, leading to a favorable safety profile.

2.4. *In vitro* chemical stability of gemcitabine prodrugs 2 and 7

The stability of representative gemcitabine prodrugs was investigated in PBS with a physiological pH of 7.4 and analyzed by using LC–MS. As shown in Fig. 3A, prodrugs **2** and **7** exhibited similar stability in PBS which is consistent with the reported stability of amino acid-conjugated amide gemcitabine prodrugs.[17]

2.5. Metabolic stability study of gemcitabine prodrugs 2 and 7 in human plasma, human liver microsomes and cytidine deaminase

The stabilities of representative prodrugs **2** and **7** were evaluated in parallel with gemcitabine in human plasma and human liver microsomes. Fig. 3B shows that 87% of gemcitabine remained when treated with human plasma at 120 min, while prodrugs **2** and **7** were metabolically stable

Fig. 3 Stability profiles of gemcitabine and representative prodrugs **2** and **7**. (A) The compounds (2 μM) were treated with PBS at 37 °C for 1, 2, 6 and 24 h; (B) the compounds tested (2 μM) were exposed to human plasma and incubated at 37 °C for 0, 10, 30, 60 and 120 min; (C) the compounds (1 μM) were treated with human liver microsomes at 37 °C for 5, 15, 30, 45 and 60 min; (D) the compounds (100 μM) were exposed to cytidine deaminase at 37 °C for 5, 20, 40 and 60 min.

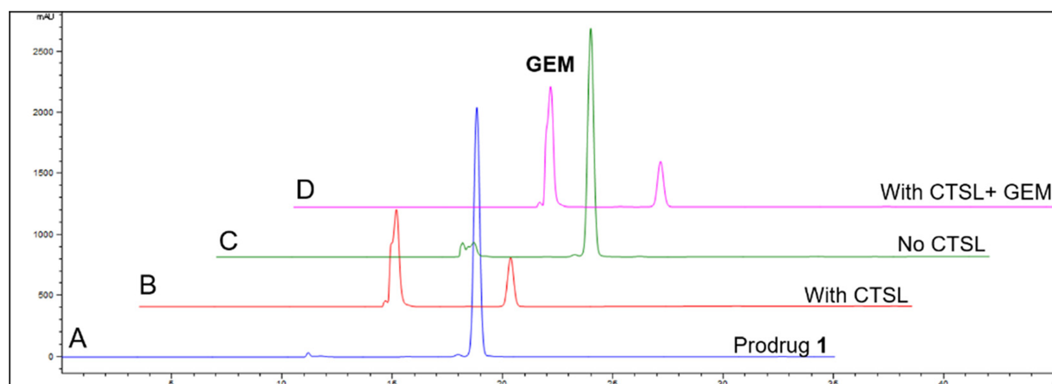

Fig. 4 Bioactivation of prodrug **1** with CTSL. (A) HPLC analysis of the prodrug **1** control. (B) Prodrug **1** incubated with CTSL at 37 °C for 24 h. (C) Prodrug **1** incubated in buffer solution without CTSL at 37 °C for 24 h. (D) Mixture of prodrug **1** after reaction with CTSL and with the addition of gemcitabine as a control.

after 120 min. Surprisingly, selected prodrugs **2** and **7** were stable in human liver microsomal fractions with $t_{1/2} > 145$ min (88% and 80% remaining at 60 min, respectively), whereas only 59% of gemcitabine remained after 60 min ($t_{1/2}$ = 77.9 min) (Fig. 3C). No significant difference in stability was found in L-configuration prodrug **2** and D-configuration prodrug **7**. The results indicated that the metabolic stability was enhanced with the tested prodrugs in comparison with gemcitabine.

Cytidine deaminase (CDA) is responsible for the rapid deamination of gemcitabine and reducing its efficacy.[27] An *in vitro* CDA assay was performed with prodrugs **2** and **7** to evaluate their resistance to this enzyme by monitoring the UV absorption at 280 nm.[28] The decrease in the absorbance intensity was indicative of deamination. As shown in Fig. 3D, gemcitabine was rapidly deaminated by a significant decrease in its absorbance intensity in the presence of CDA, which is consistent with the high susceptibility of free gemcitabine to this enzyme. However, prodrugs **2** and **7** were unaffected by CDA, as shown by the similar results obtained in the presence and in the absence of CDA. The enhanced stability can be explained by the protection of the 4-*N*-amino group in gemcitabine *via* amide bond formation.

