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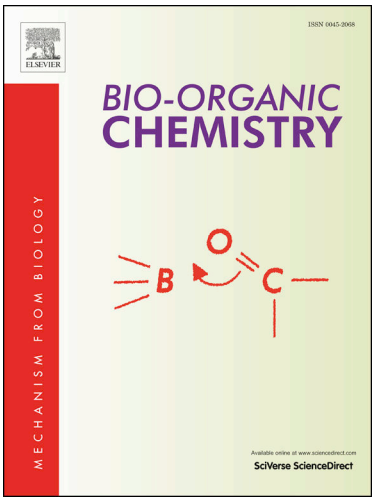


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# Design, Synthesis and Biological Evaluation of Hybrid of Ubenimex-Fluorouracil for Hepatocellular Carcinoma Therapy

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**ABSTRACT**

In our previous study, we discovered a ubenimex-fluorouracil (5FU) conjugates BC-02, which displays significant *in vivo* anti-tumor activity, however, the instability of BC-02 in plasma limits its further development as a drug candidate. Herein, we designed and synthesized four novel ubenimex-5FU conjugates by optimizing the linkers between ubenimex and 5FU based on BC-02. Representative compound **20** is more stable than BC-02 in human plasma and displays about 100 times higher CD13 inhibitory activity than the positive control ubenimex. Meanwhile, the antiproliferative activity of **20** was comparable with 5FU *in vitro*. The preliminary mechanism study indicated that compound **20** exhibited significant anti-invasion and

anti-angiogenesis activities *in vitro*. Furthermore, compound **20** obviously inhibits tumor growth and metastasis in vivo and prolong the survival time of tumor-bearing mice. Our study may have an important implication reference for the design of more druglike mutual prodrug, and compound **20** can be used as a lead compound for further design and development.

Keywords: Anti-cancer, APN/CD13 inhibitor, Mutual prodrug, angiogenesis, metastasis.

## 1. Introduction

CD13, also known as aminopeptidase N (EC 3.4.11.2), is a zinc dependent type 2 transmembrane ecto-peptidase, which expresses on various cell types, especially on the surface of tumor cells [1]. CD13 is involved in a variety of biological functions [2], including invasion, metastasis [3], neovascularization [4, 5], chemotherapy resistance[6, 7] and so on. Recently, it has been reported that CD13 is a biomarker of liver cancer stem cells, which is closely related to drug resistance and cancer recurrence [8]. Moreover, several studies have found that fluorouracil, doxorubicin, gemcitabine and sorafenib treatment can increase the expression of CD13, which is one of the most important reasons for cytotoxic drug resistance [9, 10]. More recent studies have revealed that high expression of CD13 is associated with poor prognosis and patient survival in a variety of cancers, such as hepatocellular carcinoma (HCC) [11], multiple myeloma [12], pancreas and colon cancers [13, 14], and non-small cell lung cancer [15]. Therefore, CD13 is regarded as an important target for the treatment of cancer [16].

In recent years, CD13 has been used as a ligand target in antibody conjugated drugs (ADCs) and peptide conjugated drugs (PDCs). In 2020, Zapata et al. reported the first antibody conjugated drug MI130110 using CD13 monoclonal antibody as ligand, which is tumor tissue specific and displays remarkable anti-tumor activity [17]. CD13 inhibitor NGR (ASN-GLY-ARG) based peptide conjugated drugs NGR-hTNFα and tTF-NGR specifically recognize CD13, and are currently in phase III and phase I clinical stages respectively [18, 19]. Therefore, the development of CD13 targeted conjugated drugs (TDC) has become a promising field in development of anti-tumor drugs.

Recent studies have found that the combination of 5FU and CD13 inhibitor ubenimex synergistically inhibits the proliferation of liver cancer cells *in vitro* and *in vivo* [8]. Our previous studies have reported a series of 5FU and ubenimex mutual prodrugs [20, 21], among which, compound BC-02 displayed significant *in vivo* anti-tumor activity, even in 5FU-resistant liver cancer model. We further revealed that the promising activity of BC-02 in 5FU-resistance tumor cells may due to the down-regulation of CD13 and increase of Reactive oxygen species (ROS) level [22]. Moreover, BC-02 effectively suppressed self-renewal and malignant proliferation of cancer stem cells (CSCs) by targeting CD13 and intracellular ROS-induced DNA damage[23]. Despite the outstanding anti-tumor activity, BC-02 has been found to degrade rapidly in plasma, which limits its further study as a drug candidate. Therefore, to improve its plasma stability, we optimize the chemical structure of BC-02 by modifying the linker between ubenimex and 5FU in this current study (**Fig. 1**). We also conduct detailed in vivo and in vitro anti-tumor study and human plasma stability study of the newly designed compounds. ~~Representative compound **20** slowly cleaves to 5FU in human plasma with a $t_{1/2}$ of 12 h, which is much longer than that of BC-02 ($t_{1/2}$: 1.5 h), which indicates the significantly improved plasma stability of **20**. Slow release of 5FU can prolong the time of 5FU *in vivo*, reduce the metabolic inactivation of 5FU, so that improve the anti-tumor efficacy. The *in vitro* and *in vivo* antitumor activity studies also showed that **20** could inhibit tumor growth, metastasis and prolong survival.~~

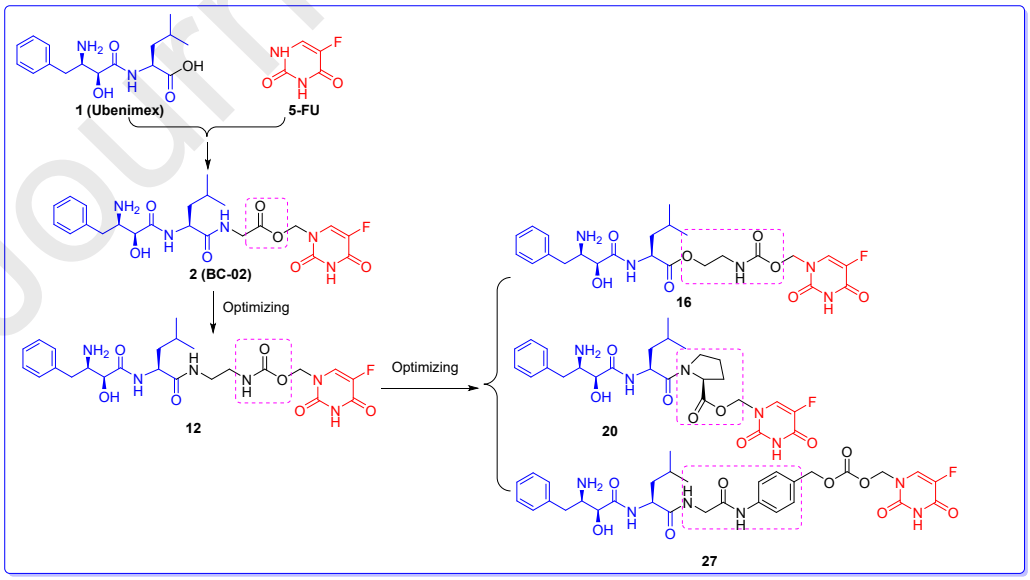



**Figure 1.** Drug design and chemical structure of targeted compounds.

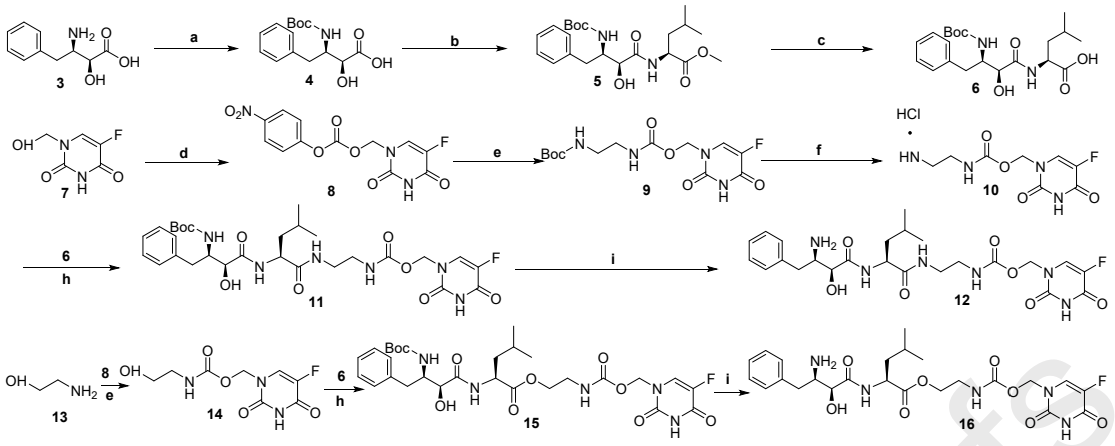
## 2. Results and discussion

### *2.1. Chemistry*

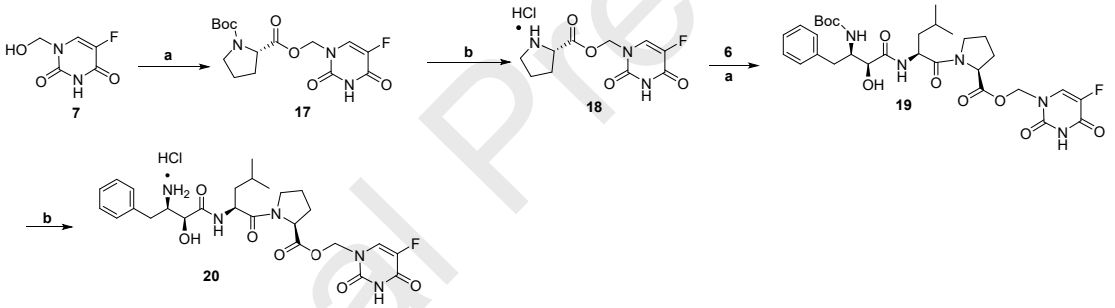
Compounds **12** and **16** were synthesized following the procedures showed in **Scheme 1**. The start material **3** was protected by $(Boc)_2O$, then coupled with methyl *L*-leucinate hydrochloride in the presence of 1-hydroxybenzotriazole (HOBt) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) to obtain compound **5.** Cleavage of the protecting group with base got compound **6**. Compound **7** was coupled with acyl chloride to get intermediate **8**. Boc-ethylenediamine and **8** generated carbamate **9** in alkaline conditions, and intermediate **9** was converted to **10** in the presence of HCl in ethyl acetate. Condensation of **10** and **6** in the presence of HOBt and EDCI gave the amide **11**. Cleavage of the Boc protecting group with HCl in ethyl acetate and alkalified afforded the target compound **12**. Compound **16** was prepared by the same procedure described for **12** except using 2-aminoethanol.

