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Design, synthesis and antitumor activity evaluation of pretubulysin analogs

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Abstract Pretubulysin, a biosynthetic precursor of the tubulysins, shows potent biological activity in a variety of tumor cell lines. Although there are several total synthesis routes to tubulysin and pretubulysin reported, the commercialization still has been hampered due to the complexity of the structure. To find structurally simpler pretubulysin analogs, a series of 2-(3-(methylamino)propyl)thiazole-4-carboxamides are designed and synthesized, and their anticancer activities are screened using MCF-7 (breast cancer), and NCI-H157 (lung cancer) cell lines. Taxol (IC_{50} = 0.01μM) and pretubulysin are used as the control. Compounds **8c** (IC_{50} = 0.05 μM, MCF-7; 0.09 μM, NCI-H157) and **8h** (IC_{50} = 0.01 μM, MCF-7; 0.02 μM, NCI-H157) exhibited certain antitumor activities comparable to those of Taxol. The urea analogs of

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pretubulysin might represent a promising scaffold for the further development of novel antitumor drugs.

Key words： antitumor activity; pretubulysin; structural modification; tubulysin; urea

1. Introduction

Tubulysins, a family of tetrapeptides isolated from extracts of myxobacteria cultures, showed extraordinary antitumor activities by binding to tubulin near the vinca alkaloid binding site and inhibiting tubulin polymerization.[1] Even its structure was simpler than tubulysins, pretubulysin (**1, Fig. 1**), a presumed biosynthetic precursor of the tubulysins,[2-4] was still bearing similar antitumor efficacy in the nanomolar region.[5] Recently, Pretubulysin also displayed potential use in the treatment for tumor growth,[6-8] metastasis,[9] and angiogenesis reducing.[2] However, the structural complexity and low yield of pretubulysin led to the commercial supply a challenging issue. Since the first total synthesis of Tubulysin D reported by Ellman,[10] numerous medicinal chemists had put more effort into pursuing concise total synthesis routes,[11,12] and simpler structural analogs.[13,14]

It was reported that the isopropyl group of the Tuv moiety played an essential role in maintaining the antitumor activity of pretubulysin.[15,16] Compounds would lose antitumor activity when the isopropyl group was replaced by an aromatic cyclic group or a piperidine group.[17,18] Meanwhile, The Mep and Tup parts were tolerant of many structural modifications.[13,19,20] In our lab, we previously found that the compounds with urea group in Mep part would still possess certain antitumor activity.[18, 21] For example, Compounds **2** and **3** (**Fig. 1**) exhibited antiproliferative activity against MCF-7 human breast cell lines with an IC_{50} value of 0.2 μM and 0.47 μM, respectively. To simplify the pretubulysin synthesis and increase the hydrophobicity by introducing aromatic ring(s), a series of 2-(3-(methylamino)propyl)thiazole-4-carboxamides (**I**,

Fig.1), which combines both the urea moiety and Tuv fragment of pretubulysin, were designed and synthesized, and the *in vitro* antitumor activity was measured by MTT assay.

2. Results and discussion

2.1 Chemistry

The synthesis of 2-(3-(methylamino)propyl)thiazole-4-carboxamides was depicted in **Scheme 1**. A three-step synthesis process was employed to transform commercially available ethyl bromopyruvate **4** into thiazole ester compound **6**. Firstly, a thiazole ring was formed by a cyclization reaction of **4** with thioacetamide in ethanol. Secondly, bromination of the methyl group afforded the compound ethyl 2-(bromomethyl)thiazole-4-carboxylate **5**, by the bromine radical generated from NBS and dibenzoyl peroxide (BPO). Finally, after converting compound **5** to the corresponding phosphonium bromide by treatment with triphenylphosphine, then a Witting reaction with (*S*)-*N*-tert-Butoxycarbonyl-*N*-methylvalinal[22]afforded the thiazole ester **6** in moderate yield. The olefin group of intermediate **6** was reduced under hydrogen conditions, and the ester group was then hydrolyzed under alkaline conditions to afford the corresponding carboxylic acid. The resultant acid and a variety of amines, including methylamine, benzylamine, and phenylethylamine, yielded amides **7a–7c** under classic peptide coupling condition (HOBt, EDCI, DIPEA). Removal of the Boc group in compounds **7a–7c**, followed by coupling with a series of 2-ureidoacetic acid (prepared from *L*-valine or *L*-leucine with isocyanates)[23] afforded **8a–8q** in moderate yields.