2.6. Bioactivation mechanism of gemcitabine prodrug 1

In an attempt to characterize the specific mechanism of bioactivation of these prodrugs, human cysteine cathepsin L protease was used as it is overexpressed in various cancers and is associated with a number of prodrug activation processes.[20–23] Prodrug **1** was incubated with CTSL at 37 °C for 24 h and then analyzed by analytical HPLC chromatography. As shown in Fig. 4, prodrug **1** was metabolized to release gemcitabine in the presence of CTSL, while it was relatively stable in the reaction buffer without CTSL. The results indicated that **1** can serve as a substrate of CTSL.

3. Conclusions

In summary, a series of L- and D-configuration Lys(Ac) conjugated gemcitabine prodrugs were synthesized and evaluated for their anticancer activity. Among them, prodrugs **1–3** and **6–8** that contain a Boc, an indole-3-acetyl or a free amino group showed potent antiproliferative activity in A549 and BxPC-3 cells, while they exhibited less potent activity against Caco-2 cells. Moreover, these prodrugs were found to be less toxic to human normal HEK-293T cells compared to gemcitabine, leading to an improved safety profile. The chemical and metabolic stability study revealed that representative prodrugs **2** and **7** were stable in PBS buffer and showed enhanced stability in human plasma, human liver microsomes and CDA compared to gemcitabine. Prodrug **1** can be activated by tumor cell-enriched cathepsin L to generate gemcitabine. The results suggest that these Lys(Ac) conjugated gemcitabine prodrugs deserve further investigation as new anticancer drugs.

4. Experimental section

4.1. Chemistry

Reagents and solvents were obtained from commercial sources and used without further purification. Reactions were monitored using silica gel plates (GF-254, Qingdao Marine Chemical Co, China) and detected under UV light. Silica gel (200–300 mesh) was utilized for column chromatography purifications. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker Ascend 400 Avance III HD and high-resolution mass spectrometry (HRMS) data were acquired on an Agilent QTOF-6520 (Agilent Technologies, US). Preparative high-performance liquid chromatography (HPLC) was performed on an Agilent HPLC system with a C18 reverse phase column (YMC-Pack ODS-A, 250 × 10 mm, 5 μm) using H_2O/MeOH at a flow rate of 1 mL min^{-1} over 40 min. The purity of all prodrugs was confirmed by HPLC (purity is >95%).

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4.1.1. *Tert*-butyl((*S*)-6-acetamido-1-((1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)amino)-1-oxohexan-2-yl) carbamate (1). *N*-Methylmorpholine (NMM, 213 μL, 1.90 mmol), 1-hydroxybenzotriazole (HOBt, 258 mg, 1.90 mmol), Boc–L-Lys(Ac)–OH (548 mg, 1.90 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI, 364 mg, 1.90 mmol) were sequentially added to a stirred solution of gemcitabine (500 mg, 1.90 mmol) in DMF (2 mL) at room temperature under N_2 protection. The reaction was heated to 55 °C and was kept stirring for 17 h. After the reaction was completed, the reaction mixture was cooled to room temperature and extracted with brine and EtOAc. The organic phase was separated, and the aqueous layer was extracted with fresh portions of EtOAc (3 × 10 mL). The combined organic layers were then sequentially washed with sat. $NaHCO_3$ and brine and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by column chromatography (DCM/MeOH, 20 : 1) to afford **1** (584 mg, 58%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.27 (d, *J* = 9.27 Hz, 1H), 7.83–7.80 (m, 1 H), 7.26 (d, *J* = 6.69 Hz, 1H), 7.13 (d, *J* = 6.69 Hz, 1H), 6.38–6.37 (m, 1H), 6.18 (t, *J* = 14.60 Hz, 1H), 5.35 (br, 1H), 4.20–4.08 (m, 2H), 3.91–3.88 (m, 1H), 3.83–3.80 (m, 1H), 3.67–3.61 (m, 1H), 3.17 (s, 1H), 3.02–2.97 (m, 2H), 1.78 (s, 3H), 1.62–1.51(m, 2H), 1.37–1.28 (m, 13H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 174.79, 169.55, 163.99, 156.04, 154.64, 145.38, 123.39 (t, *J* = 257 Hz, C2′), 96.41, 95.06, 84.62 (t, *J* = 31.0 Hz, C1′), 81.56, 78.74, 68.80 (t, *J* = 22.0 Hz, C3′), 59.20, 55.77, 55.35, 49.06, 31.13, 29.20, 28.62, 23.52, 23.03. HRMS calcd. for $C_{22}H_{34}F_2N_5O_8$ [M + H]$^+$ 534.2375, found 534.2372.