The synthesis of compound **20** was described in **Scheme 2**. In the presence of HOBt and EDCI, **7** reacted with *N*-Boc-*L*-proline to obtain ester **17**. Using the HCl in ethyl acetate to remove the protecting group of intermediate **17** to afford **18**, which was condensed with compound **6** to obtain **19**. Cleavage the Boc protecting group with HCl in ethyl acetate to obtain the target compound **20**.

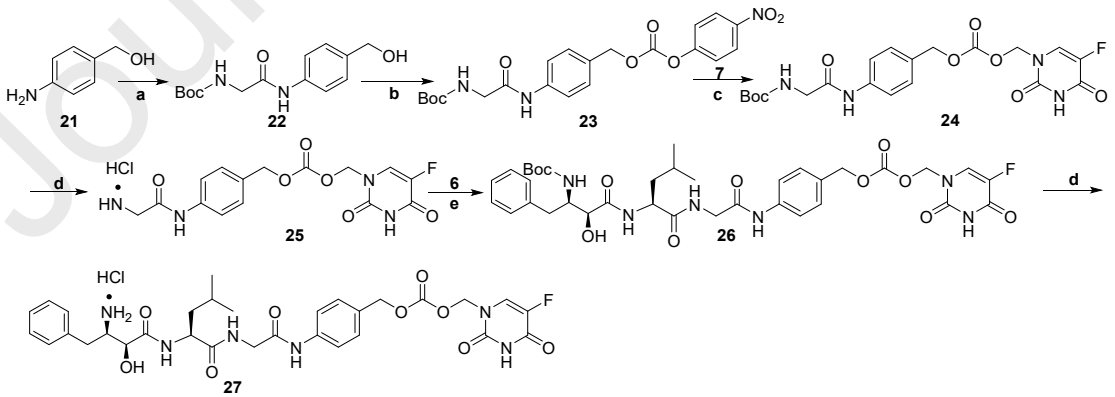
As described in **Scheme 3**, the compound **27** was synthesized from the starting material **21**. **22** was obtained by using EDCI and HOBt as condensing agent, then the hydroxyl was converted to carbonate **23**. In the presence of DMAP, the carbonate bond was replaced by **7** to obtain **24**. The remaining steps were prepared by the same procedure described for **12**.



**Scheme 1:** Synthesis of Compounds **12** and **16**. Reagents and conditions: (a) $(Boc)_2O$, 1N NaOH; (b) Methyl *L*-leucinate hydrochloride, EDCI, HOBt, DCM; (c) 1.5 N NaOH, MeOH, 80 °C; (d) *p*-nitrophenyl chloroformate, TEA, THF; (e) Boc-Ethylenediamine, TEA, DMAP, DCE, 60 °C; (f) dry HCl in EtOAc; (h) EDCI, HOBt, DCM; (i) dry HCl in EtOAc, TEA.



**Scheme 2:** Synthesis of Compound **20**. Reagents and conditions: (a) EDCI, HOBt, DCM; (b) dry HCl in EtOAc.



**Scheme 3:** Synthesis of Compound **27**. Reagents and conditions: (a) Boc-glycine, EDCI, HOBt, DCM; (b) *p*-Nitrophenyl chloroformate, TEA, THF; (c) TEA, DMAP, DCE, 60 °C; (d) dry HCl in EtOAc; (e) EDCI, HOBt, TEA, DCM.

### *2.2. Compounds design*

Carbamate is a frequent used group in the design of many prodrugs [24, 25]. Capecitabine, a 5FU prodrug, is designed using carbamate group, which has better stability than ester group [26]. Therefore, in order to improve the stability of BC-02, the carbamate was used to replace of the ester bond of BC-02 to afford compound **12** (**Fig. 1**).

### *2.3. Biological evaluation of compound 12*

Firstly, the CD13 inhibitory activity of compound **12** was determined using ubenimex as the positive control. As showed in **table 1**, the $IC_{50}$ value of potency of compound **12** against CD13 from porcine kidney is 6.8-fold higher than ubenimex, with an $IC_{50}$ value of 1.35 μM.

**Table 1**

**CD13 inhibitory activity of compound 12.**

| Cpd | $IC_{50}$(μM)[a] toward CD13 from porcine kidney |
|---|---|
| **12** | 1.35 |
| **Ubenimex** | 9.21 |

[a]Assays were performed in replicate (n ≥ 2); the standard deviation (SD) values are <20% of the mean.

Four cancer cell lines including breast cancer cell MDA-MB-231, leukemia cell HEL and KG1 and prostate cancer cell PC-3 were chosen to evaluate the antiproliferative activity of compound **12**, 5FU and ubenimex. Results illustrate that compound **12** exhibits no antiproliferative effect against all the cell lines up to 200 μM (**Table 2**). It is not surprising because the carbamate linker may stable *in vitro* and could not cleave and release 5FU during cell culture.

**Table 2**

**Antiproliferative Activities of compound 12, 5-FU and Ubenimex ($IC_{50}$ in μM[a])**

| Cpd | MDA-MB-231 | HEL | KG1 | PC-3 |
|---|---|---|---|---|
| **12** | >200 | >200 | >200 | >200 |

| | | | | |
|---|---|---|---|---|
| **5-FU** | 24.45 | 27.55 | 96.03 | 41.05 |
| **Ubenimex** | >500 | >500 | >500 | >500 |

[a]Assays were performed in replicate (n ≥ 2); the SD values are <20% of the mean.

As a frequently used group of prodrugs, carbamate was reported can be metabolized *in vivo* [24-26]. Therefore, we next investigated the anti-tumor effect of compound **12** *in vivo* in a mouse HCC H22 cell transplant model and human hypopharyngeal adenocarcinoma cell line FaDu xenograft model. It is disappointed to find that compound **12** exhibits no obvious antitumor activity in both animal models with i.p. or oral administration (**Fig. 2a-2d**). We suppose the ineffective results may due to the high structural stability of carbamate in plasma. Then we checked the stability of compound **12** in human and rat plasma using a HPLC method. To our expectation, compound **12** can not cleave in both human and rat plasma within 24 h (**Fig. S1**), which meets our speculation that carbamate is too stable to cleave.

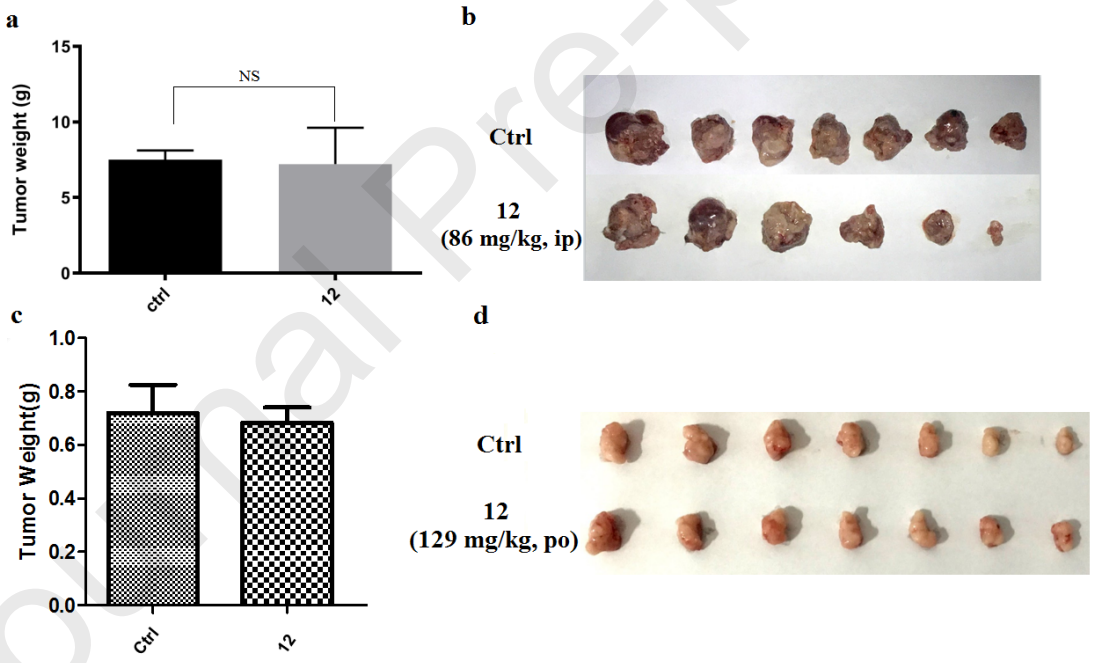



**Figure 2. In vivo antitumor activities of compound 12.** (a) Weights of tumors in HCC H22 subcutaneous model (Student's t-test). Error bars indicate standard error of the mean (SEM); (b). Photographs of H22 tumors tissue; (c) Weights of tumors in Fadu xenograft model, Error bars indicate SEM; (d). Photographs of Fadu tumor tissues.

### *2.4. Biological evaluation of compound 16, 20 and 27*

Although compound **12** is more stable than BC-02, the carbamate structure is too stable to cleave both *in vitro* and *in vivo*, which results in ineffective anti-tumor activity. Therefore, we aim to find a degradable linker which is more stable than the ester of BC-02 as well as releases 5FU in a suitable speed for the further modification. Firstly, replacement of amide bond of compound **12** by ester bond afforded compound **16** (**Fig. 1**). We speculated that the hydrolysis of ester bond of compound **16** can accelerate the further hydrolysis of carbamate. On the other hand, we also designed and synthesized compounds **20** and **27** by using the cyclic substituted ester and carbonates.

Firstly, the CD13 inhibitory activity of compound **16**, **20**, **27** was screened using the commercial CD13 enzyme. All of the compounds display higher inhibitory activity than the positive control ubenimex (**Table 3**). Among which, compound **20** ($IC_{50}$=0.096 μM) is about 100-fold more potent than ubenimex ($IC_{50}$=9.21 μM). Moreover, the CD13 inhibitory activity conducted with ES-2 cells (**Table 3**) also confirms the superior activities of compound **20**.

**Table 3**

**CD13 inhibitory activity of compound 16, 20 and 27.**

| *Compds* | $IC_{50}$(μM)[a] toward CD13 from porcine kidney | $IC_{50}$(μM)[a] toward CD13 on the surface of ES-2 cells |
|---|---|---|
| **16** | 1.29 | - |
| **20** | 0.096 | 0.24 |
| **27** | 0.787 | - |
| **Ubenimex** | 9.21 | 16.27 |
| **BC-02** [21] | 0.18 | 1.68 |

[a]Assays were performed in replicate (n ≥ 2); the SD values are <20% of the mean.