2.2 Cell viability assay

The structures of all compounds and their antitumor activities were listed in **Table 1**. This series of compounds only had 2 or 3 stereocenters compared to pretubulysin which had 6. Although the antitumor activities of these compounds were discovered to be 10-fold less than that of pretubulysin, certain compounds exhibited potent antitumor activity comparable to those of Taxol. Compound **8c** showed the antitumor activities, with IC_{50} values of 0.05 μM against MCF-7 breast cancer cell, and 0.09 μM against NCI-H157 lung cancer cell. The most potent compound **8h** showed the antitumor activities, with IC_{50} values of 0.01 μM against MCF-7 breast cancer cells, and 0.02 μM against NCI-H157lung cancer cells. Previous studies including our lab's work

showed that the central Tuv part played a critical role in maintaining the antitumor activity of tubulysins and pretubulysin.[17, 24] Both the right end (Tup part), and the left end (Mep part) were tolerant of many modifications. When comparing the three R_1 groups of these analogs (Tup part of pretubulysin), those compounds containing phenylethylamine fragments generally possessed better activity than those with methylamine and benzylamine fragments (e.g., **8i** *vs*. **8b** and **8a**). It might suggest that the phenyl group in the Tup was important for maintaining the activity. When comparing the four R_3 groups of these analogs (Mep part of pretubulysin), those compounds with halogen-substituted phenyl group generally possessed better activity. For example, compounds **8b** (IC_{50} = 1.05 μM, MCF-7) and **8c** (IC_{50} = 0.15 μM, MCF-7) were more potent than compound **8a** (IC_{50} = 1.75 μM, MCF-7); compounds **8e** (IC_{50} = 0.79 μM, MCF-7) and **8f** (IC_{50} = 0.53 μM, MCF-7) were more potent than compound **8d** (IC_{50} = 1.27 μM, MCF-7). These phenomena were often observed in synthesized small-molecule antitumor agents, especially in the receptor tyrosine kinases (RTK) inhibitors.[25,26] The halogen group was recognized as the second favorite heteroatom in drug design, and a large number of halogen-containing drugs were approved by FDA or under investigation.[27,28] Regarding the R_2 group, it seemed that the leucine analogs were much better than valine ones in most cases, and at least the similar effect in some cases. For example, Compound **8c** (IC_{50} = 0.05 μM) were more potent than compound **8m** (IC_{50} = 0.76 μM) against MCF-7 and NCI-H157 cancer cells, and compound **8h** (IC_{50} = 0.01 μM) was more potent than compound **8p** (IC_{50} = 0.38 μM). Compounds **8n** (IC_{50} = 1.02 μM, MCF-7) and **8d** (IC_{50} = 1.27 μM, MCF-7) had similar antitumor activity; compounds **8j** (IC_{50} = 0.09 μM) and **8q** (IC_{50} = 0.11 μM) exhibited almost the same antitumor activity against MCF-7 breast cancer cells. Although these urea analogs were less potent than that of pretubulysin, compound **8h** exhibited potent antitumor activity comparable to that of Taxol. Also, these compounds were more potent than our previously prepared Tuv aromatic or cyclization analogs.[17] The lipophilicity as calculated by the ClogP (4.4-8.3) of these compounds was higher than that of pretubulysin (3.8). There were only 2 or 3 stereocenters in these structures, and it was easier to obtain from commercial starting materials. The stereocenters decrease meant lots of saving on the studies on the stereoisomers and related substances.