4.1.2. (*S*)-6-Acetamido-2-amino-*N*-(1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)hexanamide (2). To a solution of **1** (500 mg, 937 μmol) in DCM (3 mL) was added TFA (209 μL, 2.81 mmol), and the reaction was stirred at room temperature for 2 h. The reaction was neutralized by adding sat. $NaHCO_3$ and the water layer was lyophilized. The residue was purified by column chromatography (DCM/MeOH, 10 : 1) to give **2** (304 mg, 75%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.04 (d, *J* = 8.41 Hz, 1H), 7.82–7.80 (m, 1H), 7.68 (d, *J* = 7.15 Hz, 1H), 7.52 (s, 1H), 7.05 (s, 1H), 6.14–6.10 (m, 1H), 5.99 (d, *J* = 7.57 Hz, 1H), 5.50–4.46 (m, 1H), 4.18–4.11 (m, 1H), 3.81–3.75 (m, 2H), 3.64–3.60 (m, 1H), 3.01–2.97 (m, 2H), 1.77 (s, 3H), 1.72–1.66 (m, 1H), 1.62–1.54 (m, 1H), 1.39–1.26 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 173.86, 169.53, 163.68, 155.08, 140.12, 123.59 (t, *J* = 256 Hz, C2′), 95.96, 84.01 (t, *J* = 34.0 Hz, C1′), 80.94, 69.09 (t, *J* = 22.0 Hz, C3′), 59.42, 53.58, 38.83, 32.29, 29.34, 23.30, 23.10. HRMS calcd. for $C_{17}H_{26}F_2N_5O_6$ [M + H]$^+$ 434.1850, found 434.1841.

4.1.3. (*S*)-2-(2-(1*H*-Indol-3-yl)acetamido)-6-acetamido-*N*-(1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl) tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl) hexanamide (3). **3** was prepared as described for compound **1**. The reaction was stirred at room temperature for 2 h. Obtained from compound **2** (60 mg, 138 μmol) and indole-3-acetic acid (24 mg, 138 μmol). It was purified by column chromatography (DCM/MeOH, 20 : 1) to afford a white solid (33 mg, 40%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.08 (d, *J* = 16.38 Hz, 1H), 7.84 (br, 1H), 7.65 (d, *J* = 7.37 Hz, 1H), 7.53–7.49 (m, 2H), 7.38–7.27 (m, 2H), 7.10 (t, *J* = 53.24 Hz, 2H), 6.27–6.22 (m, 1H), 6.02–5.98 (m, 1H), 5.39–5.27 (m, 1H), 4.50–4.44 (m, 1H), 4.18–4.10 (m, 1H), 3.92 (s, 1H), 3.73 (d, *J* = 12.69 Hz, 1H), 3.61–3.56 (m, 2H), 3.00–2.99 (m, 2H), 1.71 (s, 3H), 1.62–1.51 (m, 2H), 1.39–1.23 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 173.74, 170.88, 169.43, 163.71, 154.83, 136.56, 130.13, 127.41, 124.78, 124.61 (t, *J* = 234 Hz, C2′), 121.64, 119.06, 118.83, 111.96, 106.56, 96.16, 84.70 (t, *J* = 34.0 Hz, C1′), 79.02, 70.59 (t, *J* = 27.0 Hz, C3′), 59.64, 53.62, 38.84, 32.28, 30.59, 29.38, 23.32, 23.12. HRMS calcd. for $C_{27}H_{33}F_2N_6O_7$ [M + H]$^+$ 591.2378, found 591.2376.