In order to explore the interaction between target compound **20** and CD13, we conducted the docking study using a Sybyl/Sketch module. Similar with ubenimex,

the hydroxyl group of **20** chelates with the zinc ion of CD13, the benzene ring and isobutyl of **20** insert into the S1 and S1' hydrophobic pockets respectively (**Fig. 3**). It should be noted that the restricted conformation of 5-membered ring linker of **20** makes the 5FU moieties effectively occupy the S2' bigger pocket (**Fig. 3**). This might be the reason why **20** displayed a better inhibitory efficacy against CD13 than ubenimex (**Table 3**).

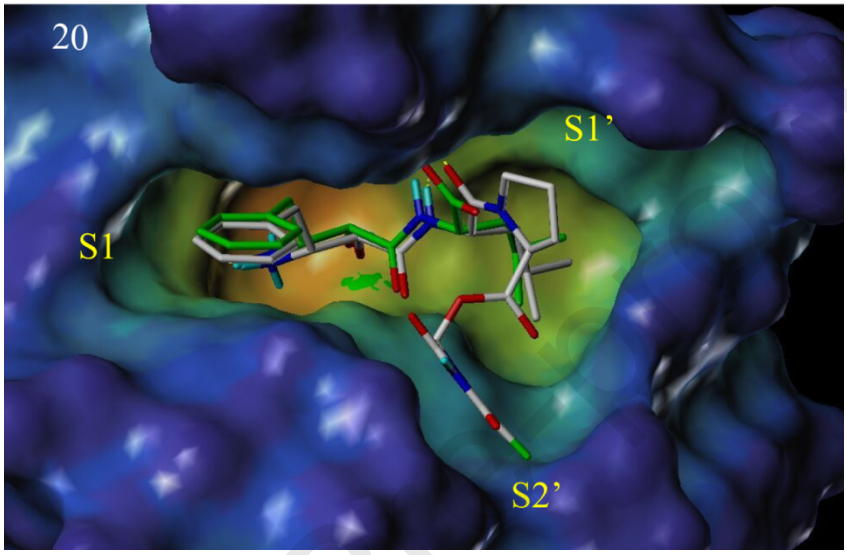



**Figure 3**. Proposed binding preferences comparison of compound **20** at CD13 (derived by modification of PDB code 2DQM using Tripos SYBYL 8.0). **20** (white) aligned with ubenimex (green) in a CD13 homology model. The green sphere is zinc ion, and the dashed lines stand for the hydrogen bonds (atom types: H, white; N, blue; O, red).

Six human solid tumor cell lines PLC/PRF/5, HUH7, HepG2, MHCC97H, MCF-7 and ES-2 as well as three of haematological tumor cell lines HL-60, MV4-11 and RS4;11 were chosen for the *in vitro* anti-tumor assay of compounds **16**, **20** and **27**. The antiproliferative activities of **20** and **27** are comparable with that of 5FU while the compound **16** displays much weaker activity (**Table 4**). The positive result displays these three compounds can cleave and release 5FU during cell culture *in vitro*. Then we tested the stability of these three compounds in human plasma. As shown in **figure 4a-4c**, compound **27** and **16** are completely cleaved within 2 h and 12 h, respectively. Compound **20** cleaves much more slowly, with a total cleavage time of more than 24 h. It should be noted that **27** and **20** release 5FU as degradation product, while **16** releases compound **14** (structure shown in scheme 1) as degradation product. The *in*

*vitro* antiproliferative and stability study shows compound **20** owns a suitable degradation linker, which makes it more stable than BC-02 in human plasma (the completely cleavage time of BC-02 was 8 h) [21] and release 5FU in a proper rate. Similar result is also observed in rat plasma. Therefore, compound **20** was chosen for further *in vivo* biological evaluation.

**Table 4**

**Antiproliferative Activities of compounds 16, 20, 27 and 5FU ($IC_{50}$ in μM[a]).**

| Cell lines | 16 | 20 | 27 | 5-FU | Ubenimex[20,21] |
|---|---|---|---|---|---|
| **PLC/PRF/5** | - | 29.05 | - | 35.66 | >500 |
| **HUH7** | >200 | 34.89 | 7.42 | 14.53 | >500 |
| **HepG2** | >200 | 12.02 | 6.03 | 4.13 | >500 |
| **MHCC97H** | >200 | 53.31 | 11.77 | 12.31 | - |
| **MCF-7** | 52.91 | 9.92 | 4.14 | 3.06 | >500 |
| **ES-2** | - | 65.63 | - | 71.47 | >500 |
| **HL-60** | 184.70 | 13.16 | 3.62 | 2.79 | >500 |
| **MV4-11** | 119.90 | 2.68 | 1.03 | 0.85 | >500 |
| **RS4;11** | >200 | 10.05 | 3.63 | 2.81 | - |

[a]Assays were performed in replicate ($n \geq 2$); the SD values are <20% of the mean.

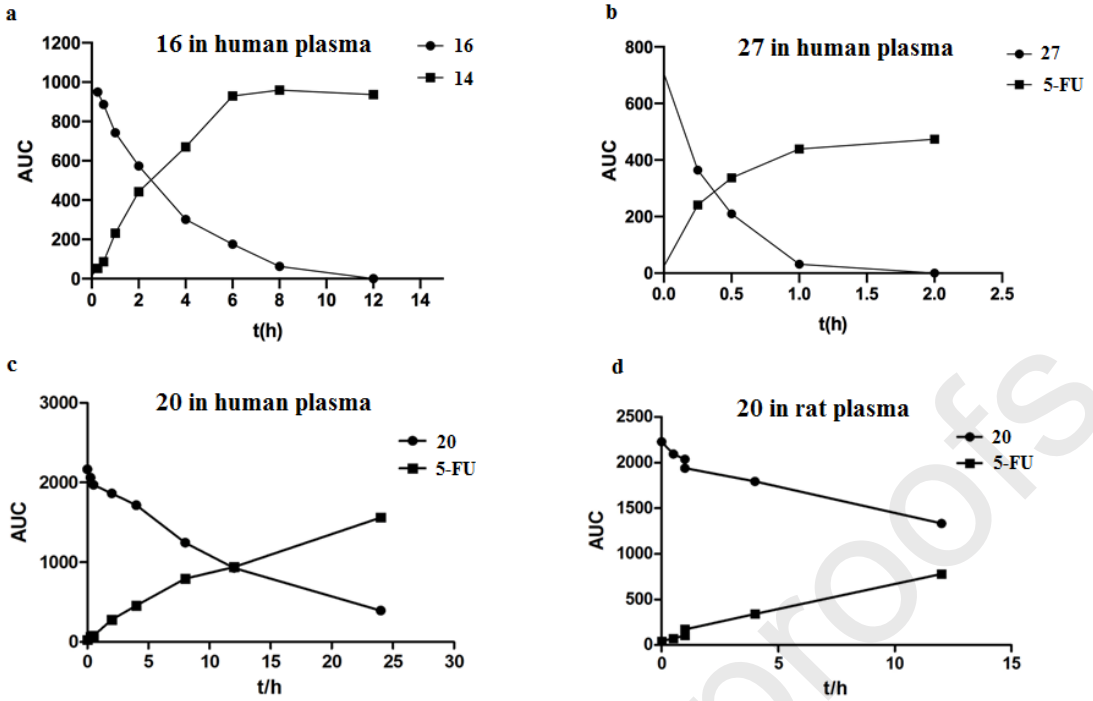



**Figure 4**. Stability of compound **16**, **20** and **27** in human plasma or rat plasma; (a). Points of compound **16** were obtained after 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h, respectively; (b). Points of compound **27** were obtained after 0, 0.25, 0.5, 1 and 2 h, respectively; (c). Points of compound **20** were obtained after 0, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h, respectively; (d). Points of compound **20** were obtained after 0, 0.25, 0.5, 1, 2, 4 and 12 h, respectively.

The mouse heptoma H22 tumor transplant model was utilized to evaluate the antitumor efficacy of compound **20** *in vivo*. Capecitabine is the most widely used and successful 5FU prodrug in clinical. Therefore, capecitabine and 5FU were used as positive controls in this experiment. Compound **20** (100 μmol/kg, iv) displays the comparable anti-tumor activity with positive control capecitabine (300 μmol/kg, po), while it shows better antitumor activity than that of 5FU (150 μmol/kg, iv) (**Fig. 5**). No significant toxicity or body weight loss is observed in the compound **20** treated animals, one tumor tissue disappeared during compound **20** treatment. Two mice died in 5FU treated group, which indicates its highly toxicity at dose of 150 μmol/kg.

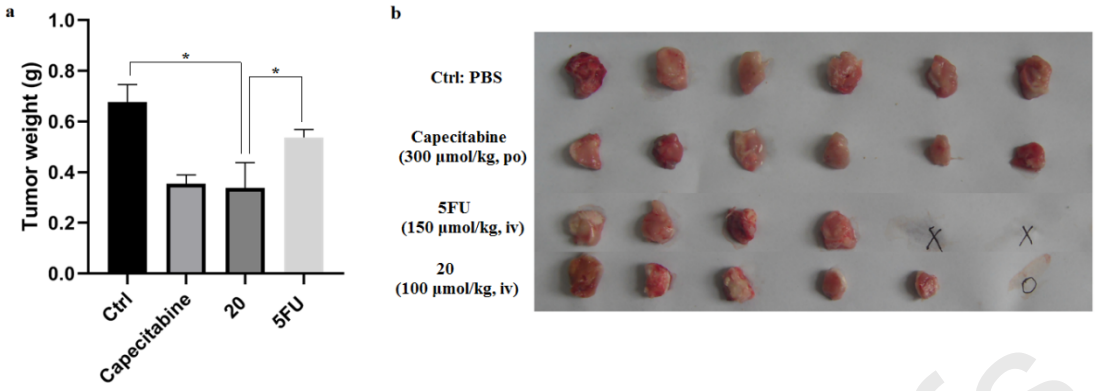



**Figure 5. In vivo antitumor activities of 20.** (a) Weights of tumors in subcutaneous model (*$P$ < 0.05; Student's t-test). Error bars indicate SEM. (b). Photographs of tumor tissues. The "O" means no tumor detected, while the "X" means mice died in the experiment.