2.3 Molecular docking

To better understand how these compounds interact with tubulin, molecular docking study was performed using the co-crystal structure of microtubules and tubulysin M (PDB code: 4ZOL) by Sybyl 2.1.1 software.[29] Pretubulysin, compound **8c**, and compound **8h** were chosen for the molecular docking study, according to the general protocol. The predicted binding mode was shown in **Fig. 2**.

For pretubulysin, the two carbonyl groups of Tup and Mep had hydrogen bond interaction with the amino acid residue Tyr224, Lys 352, and Val 353, with the total scores of 9.8140 (A, **Fig. 2**). However, compounds **8c** and **8h** adopted different conformers to interact with the tubulin. As for compound **8c**, the urea group had hydrogen bond interaction with Asp179 and Asn329. Meanwhile, the trifluoromethyl group had additional interaction with the Ala333, with the total scores of 9.2288 (B, **Fig. 2**). These phenomena might partially explain that compound **8c** had a better antitumor potency, even with a methyl group in the Tup part. As for compound **8h**, the Asn329 amino acid had interactions with the urea group and carbonyl group, with total scores of 9.1030. Both compounds **8c** and **8h** lacked the hydrogen bond interaction of Tyr224 with the carbonyl group of Tup, which might be the reasons for the reducing potency of antitumor activity of these urea analogs.

3. Conclusion

In summary, a number of new 2-(3-(methylamino)propyl)thiazole-4-carboxamides were synthesized, and their anticancer activities were evaluated by MTT method using MCF-7-breast cancer and NCI-H157-lung cancer cell lines. Compound **8c** showed the antitumor activities, with IC_{50} values of 0.05 μM against MCF-7 breast cancer cells, and 0.09 μM against NCI-H157 lung cancer cells. The most potent compound **8h** showed the antitumor activities, with IC_{50} values of 0.01 μM against MCF-7 breast cancer cell, and 0.02 μM against NCI-H157 lung cancer cell. Although the antitumor activities of these urea compounds were less potent than that of the pretubulysin, compound **8h** showed potent antitumor activity comparable to that of Taxol. It was relatively easier to synthesize this series of more lipophilic compounds to enhance oral absorption. This scaffold might represent a promising lead for the further development of novel antitumor

drugs.

4. Experimental Section

4.1 Chemistry

General

Unless otherwise stated, all reactions were performed in flame-dried glassware equipped with glass stoppers under positive pressure of Ar with magnetic stirring. The NMR spectra of the intermediates and final products in deuterated chloroform were detected on a Bruker 400 or 600 MHz spectrometer (see Supporting Information). High-resolution mass spectra (HRMS) were recorded on an Agilent 6210 ESI/TOF mass spectrometer. Melting points were determined using a digitizing melting point apparatus (WRS-1B, Shanghai Precision & Scientific Instrument Co., Ltd.) and were uncorrected. All synthesized compounds were purified by column chromatography on silica gel from Qingdao Ocean Chemical Co. (200 to 300 mesh) with particle size from 54 to 74 μm using ethyl acetate and hexane (or petroleum ether) as the eluent. Analytical TLC was carried out on Merck pre-coated silica gel 60 GF-254 using 0.25-mm-thick TLC plates. CLogP was obtained by ChemBioDraw Ultra 12.0 software.

*General procedure for compound **8***

*2-((R)-3-((S)-2-(3-(4-Methoxylphenyl)ureido)-N,4-dimethylpentanamido)-4-methylpentyl)-N-methylthiazole-4-carboxamide (**8a**).*

Step 1: To a solution of **7a** (2.35 g, 6.6 mmol) in DCM (30 mL), trifluoroacetic acid (TFA, 15 mL) was added at room temperature. The reaction mixture was stirred at room temperature for 3 h. Then the mixture was concentrated under reduced pressure, and the obtained residue was washed with water. The aqueous solution was cautiously neutralized with excess, solid $NaHCO_3$ and extracted with ethyl acetate (3×30 mL). The extracted organic layers were dried over anhydrous Na_2SO_4, filtered, concentrated to yield (1.48 g, 87%) the amine intermediated as a yellow oil and used in the next step directly.