4.1.4. *N*-((*S*)-6-Acetamido-1-((1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)amino)-1-oxohexan-2-yl)adamantane-1-carboxamide (4). **4** was prepared as described for compound **3**. Obtained from compound **2** (60 mg, 138 μmol) and 1-adamantane carboxylic acid (25 mg, 138 μmol). It was purified by column chromatography (DCM/MeOH, 20 : 1) to afford a white solid (30 mg, 36%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.02 (d, *J* = 7.75 Hz, 1H), 7.78–7.76 (m, 1H), 7.62 (d, *J* = 7.75 Hz, 1H), 7.49 (s, 1H), 7.01 (s, 1H), 6.23–6.20 (m, 1H), 6.01 (d, *J* = 7.16 Hz, 1H), 5.33–5.22 (m, 1H), 4.49–4.45 (m, 1H), 4.14–4.12 (m, 1H), 3.74–3.71 (m, 2H), 3.61–3.57 (m, 2H), 3.00–2.97 (m, 2H), 2.02–1.99 (m, 3H), 1.93–1.84 (m, 4H), 1.77 (s, 3H), 1.71–1.57 (m, 6H), 1.39–1.23 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 182.58, 175.52, 173.76, 169.45, 163.71, 154.83, 128.25 (t, *J* = 188 Hz, C2′), 124.46, 119.31, 110.50, 96.16, 79.08, 55.25, 53.61, 38.84, 38.55, 36.25, 32.28, 31.75, 30.85, 29.37, 27.63, 27.02, 23.77, 23.32, 23.07, 22.55, 14.41. HRMS calcd. for $C_{28}H_{40}F_2N_5O_7$ [M + H]$^+$ 596.2895, found 596.2895.

4.1.5. *N*-((*S*)-6-Acetamido-1-((1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)amino)-1-oxohexan-2-yl)octanamide (5). **5** was prepared as described for compound **3**. Obtained from compound **2** (60 mg, 138 μmol) and octanoic acid (22 μL, 138 μmol). It was purified by column chromatography (DCM/MeOH, 20 : 1) to afford a white solid (33 mg, 43%). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.06 (d, *J* = 9.47 Hz, 1H), 7.83–7.80 (m, 1H), 7.64 (d, *J* = 8.52 Hz, 1H), 7.53 (br, 1H), 7.06 (br, 1H), 6.24–6.20 (m, 1H), 6.01 (d, *J* = 6.63 Hz, 1H), 5.36–5.25 (m, 1H), 4.49–4.44 (m, 1H), 4.14–4.10 (m, 2H), 3.76–3.72 (m, 1H), 3.64–3.60 (m, 1H), 3.02–2.97 (m, 2H), 2.46–2.42 (m, 2H), 1.78 (s, 3H), 1.72–1.54 (m, 4H), 1.40–1.26 (m, 12H), 0.87–0.84 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 173.71, 172.20, 169.40, 163.70, 154.82, 140.65, 122.23 (t, *J* = 251 Hz, C2′), 96.14, 84.44, 79.00, 70.28 (t, *J* = 34.0 Hz, C3′), 59.68, 53.56, 38.85, 33.42, 32.31, 31.55, 29.39, 28.73, 28.68, 24.76, 23.31, 23.07, 22.46, 14.38. HRMS calcd. for $C_{25}H_{40}F_2N_5O_7$ [M + H]$^+$ 560.2895, found 560.2890.