CD13 is regarded as a key target in cancer migration, metastasis and angiogenesis. Therefore, in this study we checked the anti-metastasis and anti-angiogenesis of compound **20**. The in vitro anti-invasion assay demonstrated that compound **20** significantly inhibits ES-2 cell invasion at 5 μg/mL, which is more potent than ubenimex at the same concentration (**Fig. 6a**). Data shown in figure 6b demonstrated that compound **20** exhibits superior inhibitory effect on human umbilical vein endothelial cells (HUVECs) tube formation than the positive control ubenimex at the same dose (30 μM) (**Fig. 6b**). On the other hand, as described in **figure 6c,** the number of microvessels sprouting from aortic rings treated with compound **20** were much fewer than that of ubenimex (20 μg/mL). Together, our data reveals the remarkble anti-angiogenesis activities of **20**.

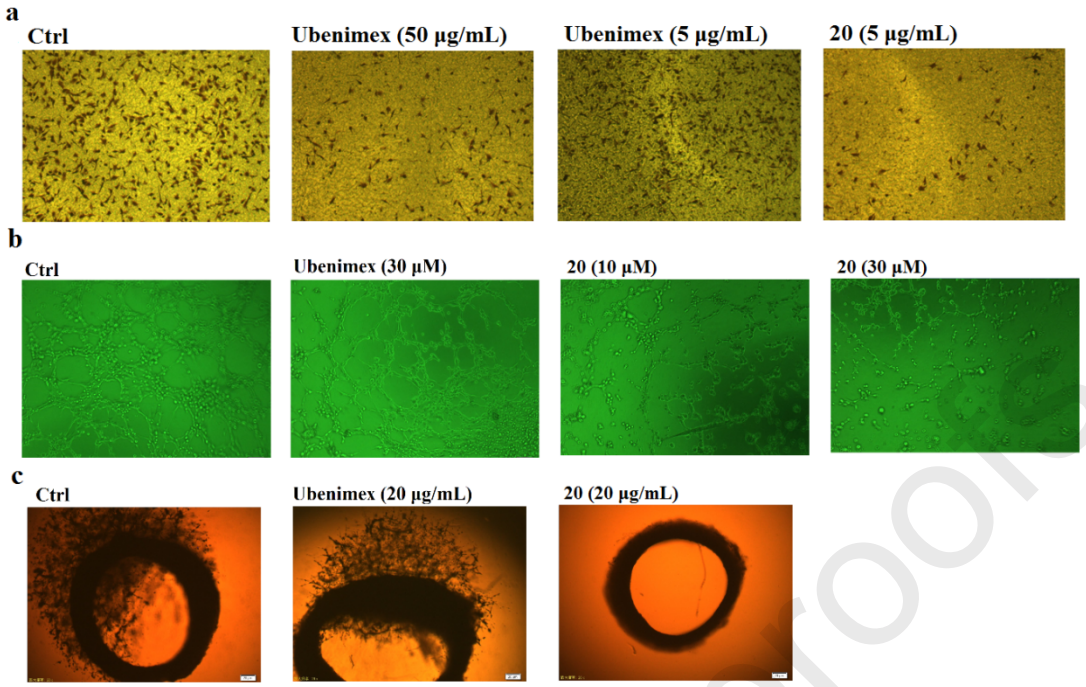



**Figure 6**. (a) Representative photographs of anti-invasion results after treatment of **20** and Ubenimex (positive control), the assay was performed on transwell chambers coated with Matrigel using ES-2 cells; (b) Representative photographs of the HUVEC capillary-like tubular network after treatment of **20** and ubenimex. (c) Representative photographs of rat aortic rings after treatment of **20** and ubenimex.

In vivo anti-metastasis effect of compound **20** was performed in a H22 pulmonary metastasis tumor model. A significant decrease of H22 pulmonary nodes is detected in compound **20** (150 μmol/kg, iv) treated group, revealing remarkable anti-metastasis effect (**Fig. 7**). Capecitabine also decreases the number of H22 pulmonary nodes, while its anti-metastasis activity is much weaker than compound **20** (**Fig. 7**).

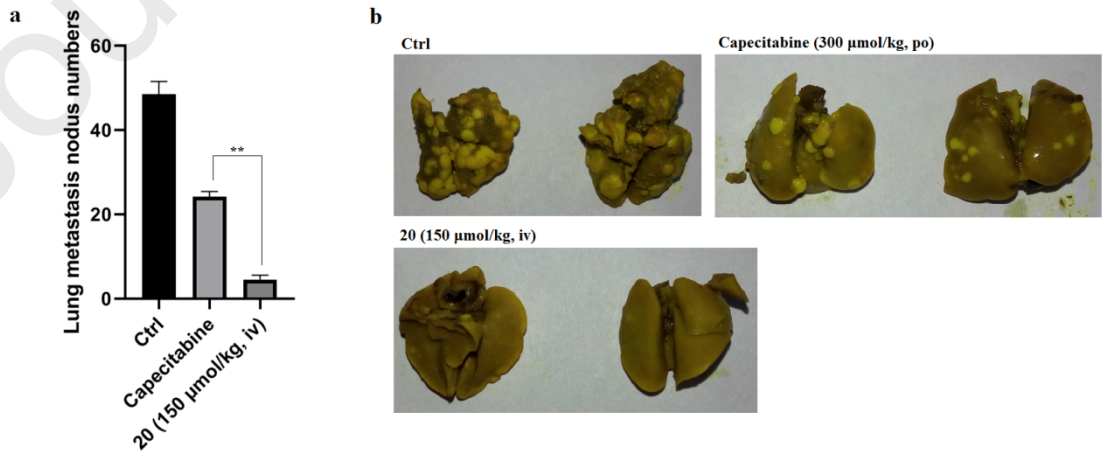


**Figure 7. In vivo anti-metastasis activity of 20.** (a) Lung metastasis nodus numbers after treatment of **20** and capecitabine (positive control) in the H22 hepatoma pulmonary metastasis model (** $P < 0.01$; Student's t-test). Error bars indicate SEM (b) Representative photographs of lungs.

Based on the above *in vitro* and *in vivo* results of the potential anticancer effect of compound **20**, we next investigated whether it could be extending the lifespan of tumor bearing mice. Survival curves suggested that treatment with compound **20** at 150 μmol/kg obviously increases the survival time with statistically significant differences regarding the control group. In detail, the median survival time (MST) of mice treated with **20** is increased 17 days, which is to 1.5-fold longer than control group (11days) and comparable with that of capecitabine (300 μmol/kg) (**Fig. 8**).

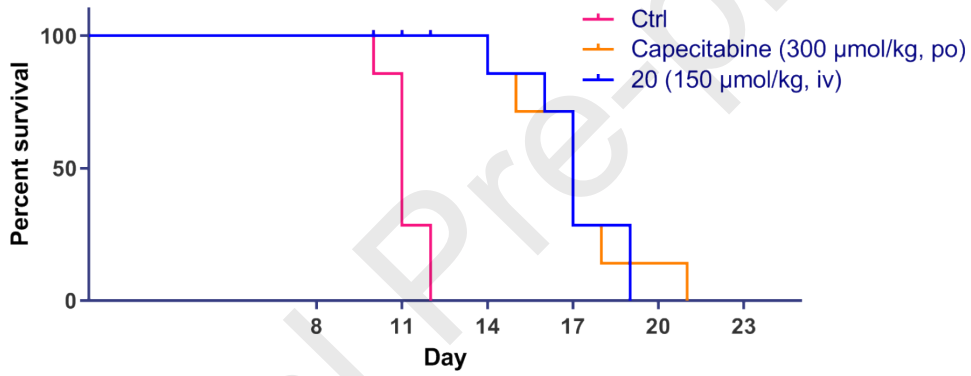



**Figure 8.** Kaplan-Meier curves showing survival of mice treated with normal PBS (pink), **20** (blue) and Capecitabine (yellow, positive control).

Previous studies demonstrated that CD13 was a CSC marker in HCC and upregulation of CD13 was related to tumor resistance [8]. After treatment with 5FU, the expression of CD13 was upregulated. And ubenimex can reduce the number of CD13-positive cells thereby sensitizing cancer cells to the 5FU [8-10]. Therefore, flow cytometry was utilized to evaluate the influence of compound **20** on CD13-positive fraction of PLC/PRF/5 cells. As shown in **figure 9**, the number of CD13-positive fraction of PLC/PRF/5 cells is increased by 5FU treatment, while compound **20** dramatically decreases the CD13-positive PLC/PRF/5 cells, revealing compound **20** might reduce the population of the CSC marker positive cells.

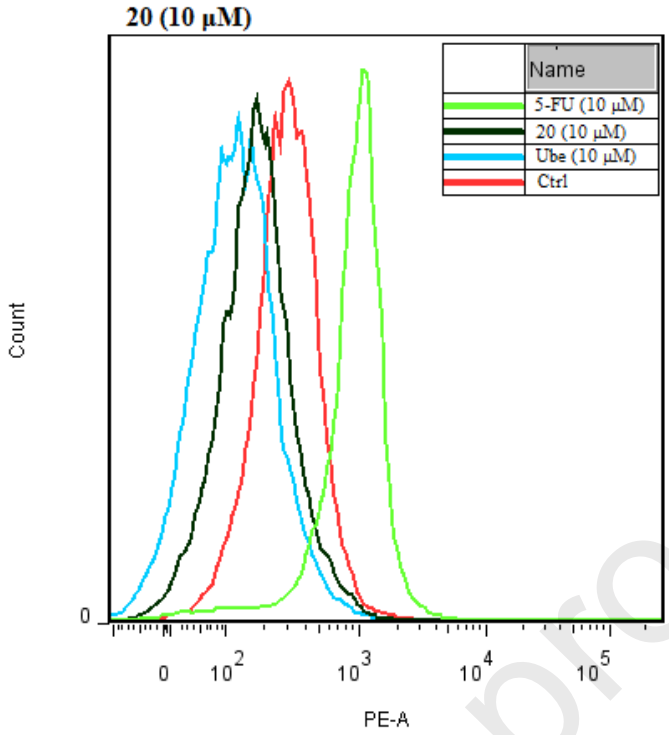



**Figure 9.** Expression of CD13 in PLC/PRF/5 cells after treatment of 5FU, ubenimex and **20** for 48 h at 10 µM.

## 3. Conclusion

In our previous study, we reported that the hybrid compound BC-02 exhibited remarkable *in vitro* and *in vivo* anti-tumor activity. However, the poor plasma stability of BC-02 hindered its further development. In the present study, we optimize the stability of BC-02 by modifying the linker between ubenimex and 5FU and get four newly designed compounds. ~~Among which, compound **20** is more stable than BC-02 and release 5FU in a proper rate.~~ The CD13 inhibitory activity of compound **20** is more potent than ubenimex and BC-02. The plasma stability study demonstrated that compound **20** was more stable than that of BC-02. The *in vitro* and *in vivo* anticancer study revealed that compound **20** exhibited moderate antitumor activity, which is comparable with positive control 5-FU and capecitabine. Furthermore, compound **20** exhibits significant anti-metastasis, anti-angiogenesis and CD13-positive cell

elimination effects. In this study, we found that the stability of the linkers plays an important role in the antitumor activity. If the linker is too stable to degrade, the antitumor activity will be decreased even eliminated (compound **12**), which means the cytotoxicity activity mainly comes from the released 5FU. Our study provides an important reference for the design of more druglike mutual prodrug for the purpose of clinical application. Altogether, these results also suggest that compound **20** can be deemed as a potential lead for further development of more potent analogs as potential anticancer agents.