Step 2: (3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.14 g, 0.72 mmol) and 1-hydroxybenzotriazole (0.09 g, 0.60 mmol) was added to a solution of ((4-methoxyphenyl)carbamoyl)-*L*-leucine (0.13 g, 0.44 mmol) in DMF (2 mL). After the mixture

was stirred at room temperature for 30 min, the above amine intermediate (0.1 g, 0.4 mmol) was added. Then the reaction mixture was stirred at room temperature for 6 h before water (6 mL) was added to quench the reaction. The aqueous solution was extracted with ethyl acetate (3×15 mL), and the extracted organic layers were dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography to yield **8a** (0.08 g, 40%) as a yellow solid. mp 83.3-85.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.82 (s, 1H, CH), 7.60 (br s, 1H, NH), 7.32-7.31 (m, 1H, NH), 7.02 (d, *J* = 8 Hz, 2H, ArH), 6.74-6.68 (m, 1H, ArH), 6.53 (d, *J* =8 Hz, 2H, ArH), 4.90-4.86 (m, 1H, CH), 4.34-4.30 (m, 1H, CH), 3.63 (s, 3H, OCH_3), 3.00 (s, 3H, NCH_3), 2.94-2.91 (m, 1H, CH), 2.89 (s, 3H, CH_3), 2.75-2.71 (m, 1H, CH), 2.51-2.49 (m, 1H, CH), 2.10-2.03 (m, 1H, CH), 1.83-1.79 (m, 2H, CH_2), 1.71-1.67 (m, 1H, CH), 1.54-1.50 (m, 1H, CH), 1.44-1.40 (m, 1H, CH), 0.98 (d, *J* = 8.0 Hz, 3H, CH_3), 0.94 (d, *J* = 8.0 Hz, 6H, 2XCH_3), 0.80 (d, *J* = 8.0 Hz, 3H, CH_3); ^{13}C NMR (101 MHz, $CDCl_3$): δ 176.5, 170.2, 161.9, 156.4, 155.3, 149.5, 132.0, 122.3, 121.8, 113.8, 55.3, 49.0, 41.6, 30.2, 30.0, 29.3, 26.0, 24.8, 23.4, 21.8, 20.1, 19.8. HRMS (m/z): $[M+H]^+$ calcd for $C_{26}H_{39}N_5O_4S$: 517.2723; found: 517.2728.

*2-((R)-3-((S)-2-(3-(4-Chlorophenyl)ureido)-N,4-dimethylpentanamido)-4-methylpentyl)-N-methylthiazole-4-carboxamide (**8b**)*

Prepared from **7a** by the same method as described in **8a**. White solid (0.07 g, 35%); mp 82.4-84.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88 (s, 1H, CH), 7.78-7.74 (m, 1H, NH), 7.28-7.25 (m, 1H, NH), 7.03 (d, *J* = 8.0 Hz, 2H, ArH), 6.99-6.97 (m, 1H, NH), 6.91 (d, *J* = 8.0 Hz, 2H, ArH), 4.88-4.84 (m, 1H, CH), 4.36-4.28 (m, 1H, CH), 3.02 (s, 3H, NCH_3), 2.95-2.94 (m, 1H, CH), 2.92 (s, 3H, CH_3), 2.80-2.77 (m, 1H, CH), 2.30-2.27 (m, 1H, CH), 2.17-2.10 (m, 1H, CH), 1.89-1.79 (m, 2H, CH_2), 1.57-1.51 (m, 1H, CH), 1.45-1.39 (m, 1H, CH), 0.99 (d, *J* = 8.0 Hz, 6H, 2XCH_3), 0.92 (d, *J* = 8.0 Hz, 3H, CH_3), 0.82 (d, *J* = 8.0 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.8, 170.1, 161.9, 155.8, 149.6, 137.7, 128.3, 127.1, 122.4, 120.3, 49.3, 41.2, 30.2, 29.9, 29.1, 26.1, 24.9, 23.5, 21.6, 20.2, 19.8; HRMS (m/z): $[M+H]^+$ calcd for $C_{25}H_{36}ClN_5O_3S$: 521.2227; found: 521.2230.