4.1.6. *Tert*-butyl((*R*)-6-Acetamido-1-((1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl) amino)-1-oxohexan-2-yl) carbamate (6). To a solution of D-Lys(Ac) (350 mg, 1.86 mmol) in THF (1 mL) was added NaOH solution (3 M, 1 mL) at 0 °C, and the mixture stirred for 5 min. BOC acid anhydride (534 μL, 2.32 mmol) was then added and it was stirred at room temperature for 18 h. The product was neutralized with 2 M dilute hydrochloric acid solution and extracted with EtOAc. The organic layer was filtered and evaporated under reduced pressure to afford Boc–D-Lys(Ac)–OH (460 mg, 86%) as a white solid. ^{1}H-NMR (DMSO-d_6, 400 MHz) *δ*: 7.86–7.83 (m, *J* = 12.29 Hz, 1H), 5.76 (s, 1H), 3.81–3.75 (m, *J* = 21.08 Hz, 1H), 3.17 (s, 1H), 3.00–2.96 (m, *J* = 17.56 Hz, 2H), 1.78 (s, 3H), 1.66–1.50 (m, *J* = 59.71 Hz, 2H), 1.38–1.23 (m, *J* = 57.96 Hz, 13H). Then **6** was prepared as described for compound **1**. Obtained from gemcitabine (500 mg, 1.90 mmol) and Boc–D-Lys(Ac)–OH (548 mg, 1.90 mmol) as a white solid (788 mg, 78%). ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 8.27 (d, *J* = 8.00 Hz, 1H), 7.82–7.80 (m, 1H), 7.25 (d, *J* = 8.00 Hz, 1H), 7.14 (d, *J* = 8.00 Hz, 1H), 6.38–6.36 (m, 1H), 6.18 (t, *J* = 15.39 Hz, 1H), 5.35–5.32 (m, 1H), 4.22–4.18 (m, 1H), 3.91–3.80 (m, 2H), 3.68–3.61 (m, 1H), 3.00–2.99 (m, 2H), 1.78 (s, 3H), 1.62–1.52 (m, 2H), 1.38–1.29 (m, 13H). ^{13}C NMR (100 MHz, DMSO-d_6) *δ*: 174.83, 169.42, 163.38, 156.06, 154.61, 123.42 (t, *J* = 252 Hz, C2′), 96.41, 84.62 (t, *J* = 33.0 Hz, C1′), 81.53, 78.74, 68.82 (t, *J* = 22.0 Hz, C3′), 59.25, 55.80, 38.74, 31.08, 29.22, 28.63, 23.55, 23.07. HRMS calcd. for $C_{22}H_{34}F_2N_5O_8$ $[M + H]^+$ 534.2375, found 534.2373.

4.1.7. (*R*)-6-Acetamido-2-amino-*N*-(1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)hexanamide (7). **7** was prepared as described for compound **2**. Obtained from compound **6** (500 mg, 937 μmol) as a white solid **7** (271 mg, 67%). ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 8.01 (d, *J* = 7.48 Hz, 1H), 7.85–7.82 (m, 1H), 7.67 (d, *J* = 6.41 Hz, 1H), 7.52 (br, 1H), 7.04 (br, 1H), 6.29–6.28 (m, 1H), 6.13–6.09 (m, 1H), 5.98 (d, *J* = 7.48 Hz, 1H), 5.27–5.24 (m, 1H), 4.48–4.43 (m, 2H), 3.80–3.75 (m, 2H), 3.65–3.59 (m, 2H), 3.03–2.98 (m, 2H), 1.78 (s, 3H), 1.71–1.57 (m, 2H), 1.40–1.23 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) *δ*: 173.82, 169.51, 163.69, 155.06, 140.13, 123.59 (t, *J* = 273 Hz, C2′), 95.96, 83.21(t, *J* = 31.0 Hz, C1′), 80.92, 69.13 (t, *J* = 22.0 Hz, C3′), 59.43, 53.63, 38.85, 32.25, 29.38, 23.34, 23.03. HRMS calcd. for $C_{17}H_{26}F_2N_5O_6$ $[M + H]^+$ 434.1850, found 434.1844.

4.1.8. (*R*)-2-(2-(1*H*-Indol-3-yl)acetamido)-6-acetamido-*N*-(1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl) tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl) hexanamide (8). **8** was prepared as described for compound **3**. Obtained from compound **7** (60 mg, 138 μmol) and indole-3-acetic acid (24 mg, 138 μmol). It was purified by column chromatography (DCM/MeOH, 20 : 1) to afford a white solid (35 mg, 43%). ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 8.06 (d, *J* = 7.95 Hz, 1H), 7.86–7.83 (m, 1H), 7.52–7.49 (m, 2H), 7.38–7.36 (m, 2H), 7.27–7.26 (m, 1H), 7.11–7.07 (m, 2H), 7.00–6.96 (m, 1H), 6.46–6.44 (m, 1H), 6.16–6.11 (m, 1H), 5.99 (d, *J* = 7.66 Hz, 1H), 4.49–4.30 (m, 3H), 4.03–4.01 (m, 1H), 3.82 (s, 2H), 3.03–2.98 (m, 2H), 1.78 (s, 3H), 1.72–1.59 (m, 2H), 1.41–1.24 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) *δ*: 173.74, 171.85, 169.44, 163.70, 154.89, 136.56, 130.13, 127.50, 124.61 (t, *J* = 234 Hz, C2′), 121.59, 119.01, 118.89, 111.93, 107.16, 96.31, 84.70, 77.78, 70.52 (t, *J* = 24.0 Hz, C3′), 59.44, 53.64, 38.84, 32.26, 30.99, 29.39, 23.34, 23.11. HRMS calcd. for $C_{27}H_{33}F_2N_6O_7$ $[M + H]^+$ 591.2378, found 591.2368.