## 4. Experimental section

### *4.1. Materials and Methods.*

All starting materials, reagents, and solvents were purchased from reagent company and used without further purification unless otherwise stated. TLC plates (60GF-254) was used to monitor all reactions. Silica gel or C18 silica gel was used for column chromatography purification. NMR spectra were obtained on a JOE 400YH 400 MHz instrument with TMS as an internal standard, δ in parts per million and *J* in hertz. High-resolution mass spectrometry was gathered by ocean university of China in qingdao, China. The HPLC (Agilent 1260 Infinity, column: Agilent 5 TC-C18 column (5 μm, 250 mm × 4.6 mm) was used for purity detection of compounds. The purity of all the target compounds were more than 95%.

The compounds **4** and **7** was prepared using the same procedures with our previously work [20].

*4.1.1. Methyl ((2S,3R)-3-((tert-butoxycarbonyl)amino)-2-hydroxy-4-phenylbut-anoyl)-L-leuc-inate (**5**).*

To a solution of compound **4** (1.0 g, 3.39 mmol) in dry DCM, EDCI (0.78 g, 4.06 mmol) and HOBt (0.55 g, 4.06 mmol) were added at 0 °C. After 30 min, methyl *L*-leucinate hydrochloride (737.5 mg, 4.06 mmol) and TEA (0.75 g, 7.5 mmol) were

added. The reaction was allowed to stir overnight, then was washed with 1 M HCl aqueous solution, and brine for 3 times, and dried over anhydrous $MgSO_4$. Volatiles were removed under vacuum to get the crude residue which was purified via flash chromatography to afford the compound **5**, a white solid powder (0.89 g, yield: 62%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.65 (d, $J$ = 8.6 Hz, 1H), 7.33-7.05 (m, 5H), 6.23 (d, $J$ = 9.4 Hz, 1H), 6.02 (d, $J$ = 6.5 Hz, 1H), 4.34-4.15 (m, 1H), 4.00-3.85 (m, 1H), 3.84-3.77 (m, 1H), 3.60 (s, 3H), 2.82-2.71 (m, 1H), 2.66-2.57 (m, 1H), 1.89-1.72 (m, 1H), 1.42-1.31 (s, 9H), 0.86-0.68 (m, 6H).

*4.1.2. ((2S,3R)-3-((tert-butoxycarbonyl)amino)-2-hydroxy-4-phenylbutanoyl)-L-leucine (**6**).*

Compound **5** (0.80 g, 1.89 mmol) was dissolved in 15 mL of MeOH, then 2 mL of 3 M KOH aqueous solution was added. The mixture was refluxed at 85 °C for 2 h. MeOH was evaporated under vacuum after the reaction completed. The residue was acidified with 1 M HCl to pH 5-6, then filtered. The precipitate was corresponding acid compound **6**, a white solid (0.69 g, yield: 90%). The crude material was used directly for the next step without further purification. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.72 (d, $J$ = 8.6 Hz, 1H), 7.25-7.14 (m, 5H), 6.10 (d, $J$ = 9.3 Hz, 1H), 4.30-4.22 (m, 1H), 3.92-3.84 (m, 1H), 3.79 (d, $J$ = 2.8 Hz, 1H), 2.80-2.72 (m, 1H), 2.65-2.56 (m, 1H), 1.63-1.53 (m, 2H), 1.48-1.40 (m, 1H), 1.25 (s, 9H), 0.81 (dd, $J$ = 21.4, 6.3 Hz, 6H).

*4.1.3. (5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl (4-nitrophenyl) carbonate (**8**).*

Compound **7** (1.0 g, 6.25 mmol) and TEA (0.95 mg, 9.38 mmol) were dissolved in dry THF, then the solution of *p*-nitrophenyl chloroformate (1.9 g, 9.38 mmol) in dry THF was added dropwise into the mixture at 0 °C. The reaction was allowed to

stir at room temperature overnight. The precipitate was filtered out, then filtrate was concentrated to get the crude residue which was purified via flash chromatography to afford the compound **8**, a white solid powder (1.3 g, yield: 65%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.06 (d, $J$ = 5.0 Hz, 1H), 8.33-8.27 (m, 2H), 8.13 (d, $J$ = 6.6 Hz, 1H), 7.60-7.52 (m, 2H), 5.72 (s, 2H).

*4.1.4. tert-butyl ((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl)methyl) ethane-1,2-di-yldicarbamate (**9**).*

To a solution of compound **8** (1.0 g, 3.08 mmol) in DCE, *Boc*-ethylenediamine (0.59 g, 3.7 mmol), TEA (0.37 g, 3.7 mmol) and 4-dimethylaminopyridine (DMAP) (0.45 g, 3.7 mmol) were added. The reaction was allowed to stir at 60 °C for 2 h. 15 mL of DCM was added to the mixture, then the DCM layer was washed with saturated brine (2 × 30 mL), and dried over anhydrous $MgSO_4$. Volatiles were removed under vacuum to get the crude residue which was purified via flash chromatography to afford the compound **9**, a white solid powder (0.8 g, yield: 75%). $^1$H NMR (600 MHz, DMSO-$d_6$) δ 11.95 (s, 1H), 8.06 (d, $J$ = 6.5 Hz, 1H), 7.51 (t, $J$ = 5.5 Hz, 1H), 6.81 (t, $J$ = 5.7 Hz, 1H), 5.49 (s, 2H), 3.08-2.91 (m, 4H), 1.36 (s, 9H).

*4.1.5. (5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl (2-(λ$^2$-azane-yl)ethyl)car-bamate hydrochloride (**10**).*

Compound **9** (0.73 g, 2.1 mmol) was dissolved in a solution of EtOAc (20 mL) saturated by dry HCl gas, then the reaction was stired at room temperature for 30 min. The filtered precipitate was washed by diethyl ether to give compound **10**, a white solid powder (0.54 g, yield: 92%). The crude material was used directly for the next step without further purification. $^1$H NMR (600 MHz, DMSO-$d_6$) δ 11.98 (s, 1H), 8.22-8.01 (m, 4H), 7.73 (t, $J$ = 5.7 Hz, 1H), 5.51 (s, 2H), 3.27 (q, $J$ = 6.2 Hz, 2H), 2.84 (s, 2H).

*4.1.6. (5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl ((6R,7S,10S)-6-benzyl-7-hydroxy-10-isobutyl-2,2-dimethyl-4,8,11-trioxo-3-oxa-5,9,12-triazatetradec-na-14-yl)carbamate (**11**).*

Compound **6** (0.41 g, 1 mmol) was dissolved in dry DCM (15 mL), EDCI (0.23 g, 1.2 mmol) and HOBt (0.16 g, 1.2 mmol) were added at 0 °C. 30 min later, compound **10** (0.34 g, 1.2 mmol) and TEA (0.22 g, 2.2 mmol) were added. The reaction was stirred at room temperature overnight. Then was washed with 1 M HCl aqueous solution (2 × 30 mL), and brine (2 × 30 mL), and dried over anhydrous $MgSO_4$. Volatiles were removed under vacuum to get the crude residue which was purified via flash chromatography to afford the compound **11**, a white solid powder (0.34 g, yield: 53%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 11.95 (s, 1H), 8.07-7.94 (m, 2H), 7.57 (d, $J$ = 9.0 Hz, 1H), 7.49 (t, $J$ = 4.8, 3.8 Hz, 1H), 7.27-7.12 (m, 5H), 6.15 (d, $J$ = 9.3 Hz, 1H), 5.93 (d, $J$ = 6.1 Hz, 1H), 5.43 (s, 2H), 4.33-4.18 (m, 1H), 3.91 (q, $J$ = 9.2, 8.5 Hz, 1H), 3.82-3.74 (m, 1H), 3.01 (dt, $J$ = 21.9, 6.4 Hz, 4H), 2.74 (dd, $J$ = 13.5, 7.3 Hz, 1H), 2.62 (dd, $J$ = 13.5, 7.8 Hz, 1H), 1.55-1.34 (m, 3H), 1.24 (s, 9H), 0.79 (dd, $J$ = 17.8, 6.2 Hz, 6H).

*4.1.7. (5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl(2-((S)-2-((2S,3R)-3-amino-2-hydroxy-4-phenylbutanamido)-4-methylpentanami-do)ethyl)carbamate (**12**).*

Compound **11** (0.30 g, 0.47 mmol) was dissolved in a solution of EtOAc (10 mL) saturated by dry HCl gas. The solution was stirred at room temperature for 3 h. The filtered precipitate was washed by diethyl ether, then the residue was alkalified with TEA to pH 8-9. The crude residue was purified via flash chromatography to afford the compound **12**, a white solid powder (0.23 g, yield: 87%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.16 (t, $J$ = 5.5 Hz, 1H), 8.03 (d, $J$ = 6.5 Hz, 1H), 7.90 (d, $J$ = 8.2 Hz, 1H), 7.49 (t, $J$ = 5.5 Hz, 1H), 7.32-7.16 (m, 5H), 5.43 (s, 2H), 4.24-4.15 (m, 1H), 3.90 (d, $J$ = 3.1 Hz, 1H), 3.10-3.00 (m, 2H), 3.00-2.91 (m, 3H), 2.91-2.81 (m, 1H), 2.81-2.70 (m, 1H), 1.64-1.38 (m, 3H), 0.85-0.75 (m, 6H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 172.5, 171.4, 171.1, 136.8, 129.9, 129.6, 129.1, 127.4, 68.7, 60.2, 54.7, 51.5, 41.5, 35.1, 24.6, 23.4, 22.4. HRMS (AP-ESI) m/z calcd for $C_{24}H_{34}O_7N_6F$ $[M+H]^+$ 537.24634, found 537.24675. Retention time: 14.1 min, eluted with 30% acetonitrile/70% water (containing 0.1% triethylamine and 0.15% trifluoroacetic acid).