*2-((R)-3-((S)-2-(3-(4-Chloro-3-(trifluoromethyl)phenyl)ureido)-N,4-dimethylpentanamido)-4-methylpentyl)-N-methylthiazole-4-carboxamide (**8c**).*

Prepared from **7a** by the same method as described in **8a**. Yellow solid, (0.08 g, 33.3%); mp 81.3-83.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.99 (s, 1H, CH), 7.91 (s, 1H, NH), 7.38 (d, *J* = 4 Hz, 1H, NH), 7.28-7.25 (m, 1H, NH), 7.23-7.21 (m, 1H, ArH), 7.20-7.19 (m, 1H, ArH), 7.02-6.99 (m, 1H, ArH), 4.84-4.79 (m, 1H, CH), 4.40-4.32 (m, 1H, CH), 3.04 (s, 3H, NCH_3), 2.98-2.95 (m, 1H, CH), 2.93 (s, 3H, CH_3), 2.86-2.84 (m, 1H, CH), 2.82-2.78 (m, 1H, CH), 2.22-2.14 (m, 1H, CH), 1.92-1.87 (m, 1H, CH), 1.57-1.53 (m, 1H, CH), 1.49-1.40 (m, 1H, CH), 1.24-1.23 (m, 1H, CH), 1.0 (d, *J* = 8 Hz, 6H, 2XCH_3), 0.95 (d, *J* =8.0 Hz, 3H, CH_3), 0.83 (d, *J* = 8.0 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.9, 169.9, 169.6, 161.8, 161.7, 155.5, 155.4, 149.9, 149.7, 138.0, 131.3, 122.4 (CF_3), 117.5, 49.6, 40.9, 30.1, 29.2, 26.0, 24.9, 23.5, 21.3, 20.1, 19.7; HRMS (m/z): $[M+H]^+$ calcd for $C_{26}H_{35}ClF_3N_5O_3S$: 589.2101; found: 589.2105.

*N-Benzyl-2-((R)-3-((S)-2-(3-(4-methoxyphenyl)ureido)-N,4-dimethylpentanamido)-4-methylpentyl)thiazole-4-carboxamide (**8d**).*

Prepared from **7b** by the same method as described in **8a**. Yellow solid, (0.06 g, 21%); mp 65.5-66.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (s, 1H, CH), 7.72-7.65 (m, 1H, NH), 7.35-7.29 (multiple, 5H, ArH), 7.27-7.25 (m, 1H, ArH), 7.20-7.14 (m, 1H, ArH), 7.04 (d, *J* = 8 Hz,, 2H, ArH), 6.59 (d, *J* = 4 Hz, 1H, ArH), 6.44-6.40 (m, 1H, CH), 4.91-4.85 (m, 1H, CH), 4.59 (d, *J* = 4 Hz,, 1H, CH), 4.34-4.30 (m, 1H, CH), 3.72-3.70 (m, 1H, CH), 3.67-3.65 (s, 3H, OCH_3), 2.99 (s, 3H, NCH_3), 2.92-2.84 (m, 1H, CH), 2.75-2.68 (m, 1H, CH), 2.12-2.03 (m, 1H, CH), 1.84-1.79 (m, 2H, CH_2), 1.72-1.64 (m, 1H, CH), 1.56-1.49 (m, 1H, CH), 1.43-1.38 (m, 1H, CH), 1.28-1.23 (m, 1H, CH), 0.9 9 (d, *J* = 8.0 Hz, 3H, CH_3), 0.94 (d, *J* = 8.0 Hz, 6H, 2xCH_3), 0.81 (d, *J* = 8.0 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.5, 170.2, 161.2, 156.4, 155.4, 149.5, 138.5, 132.0, 128.7, 127.9, 127.4, 122.8, 122.0, 113.9, 55.4, 49.1, 43.2, 41.7, 30.2, 30.0, 29.2, 24.8, 23.5, 21.8, 20.1, 19.8; HRMS (m/z): $[M+H]^+$ calcd for $C_{32}H_{43}N_5O_4S$: 593.3036; found: 593.3039.