4.1.9. *N*-((*R*)-6-Acetamido-1-((1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)amino)-1-oxohexan-2-yl)adamantane-1-carboxamide (9). **9** was prepared as described for compound **3**. Obtained from compound **7** (60 mg, 138 μmol) and 1-adamantane carboxylic acid (25 mg, 138 μmol). It was purified by column chromatography (DCM/MeOH, 20 : 1) to afford a white solid (26 mg, 31%). ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 8.10 (d, *J* = 8.18 Hz, 1H), 7.85–7.83 (m, 2H), 7.58–7.53 (m, 2H), 7.37–7.27 (m, 2H), 4.49–4.43 (m, 1H), 4.36–4.33 (m, 1H), 4.03–3.98 (m, 2H), 3.92–3.90 (m, 2H), 3.05–3.00 (m, 2H), 2.03–1.97 (m, 3H), 1.87–1.84 (m, 2H), 1.78 (s, 3H), 1.73–1.67 (m, 4H), 1.39–1.24 (m, 12H). ^{13}C NMR (100 MHz, DMSO-d_6) *δ*: 176.66, 173.70, 169.45, 163.62, 154.83, 143.33, 128.13 (t, *J* = 188 Hz, C2′), 124.09, 119.12, 110.77, 96.22, 85.66, 55.37, 53.64, 38.84, 36.37, 35.30, 32.27, 31.75, 30.86, 29.38, 27.73, 27.02, 23.33, 23.07, 22.56, 16.01, 14.41. HRMS calcd. for $C_{28}H_{40}F_2N_5O_7$ $[M + H]^+$ 596.2895, found 596.2893.

4.1.10. *N*-((*R*)-6-Acetamido-1-((1-((2*R*,4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-2-oxo-1,2-dihydropyrimidin-4-yl)amino)-1-oxohexan-2-yl)octanamide (10). **10** was prepared as described for compound **3**. Obtained from compound **7** (60 mg, 138 μmol) and octanoic acid (22 μL, 138 μmol). It was purified by column chromatography (DCM/MeOH, 20 : 1) to afford a white solid (30 mg, 38%). ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 8.12–8.10 (m, 1H), 7.87–7.84 (m, 1H), 7.66 (d, *J* = 8.54 Hz, 1H), 7.52–7.48 (m, 1H), 7.05 (br, 1H), 6.21–6.18 (m, 1H), 6.13–6.11 (m, 1H), 6.03–5.99 (m, 1H), 5.34–5.31 (m, 1H), 4.48–4.42 (m, 1H), 3.81–3.73 (m, 1H), 3.64–3.59 (m, 1H), 3.02–2.97 (m, 2H), 2.46–2.33 (m, 2H), 1.78 (s, 3H), 1.73–1.54 (m, 4H), 1.40–1.24 (m, 12H), 1.00–0.96 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) *δ*: 173.77, 172.14, 169.47, 163.85, 158.69, 154.85, 119.24 (t, *J* = 250 Hz, C2′), 96.25, 85.07, 79.00, 70.38, 59.64, 55.17, 38.85, 36.69, 34.57, 31.54, 29.38, 28.72, 25.97, 24.75, 23.36, 23.05, 22.46, 14.36. HRMS calcd. for $C_{25}H_{40}F_2N_5O_7$ $[M + H]^+$ 560.2895, found 560.2892.

4.2. Cell viability assay

This assay was determined by the CCK8 method. All cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10 mM HEPES at 37 °C in a 5% CO_2 humidified atmosphere. For the viability assays, A549 and HEK-293T cells were seeded in 96-well plates at a density of 5 × 10^3 cells per well in DMEM 10% FBS medium, while BxPC-3 and Caco-2 cells were seeded in 96-well plates at a density

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of 8×10^3 cells per well in DMEM 10% FBS medium. After 24 h preincubation, the medium was removed and the cells were treated with different concentrations of the compounds. The final volume was 100 μL per well in DMEM 10% FBS with DMSO less than 0.1%. Then the cells were incubated for 72 h, followed by the addition of fresh diluted CCK8 (Biosharp) solution. The absorbance was detected at 450 nm. The IC_{50} values were calculated using GraphPad. All experiments were repeated three times.