*4.1.8. (5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl)methyl(2-hydroxyeth-yl)carbamate (**14**).*

The title compound was prepared by the same procedure described for **9** except using 2-aminoethanol as a white solid, 71% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 11.93 (s, 1H), 8.05 (d, $J$ = 6.6 Hz, 1H), 7.46 (t, $J$ = 5.7 Hz, 1H), 5.44 (s, 2H), 4.68 (t, $J$ = 5.6 Hz, 1H), 3.35-3.29 (m, 2H), 3.01-2.98 (m, 2H).

*4.1.9. 2-((((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl)methoxy)carbonoyl)-amino)-ethyl((2S,3R)-3-((tert-butoxycarbonyl)amino)-2-hydroxy-4-phenylbutanoyl)-L-leucinate (**15**).*

The title compound was prepared by the same procedure described for **11** as a white solid, 48% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 11.95 (s, 1H), 8.04 (d, $J$ = 6.6 Hz, 1H), 7.89 (d, $J$ = 8.6 Hz, 1H), 7.66 (t, $J$ = 5.6 Hz, 1H), 7.26-7.11 (m, 6H), 6.10 (d, $J$ = 9.3 Hz, 1H), 5.98 (d, $J$ = 6.6 Hz, 1H), 5.72 (s, 1H), 5.45 (s, 2H), 4.43-4.28 (m, 1H), 4.04-3.94 (m, 2H), 3.94-3.82 (m, 1H), 3.84-3.76 (m, 1H), 3.19 (d, $J$ = 5.6 Hz, 3H), 2.81-2.69 (m, 1H), 2.68-2.55 (m, 1H), 1.67-1.48 (m, 3H), 1.50-1.37 (m, 2H), 1.24 (s, 9H), 0.86-0.72 (m, 8H).

*4.1.10. 2-((((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl)methoxy)carbonoyl)-amino)-ethyl((2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl)-L-leucinate (**16**).*

The title compound was prepared by the same procedure described for **12** as a white solid, 85% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.30 (d, $J$ = 7.4 Hz, 1H), 8.03 (d, $J$ = 6.5 Hz, 1H), 7.36-7.20 (m, 5H), 5.44 (s, 1H), 4.30-4.17 (m, 1H), 4.09-3.88 (m, 2H), 3.51-3.40 (m, 2H), 3.21-3.15 (m, 1H), 2.93-2.83 (m, 1H), 2.83-2.74 (m, 1H), 1.66-1.37 (m, 3H), 0.83 (dd, $J$ = 9.6, 6.0 Hz, 6H). $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.62, 171.82, 155.82, 149.90, 137.01, 129.96, 129.17, 127.42, 71.23, 69.13, 63.66, 54.90, 50.93, 46.01, 24.74, 23.12, 22.08, 9.10. HRMS (AP-ESI) m/z calcd for $C_{24}H_{33}O_8N_5F$ $[M+H]^+$ 538.23102, found 538.23077. Retention time: 5.9 min, eluted with 35% acetoni-trile/65% water (containing 0.1% triethylamine and 0.15% trifluoroacetic acid).

*4.1.11. 1-(tert-butyl)2-((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl)methyl) (S)-pyrroli-dine-1,2-dicarboxylate (**17**).*

The title compound was prepared by the same procedure described for **5** except using **7** and *N*-Boc-*L*-proline as a white solid, 61% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.06 (d, $J$ = 5.0 Hz, 1H), 8.17 (d, $J$ = 6.6 Hz, 1H), 5.65 (s, 2H), 4.18 (dd, $J$ = 8.7, 3.8 Hz, 2H), 4.08-3.97 (m, 1H), 2.27-2.17 (m, 2H), 1.83-1.81 (m, 2H), 1.37 (s, 9H).

*4.1.12. (5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl)methyl-L-prolinate hydro-chloride (**18**).*

The title compound was prepared by the same procedure described for **10** as a white solid, 88% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.03 (d, $J$ = 5.0 Hz, 1H), 8.18 (d, $J$ = 6.6 Hz, 1H), 5.67 (d, $J$ = 1.4 Hz, 2H), 4.37 (t, $J$ = 8.0 Hz, 1H), 3.20-3.14 (m, 2H), 2.27-2.12 (m, 1H), 2.02-1.92 (m, 1H), 1.92-1.79 (m, 3H).

*4.1.13. (5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl)methyl ((2S,3R)-3-((tert-butoxy-carbonyl)amino)-2-hydroxy-4-phenylbutanoyl)-L-leucyl-L-prolinate (**19**).*

The title compound was prepared by the same procedure described for **11** as a white solid, 55% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 11.99 (s, 1H), 8.08 (d, $J$ = 6.7 Hz, 1H), 7.58 (d, $J$ = 8.8 Hz, 1H), 7.28-7.09 (m, 6H), 6.17 (d, $J$ = 9.5 Hz, 1H), 6.00 (d, $J$ = 6.7 Hz, 1H), 5.63 (d, $J$ = 10.2 Hz, 1H), 5.49 (d, $J$ = 10.1 Hz, 1H), 4.63-4.51 (m, 1H), 4.34 – 4.24 (m, 1H), 3.95-3.83 (m, 1H), 3.79-3.71 (m, 1H), 3.67-3.55 (m, 1H), 3.51-3.40 (m, 1H), 2.73 (dd, $J$ = 13.2, 7.1 Hz, 1H), 2.61 (dd, $J$ = 13.2, 7.7 Hz, 1H), 2.21-2.05 (m, 1H), 1.95-1.78 (m, 3H), 1.65-1.49 (m, 1H), 1.47-1.31 (m, 2H), 1.24 (s, 9H), 0.81 (dd, $J$ = 6.6, 3.4 Hz, 6H).

*4.1.14. (5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl)methyl ((2S,3R)-3-amino-2-hydro-xy-4-phenylbutanoyl)-L-leucyl-L-prolinate hydrochloride (**20**).*

The compound **20** was prepared by the same procedure described for **12** except alkalifying as a white solid, 82% yield. $^{1}$H-NMR(400 MHz DMSO-$d_6$): $\delta$ 12.01 (d, $J$=5.0 Hz, 1H), 8.15-8.12 (m, 2H), 8.02 (s, 3H), 7.36-7.25 (m, 5H), 6.69 (s, 1H), 5.72-5.51 (m, 2H), 4.52-4.46 (m, 1H), 4.34-4.31 (m, 1H), 4.04-3.97 (m, 1H), 3.74-3.68 (m, 1H), 3.53-3.50 (m, 2H), 2.95-2.86 (m, 2H), 2.21-2.12 (m, 1H), 1.99-1.82 (m, 3H), 1.69-1.62 (m, 1H), 1.52-1.36 (m, 2H), 0.92-0.87 (m, 6H). $^{13}$C NMR (101 MHz, DMSO-$d_6$): $\delta$ 171.8, 171.5, 170.9, 158.2, 149.8, 141.3, 137.0, 130.1, 129.7, 129.3, 127.6, 71.7, 68.7, 59.1, 54.9, 49.5, 47.2, 35.3, 29.1, 25.3, 24.7, 23.7, 22.4. HRMS (AP-ESI) m/z calcd for $C_{26}H_{35}O_7N_5F$ $[M + H]^+$ 548.25140, found 548.25150. Retention time: 12.6 min, eluted with 30% acetonitrile/70% water (containing 0.1% triethylamine and 0.15% trifluoroacetic acid).

*4.1.15. tert-butyl (2-((4-(hydroxymethyl)phenyl)amino)-2-oxoethyl)carbamate (**22**).*

The title compound was prepared by the same procedure described for **5** except using 4-aminobenzenemethanol and Boc-glycine as a white solid, 43% yield. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 10.00 (s, 1H), 7.58 (d, $J$ = 8.2 Hz, 2H), 7.33 (d, $J$ = 8.3 Hz, 2H), 7.04 (t, $J$ = 6.1 Hz, 1H), 5.08 (s, 2H), 3.72 (d, $J$ = 6.2 Hz, 2H), 1.39 (s, 9H).

*4.1.16. tert-butyl (2-((4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-2-oxoethyl) carbamate (**23**).*

The title compound was prepared by the same procedure described for **8** except using **22** as a white solid, 75% yield. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 10.03 (s, 1H), 8.36-8.27 (m, 2H), 7.63 (d, $J$ = 8.2 Hz, 2H), 7.62-7.53 (m, 2H), 7.42 (d, $J$ = 8.3 Hz, 2H), 7.06 (t, $J$ = 6.2 Hz, 1H), 5.25 (s, 2H), 3.73 (d, $J$ = 6.2 Hz, 2H), 1.40 (s, 9H).

*4.1.17. tert-butyl (2-((4-(((((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin1(2H)yl)meth-oxy)carb-onyl)oxy)methyl)phenyl)amino)-2-oxoethyl)carbamate (**24**).*

The title compound was prepared by the same procedure described for **9** except using **7** as a white solid, 65% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.02 (s, 1H), 10.01 (s, 1H), 8.14 (d, $J$ = 6.5 Hz, 1H), 7.60 (d, $J$ = 8.2 Hz, 2H), 7.34 (d, $J$ = 8.2 Hz, 2H), 7.05 (t, $J$ = 6.2 Hz, 1H), 5.61 (s, 2H), 5.11 (s, 2H), 3.72 (d, $J$ = 6.1 Hz, 2H), 1.39 (s, 9H).

*4.1.18. 4-(2-aminoacetamido)benzyl ((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)yl)me-thyl) carbonate hydrochloride (**25**).*

The title compound was prepared by the same procedure described for **10** as a white solid, 84% yield. The crude material was used directly for the next step without further purification.

*4.1.19. tert-butyl((2R,3S)-4-(((S)-1-((2-((4-(((((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methoxy)carbonyl)oxy)methyl)phenyl)amino)-2-oxoethyl)amino)-4-methyl-1-oxopentan-2-yl)amino)-3-hydroxy-4-oxo-1-phenylbutan-2-yl)carbamate (**26**).*

The title compound was prepared by the same procedure described for **11** as a white solid, 52% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.00 (d, $J$ = 5.0 Hz, 1H), 9.92 (s, 1H), 8.37 (t, $J$ = 5.9 Hz, 1H), 8.11 (d, $J$ = 6.5 Hz, 1H), 7.72 (d, $J$ = 8.5 Hz, 1H), 7.56 (d, $J$ = 8.1 Hz, 2H), 7.32-7.13 (m, 7H), 6.20 (d, $J$ = 9.3 Hz, 1H), 5.95 (d, $J$ = 6.2 Hz, 1H), 5.57 (s, 2H), 5.08 (s, 2H), 4.43-4.28 (m, 1H), 3.98-3.89 (m, 1H), 3.84-3.79 (m, 2H), 2.76 (dd, $J$ = 13.2, 7.2 Hz, 1H), 2.63 (dd, $J$ = 13.1, 7.9 Hz, 1H), 1.67-1.36 (m, 4H), 1.25 (s, 9H), 0.82 (dd, $J$ = 18.2, 6.4 Hz, 6H).