*N-Benzyl-2-((R)-3-((S)-2-(3-(4-chlorophenyl)ureido)-N,4-dimethylpentanamido)-4-methylpentyl)thiazole-4-carboxamide (**8e**).*

Prepared from **7b** by the same method as described in **8a**. Yellow solid, (0.07 g, 23%); mp 70.2-72.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.94 (s, 1H, CH), 7.55-7.52 (m, 1H, NH), 7.35-7.31 (m, 5H, ArH), 7.27-7.23 (m, 1H, NH), 7.0 (d, *J* = 8.9 Hz, 2H, ArH), 6.90 (d, *J* = 8.9 Hz, 2H, ArH),

5.31-5.23 (m, 1H, NH), 4.6 (t, J = 5.8 Hz, 2H, CH_2), 4.37-4.28 (m, 1H, CH), 3.01 (s, 3H, NCH_3), 2.95-2.91 (m, 1H, CH), 2.80-2.74 (m, 1H, CH), 2.17-2.10 (m, 1H, CH), 1.88-1.83 (m, 1H, CH), 1.76-1.72 (m, 1H, CH), 1.56-1.53 (m, 1H, CH), 1.46-1.39 (m, 1H, CH), 1.26-1.22 (m, 1H, CH), 1.0 (d, J = 8.0 Hz, 6H, 2XCH_3), 0.92 (d, J = 8.0 Hz, 3H, CH_3), 0.82 (d, J = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.8, 170.1, 169.8, 161.1, 155.8, 149.5, 138.4, 137.6, 128.7, 128.4, 127.9, 127.4, 123.0, 120.3, 49.3, 43.3, 41.2, 30.2, 29.7, 24.91, 23.5, 21.6, 20.2, 19.8; HRMS (m/z): $[M+H]^+$ calcd for $C_{31}H_{40}ClN_5O_3S$: 597.2540; found: 597.2544.

*N-Benzyl-2-((R)-3-((S)-2-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)-N,4-dimethylpentanamido)-4-methylpentyl)thiazole-4-carboxamide (**8f**).*

Prepared from **7b** by the same method as described in **8a**. Yellow solid, (0.06 g, 21%); mp 147.5-149.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.96 (s, 1H, CH), 7.79-7.75 (m, 1H, NH), 7.65-7.61 (m, 1H, NH), 7.33-7.31 (m, 5H, ArH), 7.25-7.24 (m, 1H, ArH), 7.20-7.17 (m, 1H, ArH), 7.12-7.10 (m, 1H, ArH), 6.98-6.96 (m, 1H, NH), 4.81-4.76 (m, 1H, CH), 4.61 (d, J = 1.9 Hz, 1H, CH), 4.37 (d, J = 8.6 Hz,, 1H, CH), 3.63-3.60 (m, 1H, CH), 3.02 (s, 3H, NCH_3), 2.98-2.94 (m, 1H, CH), 2.87-2.83 (m, 1H, CH), 2.76 (dd, J = 10.3, 5.1 Hz,, 1H, CH), 2.19-2.12 (m, 1H, CH), 1.88-1.82 (m, 1H, CH), 1.52 (dd, J = 14.3, 3.9 Hz, 1H, CH), 1.50-1.40 (m, 1H, CH), 1.24 (d, J = 1.2 Hz, 1H, CH), 0.99 (d, J = 6.8 Hz, 3H, CH_3), 0.93 (d, J = 6.8 Hz, 3H, CH_3), 0.89 (d, J = 9.2 Hz, 3H, CH_3), 0.82 (d, J = 6.5 Hz 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.8, 169.9, 169.7, 161.1, 161.0, 155.5, 149.6, 138.3, 137.9, 131.3, 128.8, 128.7, 127.9, 127.5, 123.0 (CF_3), 49.7, 43.3, 40.9, 30.2, 29.2, 24.9, 23.5, 21.3, 20.2, 19.7; HRMS (m/z): $[M+H]^+$ calcd for $C_{32}H_{39}ClF_3N_5O_3S$: 665.2414; found: 665.2417.