4.3. Chemistry stability study

To assess the pH stability of the selected compounds, the prodrug (2 μM) was incubated at 37 °C and 600 rpm in buffered solutions with a pH value of 7.4 for the appointed time. Test samples at the corresponding time point (60, 120, 360 and 1440 minutes) were removed at the end of incubation time and immediately mixed with 400 μL of cold acetonitrile containing 200 ng mL^{-1} tolbutamide and labetalol (internal standards) completely. The samples were subjected to centrifugation at 4000 rpm, 4 °C for 20 min. The supernatant was pipetted and mixed with 180 μL of 5% TCA, mixed completely for LC–MS analysis. The remaining test compound at each incubation time was calculated based on the peak area ratio of analyte/IS.

4.4. Human plasma stability study

The human plasma stability assay was carried out by Wu Xi App Tec Co. Ltd. The pooled frozen human plasma (3 male & 3 female from Bioreclamation IVT, Cat. #HUMANPLNHP2N, Batch HMN761044) was thawed in a water bath at 37 °C prior to the experiment. The plasma was centrifuged at 4000 rpm for 5 min. Using an Apricot automation workstation, the compounds tested were exposed to human plasma at a final concentration of 2 μM and incubated at 37 °C for 0, 10, 30, 60 and 120 min. At each time point, the samples were mixed with a quench solution containing internal standards tolbutamide (200 ng mL^{-1}) and labetalol (200 ng mL^{-1}) in acetonitrile and centrifuged at 4000 rpm for 20 min at 4 °C. After centrifugation, each bioanalysis plate was sealed and shaken for 10 minutes prior to LC–MS analysis.

4.5. Human liver microsome study

The stability assay was carried out by Wu Xi App Tec Co., Ltd. Human liver microsomes (Corning, cat. #452117) were preincubated with an NADPH cofactor (1 mM) in a phosphate buffer containing 10 mM $MgCl_2$ in a 96-well plate for 10 min at 37 °C with constant shaking. The reaction was initiated by adding the test compound (final concentration 1 μM) and incubated to a final volume of 500 μL in a 37 °C shaking water bath. At each time point (5, 15, 30, 45 and 60 min), 60 μL of the incubation mixture was added to a quench solution containing internal standards tolbutamide (200 ng mL^{-1}) and labetalol (200 ng mL^{-1}) in cold acetonitrile. The samples were then mixed on a plate shaker for 10 min and centrifuged at 4000 rpm for 20 min at 4 °C. After centrifugation, each supernatant was subjected to a bioanalysis plate, followed by LC–MS analysis.

4.6. Cytidine deaminase (CDA) study

An *in vitro* CDA ELISA assay was performed with the free gemcitabine serving as the control and CDA was purchased from Solarbio. Gemcitabine (100 μM) and prodrugs **2** and **7** (100 μM) were incubated with 0.25 μg of CDA at 37 °C, and the absorbance was recorded at $\lambda = 280$ nm for 5, 20, 40 and 60 min.

4.7. Bioactivation study using human recombinant cysteine cathepsin L (CTSL)

Human recombinant cathepsin L protease was purchased from Novoprotein (Catalog #C401). Compound **1** (100 μg, 1 mM) and CTSL (1.8 μg) were incubated in a buffer solution (200 μL, pH 6.0) including 50 mM MES, 5 mM DTT and 1 mM EDTA at 37 °C for 24 hours. The solution was quenched by adding 90 μL methanol to a new tube containing 30 μL reaction mixture. The samples were spun down and decanted for HPLC loading. Reactions were analyzed by analytical HPLC chromatography with the gradient from 60% to 95% of methanol over 35 minutes. Product identities were confirmed by comparing retention times with controls and mass spectral analysis.

Author contributions

Mengmeng Wang: conceptualization, design, synthesis, data analysis and writing – review & editing. Kunyu Qu: synthesis, biological studies and writing – original draft. Peipei Zhao: biological studies and writing – original draft. Xin Yin: biological studies. Yiwei Meng: biological studies. Piet Herdewijn: conceptualization. Chao Liu: conceptualization and writing – review & editing. Lixin Zhang: supervision and resources. Xuekui Xia: supervision, resources, and writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

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