*4.1.20. 4-(2-((S)-2-((2S,3R)-3-amino-2-hydroxy-4-phenylbutanamido)-4-methyl-pentanamido) acetamido)benzyl ((5-fluoro-2,4-dioxo-3,4-dihydropyri-midin-1(2H)-yl)methyl) carbo-nate hydrochloride (**27**).*

The title compound was prepared by the same procedure described for **12** except alkalifying as a white solid, 82% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.01 (d, $J$ = 4.9 Hz, 1H), 10.00 (s, 1H), 8.54 (t, $J$ = 5.9 Hz, 1H), 8.22-8.02 (m, 5H), 7.62 (d, $J$ = 8.2 Hz, 2H), 7.41-7.29 (m, 7H), 6.75 (s, 1H), 5.62 (s, 2H), 5.12 (s, 2H), 4.35-4.25 (m, 1H), 4.05-4.03 (m, 1H), 3.87 (d, $J$ = 6.4 Hz, 2H), 3.01-2.90 (m, 2H), 1.73-1.53 (m,

3H), 0.90 (dd, $J$ = 9.1, 6.4 Hz, 6H). $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 172.72, 171.68, 168.23, 158.10, 157.84, 153.99, 149.78, 141.15, 139.66, 138.85, 136.90, 130.12, 129.99, 129.84, 129.79, 129.16, 127.44, 119.43, 73.89, 69.92, 68.77, 54.73, 52.15, 43.22, 41.01, 35.20, 24.69, 23.44, 22.47. HRMS (AP-ESI) m/z calcd for $C_{31}H_{38}O_9N_6F$ $[M + H]^+$ 657.26758, found 657.26758. Retention time: 7.0 min, eluted with 35% acetonitrile/65% water (containing 0.1% triethylamine and 0.15% trifluoroacetic acid).

### *4.2. Biological assays*

#### *4.2.1. CD13 inhibition assay[27].*

The effect of target compounds on CD13 activity was determined by incubating microsomal aminopeptidase from porcine kidney microsomes (Sigma) with PBS (50 mM, pH 7.2) as a buffer at 37℃. First, the compounds were diluted to graded concentrations and added to 96-well microcrystal plates, then CD13 solution was added before incubating at 37℃ for 5 min, and finally *L*-leucine-p-nitroaniline solution was added as a substrate. The plates were incubated for 30 min at 37°C. Measured the absorbance at 405nm with a Micro-plate Reader (Thermo Fisher, Shanghai, China) to infer the inhibitory effects of the compounds on the enzyme.

The activity of CD13 on the surface of ES-2 cells was determined by incubating 2.5 × $10^6$/mL cells in 1 × PBS buffer with different concentrations of test compounds and a certain concentration of substrate for 1 hour at 37℃. Similarly, the measurement was performed in 96-well microcrystal plates, the substrate was *L*-leucine-p-nitroaniline, and the absorbance of the well was measured at 405 nm.

#### *4.2.2. Cell proliferation analysis*

The cells in the 96-well plate were incubated with serially diluted compounds in an incubator for 3 days, then 0.5% MTT solution was added to each well and incubated the plate for 4 hours. Finally, 150 μL of dimethyl sulfoxide was added to each well, gently shaking 15 min, then measured the absorbance at 570 nm. All the above cell lines were cultured in RPMI1640 or DMEM medium and incubated in an incubator at 37℃ and 5% $CO_2$. All media were supplemented with 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin.

#### *4.2.3. Stability of target compounds in human plasma*

Human plasma samples were stored at -20°C and thawed gradually to room temperature before processing. Compounds **12**, **16**, **20** and **27** were dissolved in acetonitrile/water to make a 4 mg/mL stock solution, then added to human plasma to a final concentration of 0.4 mg/mL and incubated at 37°C over a period of 24 h. The incubation was terminated at the specific time points by adding acetonitrile. The mixture was vortexed for 30 seconds and centrifuged (12,000 rpm/10 min), then was filtered through 0.22 μm filters., and injected into the HPLC system (Agilent 1260 HPLC) for analysis with an Alltima C18 (5 μm, 4.6 mm × 250 mm) column in a gradian of 25% acetonitrile/75% water (containing 0.1% triethylamine and 0.15% trifluoroacetic acid) over 15 min at 262 nm.

#### *4.2.4. In vivo activity in H22 tumor transplant model*

All experiments in vivo were performed according to the institutional ethical guidelines on animal care and approved by the Institute Animal Care and Use Committee at ocean university of China. Before conducting animal experiments, all Kuming mice were placed in a specific pathogen-free (SPF) environment and provided with sterile food and water. Cultured H22 cells with RPMI1640 medium (containing 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin) in a 37°C, 5% $CO_2$ incubator. Cells were diluted to $1\times10^7$/mL with PBS and injected into the mice subcutaneously (0.2 mL). Three days later, the mice were randomly divided into several treatment groups and vehicle group. Mice in the treatment groups were treated with the specific dose of the test compounds for five days a week. Vehicle group was given the same volume of PBS in the same way. After two weeks adminstration, the mice were sacrificed. Tumor tissues were excised from the subcutaneous part of each mouse and weighed. Tumor inhibition rate is calculated by (1-average tumor weight in treatment group/average tumor weight in PBS group) *100%.

#### *4.2.5. In vivo activity of FaDu xenograft model*

Human FaDu cells were stored in DMEM medium containing 10% FBS at 37°C in a 5% $CO_2$ humidified incubator. FaDu cells were counted, prepared into cell suspension with PBS, and inoculated into BALB/c-nu mice (female, 5-6 weeks old, Slac Laboratory Animal, Shanghai, China) subcutaneously ($5\times10^6$ cells per mouse).

When the tumor volume was rose to approximately 100 $mm^3$, the mice were randomly divided into two groups. The mice in the treatment group were given a specific concentration of the test compounds, and the mice in the vehicle group were given the same volume of PBS solution in the same way. Mice were sacrificed by cervical dislocation and tumor tissues were harvested. Tumor inhibition rate is calculated by (1-average tumor weight in treatment group/average tumor weight in PBS group) *100%.

#### *4.2.6. Docking study*

The docking study of compound **20** and CD13 (PDB code 2DQM) was performed using Tripos SYBYL 8.0. Briefly, the structure of compound **20** was constructed using the Sybyl/Sketch module and optimized using Powell's method with the Tripos force field with convergence criterion set at 0.05 kcal/(Å mol) and assigned charges with the Gasteiger-Huckel method. Molecular docking was performed by the Sybyl/FlexX module.

#### *4.2.7. Invasion assay*

At beginning, matrigel (BD Biosciences, NJ) was used to coated the transwell filters. The cells were washed 3 times with serum-free medium (RPMI-1640), counted, prepared into cell suspension, and added to the upper compartment of the chamber (400 μL per well), and then 100 μL of the test compounds at the specific concentration were added. Added 750 μL of conditioned medium from ES-2 cells to the bottom compartment of the chamber and incubated the plate in a 37°C incubator for 24 hours. The transwell was washed twice with PBS, and stained with crystal violet solution (0.1%) before observed under a microscope.

#### *4.2.8. In vitro HUVEC tuber formation assay [28]*

Matrigel, a basement membrane matrix (BD Biosciences, NJ), was polymerized at 37°C for 30 min. Human umbilical vein endothelial cells (HUVEC) were suspended in M199 medium containing 5% FBS were inoculated on Matrigel (40,000 per well), treated with the test compounds at the specified concentration, and incubated at 37°C and 5% $CO_2$ for 6 hours. The cells were photographed under a phase contrast microscope at 200 × magnification.

#### *4.2.9. Rat thoracic aorta rings (TARs) assay [27]*

The thoracic aortas of Wistar rats (male, 8 to 10 weeks old) were removed from the connective tissues, washed with sterile PBS and cut transversely into 1 mm thick rings. The aortic rings were immersed in matrigel in the wells of 96-well plate, then another 100 μL of matrigel was added. After the second layer of matrigel set, the plate was incubated at 37 °C with 5% $CO_2$ for 30 minutes. The test compounds were added to the wells every other day for treatment, and on the 10th day, the sprouting of microvessels was photographed under a phase-contrast microscope at 200 × magnification.

#### *4.2.10. In vivo activity of H22 pulmonary metastasis model*

A heat lamp was used to dilate the tail vein of mice (6 weeks old, female), and then the mice were transferred to a restraining device. Diluted H22 cells with PBS to $7.5 \times 10^6$ cells/mL according to the aforementioned method, and injected 0.1 mL into the tail vein of each mouse. Tumor cells were allowed to grow for 10 days. On day 11, the mice received specific concentrations of compounds for 2 weeks (5 days administration in one week). After that, the mice were sacrificed. The lung tissues were removed and weighed and then fixed in Bouin's fixation solution for one day before the lung metastasis lymph nodes.

#### *4.2.11. Survival time in mice bearing H22 cells*

Kunming mice (6 weeks old, female) were inoculated with H22 cells ($4.0 \times 10^6$ cells/mouse) by intraperitoneal injection. Three days later, the mice were randomly divided into treatment groups and vehicle group. The mice in the treatment group were treated with the specified dose of the test compounds for 14 consecutive days, and the mice in the control group were given the same volume of PBS by the same method. The median survival time (MST) of the mice was measured to infer the anti-tumor activity of the compound.

#### *4.2.12. Determination of CD13-positive fraction of PLC/PRF/5 cells*

$2 \times 10^5$ cells per well of PLC/PRF/5 cells were seeded in 6-well plates and allowed to grow for 12 h, then added test compounds. After 48 hours, the cells were collected, washed twice with cold PBS. After that, PE anti-human CD13 was added to

1× PBS suspension, and the mixture was incubated at 4°C for 15 minutes. Finally, the cells were washed and analyzed with flow cytometry.

**Supporting Information**. Stability of compound **20** in human and rat plasma. $IC_{50}$ curves of compound **16**, **20**, **27** and 5FU for cancer cell lines. $IC_{50}$ curves of compound **12**, **16**, **20**, **27** and Ubenimex for CD13. $^{1}H$ and $^{13}C$ NMR spectral information of target compounds**12**, **16**, **20** and **27**. HPLC analysis chromatograms of target compound **12**, **16**, **20** and **27**.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## ABBREVIATIONS

ADCs, Antibody conjugated drugs; APN, Aminopeptidase N; $(Boc)_2O$, Di-tert-butyl dicarbonate; CSCs, Cancer stem cells; DCE, Dichloroethane; DCM, Dichloromethane; DMAP, Dimethylaminopyridine; EDCI, 1-(3-Dimethylaminopropyl)-3-ethylcarbo-diimide; 5FU, Fluorouracil; HCl, Hydrochloride; HCC, Hepatocellular carcinoma; HOBt, 1-hydroxybenzotriazole; HPLC, High Performance Liquid Chromatography; HUVEC, Human umbilical vein endothelial cells; $MgSO_4$, Magnesium sulfate; MST, Median survival time; NGR, ASN-GLY-ARG; PDCs, Peptide conjugated drugs; ROS, Reactive oxygen species; SD, Standard deviation; SEM, Standard error of the mean; TARs, Thoracic aorta rings; TDC, Target conjugated drugs; TEA, Triethylamine; THF, Tetrahydrofuran; TNF, tumor necrosis factor.

**REFERENCES**


[1] B. Bauvois, D. Dauzonne, Aminopeptidase-n/cd13 (ec 3.4.11.2) inhibitors: Chemistry, biological evaluations, and therapeutic prospects, Med. Res. Rev. 26 (2006) 88-130.

[2] P. Mina-Osorio,The moonlighting enzyme cd13: Old and new functions to target, Trends Mol. Med. 14 (2008) 361-371.

[3] M. Ghosh, R. Lo, I. Ivic, B. Aguilera, V. Qendro, C. Devarakonda, L. H. Shapiro, Cd13 tethers the iqgap1-arf6-efa6 complex to the plasma membrane to promote arf6 activation, β1 integrin recycling, and cell migration, Sci. Signal. 12 (2019) eaav5938.

[4] L. Guzman-Rojas, R. Rangel, A. Salameh, J. K. Edwards, E. Dondossola, Y. G. Kim, A. Saghatelian, R. J. Giordano, M. G. Kolonin, F. I. Staquicini, E. Koivunen, R. L. Sidman, W. Arap, R. Pasqualini,Cooperative effects of aminopeptidase n (cd13) expressed by nonmalignant and cancer cells within the tumor microenvironment, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 1637-1642.

[5] E. Dondossola, R. Rangel, L. Guzman-Rojas, E. M. Barbu, H. Hosoya, L. S. St John, J. J. Molldrem, A. Corti, R. L. Sidman, W. Arap, R. Pasqualini,Cd13-positive bone marrow-derived myeloid cells promote angiogenesis, tumor growth, and metastasis, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 20717-20722.

[6] Q. Guo, X. Li, M. N. Cui, J. L. Sun, H. Y. Ji, B. B. Ni, M. X. Yan, Cd13: A key player in multidrug resistance in cancer chemotherapy, Oncol. Res. 28 (2020) 533-540.

[7] B. Hu, Y. Xu, Y. C. Li, J. F. Huang, J. W. Cheng, W. Guo, Y. Yin, Y. Gao, P. X. Wang, S. Y. Wu, J. Zhou, J. Fan, X. R. Yang, Cd13 promotes hepatocellular carcinogenesis and sorafenib resistance by activating hdac5-lsd1-nf-κb oncogenic signaling, Clin. Transl. Med. 10 (2020) e233-249.

[8] N. Haraguchi, H. Ishii, K. Mimori, F. Tanaka, M. Ohkuma, H. M. Kim, H. Akita, D. Takiuchi, H. Hatano, H. Nagano, G. F. Barnard, Y. Doki, M. Mori,Cd13 is a therapeutic target in human liver cancer stem cells, J. Clin. Invest. 120 (2010) 3326-3339.

[9] M. Yamashita, H. Wada, H. Eguchi, H. Ogawa, D. Yamada, T. Noda, T. Asaoka, K. Kawamoto, K. Gotoh, K. Umeshita, Y. Doki, M. Mori,A cd13 inhibitor, ubenimex, synergistically enhances the effects of anticancer drugs in hepatocellular carcinoma, Int. J. Oncol. 49 (2016) 89-98.

[10] S. Ji, Y. Ma, X. Xing, B. Ge, Y. Li, X. Xu, J. Song, M. Xiao, F. Gao, W. Jiang, C. Fang, X. Wang, Suppression of cd13 enhances the cytotoxic effect of chemotherapeutic drugs in hepatocellular carcinoma cells, Front. Pharmacol. 12 (2021) 660377.

[11] S. Saida, K. Watanabe, I. Kato, H. Fujino, K. Umeda, S. Okamoto, S. Uemoto, T. Hishiki, H. Yoshida, S. Tanaka, S. Adachi, A. Niwa, T. Nakahata, T. Heike,Prognostic significance of aminopeptidase-n (cd13) in hepatoblastoma, Pediatr. Int. 57 (2015) 558-566.

[12] H. Shim, J. H. Ha, H. Lee, J. Y. Sohn, H. J. Kim, H. S. Eom, S. Y. Kong, Expression of myeloid antigen in neoplastic plasma cells is related to adverse prognosis in patients with multiple myeloma, Biomed Res. Int. 2014 (2014) 893243.

[13] N. Ikeda, Y. Nakajima, T. Tokuhara, N. Hattori, M. Sho, H. Kanehiro, M. Miyake,Clinical significance of aminopeptidase n/cd13 expression in human pancreatic carcinoma, Clin. Cancer Res. 9 (2003) 1503-1508.

[14] H. Hashida, A. Takabayashi, M. Kanai, M. Adachi, K. Kondo, N. Kohno, Y. Yamaoka, M. Miyake, Aminopeptidase n is involved in cell motility and angiogenesis: Its clinical significance in human colon cancer, Gastroenterology. 122 (2002) 376-386.

[15] Q. Zhang, J. Wang, H. Zhang, D. Zhao, Z. Zhang, S. Zhang, Expression and clinical significance of aminopeptidase n/cd13 in non-small cell lung cancer, J. Cancer Res. Ther. 11 (2015) 223-228.

[16] S. A. Amin, N. Adhikari, T. Jha, Design of aminopeptidase n inhibitors as anti-cancer agents, J. Med. Chem. 61 (2018) 6468-6490.

[17] J. M. Domínguez, G. Pérez-Chacón, M. J. Guillén, M. J. Muñoz-Alonso, B. Somovilla-Crespo, D. Cibrián, B. Acosta-Iborra, M. Adrados, C. Muñoz-Calleja, C. Cuevas, F. Sánchez-Madrid, P. Avilés, J. M. Zapata, Cd13 as a new tumor target for antibody-drug conjugates: Validation with the conjugate mi130110, J. Hematol Oncol. 13 (2020) 32-47.

[18] A. Corti, F. Pastorino, F. Curnis, W. Arap, M. Ponzoni, R. Pasqualini, Targeted drug delivery and penetration into solid tumors, Med. Res. Rev. 32 (2012) 1078-1091.

[19] C. Schliemann, M. Gerwing, H. Heinzow, S. Harrach, C. Schwöppe, M. Wildgruber, A. A. Hansmeier, L. Angenendt, A. F. Berdel, U. Stalmann, B. Berning, K. Kratz-Albers, K. Middelberg-Bisping, S. Wiebe, J. Albring, C. Wilms, W. Hartmann, E.

Wardelmann, T. Krähling, W. Heindel, J. Gerss, E. Bormann, H. Schmidt, G. Lenz, T. Kessler, R. M. Mesters, W. E. Berdel, First-in-class cd13-targeted tissue factor ttf-ngr in patients with recurrent or refractory malignant tumors: Results of a phase i dose-escalation study, Cancers (Basel). 12 (2020) 1488-1510.

[20] Y. Jiang, X. Li, J. Hou, Y. Huang, Y. Jia, M. Zou, J. Zhang, X. Wang, W. Xu, Y. Zhang, Discovery of bc-01, a novel mutual prodrug (hybrid drug) of ubenimex and fluorouracil as anticancer agent, Eur. J. Med. Chem. 121 (2016) 649-657.

[21] Y. Jiang, X. Li, J. Hou, Y. Huang, X. Wang, Y. Jia, Q. Wang, W. Xu, J. Zhang, Y. Zhang, Synthesis and biological characterization of ubenimex-fluorouracil conjugates for anti-cancer therapy, Eur. J. Med. Chem. 143 (2018) 334-347.

[22] J. Zhang, C. Fang, M. Qu, H. Wu, X. Wang, H. Zhang, H. Ma, Z. Zhang, Y. Huang, L. Shi, S. Liang, Z. Gao, W. Song, X. Wang, Cd13 inhibition enhances cytotoxic effect of chemotherapy agents, Front. Pharmacol. 9 (2018) 1042-1052.

[23] C. Dou, C. Fang, Y. Zhao, X. Fu, Y. Zhang, D. Zhu, H. Wu, H. Liu, J. Zhang, W. Xu, Z. Liu, H. Wang, D. Li, X. Wang, Bc-02 eradicates liver cancer stem cells by upregulating the ros-dependent DNA damage, Int J. Oncol. 51 (2017) 1775-1784.

[24] C. Skarbek, S. Serra, H. Maslah, E. Rascol, R. Labruère, Arylboronate prodrugs of doxorubicin as promising chemotherapy for pancreatic cancer, Bioorg. Chem. 91 (2019) 103158.

[25] C. Zhang, Y. Qu, X. Ma, M. Li, S. Li, Y. Li, L. Wu, Nqo1-selective activated prodrugs of combretastatin a-4: Synthesis and biological evaluation, Bioorg. Chem. 103 (2020) 104200.

[26] S. Kobuchi, Y. Yazaki, Y. Ito, T. Sakaeda, Circadian variations in the pharmacokinetics of capecitabine and its metabolites in rats, Eur. J. Pharm. Sci. 112 (2018) 152-158.

[27] L. Su, J. Cao, Y. Jia, X. Zhang, H. Fang, W. Xu, Development of synthetic aminopeptidase n/cd13 inhibitors to overcome cancer metastasis and angiogenesis, ACS Med. Chem. Lett. 3 (2012) 959-964.

[28] F. Xu, Y. Jia, Q. Wen, X. Wang, L. Zhang, Y. Zhang, K. Yang, W. Xu, Synthesis and biological evaluation of n-(4-hydroxy-3-mercaptonaphthalen-1-yl)amides as inhibitors of angiogenesis and tumor growth, Eur. J. Med. Chem. 64 (2013) 377-388.