*2-((R)-3-((S)-N,4-Dimethyl-2-(3-(4-nitrophenyl)ureido)pentanamido)-4-methylpentyl)-N-phenethylthiazole-4-carboxamide (**8g**).*

Prepared from **7c** by the same method as described in **8a**. Yellow solid, (0.12 g, 57%); mp 73.4-75.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14-8.11 (m, 1H, CH), 7.96 (s, 1H, NH), 7.85-7.82 (m, 2H, ArH), 7.43-7.33 (m, 1H, NH), 7.29-7.27 (m, 1H, ArH), 7.25-7.22 (m, 2H, ArH), 7.21-7.18 (m, 3H, ArH), 7.11-7.09 (m, 1H, ArH), 4.85-4.80 (m, 1H, CH), 4.41-4.30 (m, 1H, CH), 3.71-3.64 (m, 2H, CH_2), 3.04 (s, 3H, NCH_3), 2.93-2.90 (m, 1H, CH), 2.87 (d, J = 8.1 Hz, 1H, CH), 2.80-2.78

(m, 1H, CH), 2.24-2.17 (m, 1H, CH), 1.88-1.82 (m, 1H, CH), 1.67 (d, *J* = 12.9 Hz, 1H, CH), 1.53 (dd, *J* = 18.4, 7.7 Hz, 1H, CH), 1.34 (d, *J* = 14.6 Hz, 1H, CH), 1.27-1.24 (m, 1H, CH), 1.03 (d, *J* = 8.0 Hz, 6H, 2XCH_3), 0.95 (d, *J* = 8.0 Hz, 3H, CH_3), 0.85 (d, *J* = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz,$CDCl_3$): δ 169.9, 161.1, 155.7, 149.7, 139.0, 137.6, 128.9, 128.6, 128.4, 127.2, 126.4, 122.5, 120.4, 49.3, 41.3, 40.7, 35.9, 30.2, 29.7, 26.5, 24.9, 23.5, 21.6, 20.2, 19.8; HRMS (m/z): $[M+H]^+$ calcd for $C_{32}H_{42}N_6O_5S$: 622.2937; found: 622.2941.

*2-((R)-3-((S)-2-(3-(4-Chlorophenyl)ureido)-N,4-dimethylpentanamido)-4-methylpentyl)-N-phenethylthiazole-4-carboxamide (**8h**).*

Prepared from **7c** by the same method as described in **8a**. Yellow solid, (0.10 g, 47%); mp 78.8-80.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (s, 1H, CH), 7.60-7.58 (m, 1H, NH), 7.38-7.35 (m, 1H, NH), 7.28-7.26 (m, 1H, NH), 7.25-7.19 (m, 5H, ArH), 7.02 (d, *J* = 8.9 Hz, 2H, ArH), 6.93 (d, *J* = 8.9 Hz, 2H, ArH), 4.86-4.84 (m, 1H, CH), 3.69-3.62 (m, 2H, CH_2), 3.03 (s, 3H, NCH_3), 2.89 (t, *J* = 7.3 Hz, 2H, CH_2), 2.81-2.72 (m, 2H, CH_2), 2.18-2.12 (m, 1H, CH), 2.10-2.03 (m, 1H, CH), 1.87-1.86 (m, 1H, CH), 1.78-1.74 (m, 1H, CH), 1.58-1.51 (m, 1H, CH), 1.47-1.41 (m, 1H, CH), 1.26-1.23 (m, 1H, CH), 1.02 (d, *J* = 6.7 Hz, 3H, CH_3), 0.98 (d, *J* = 12.0 Hz, 3H, CH_3), 0.94 (d, *J* = 12.0 Hz, 3H, CH_3), 0.84 (d, *J* = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz,$CDCl_3$): δ 169.9, 161.1, 155.8, 155.6, 149.7, 139.0, 137.6, 128.9, 128.6, 128.4, 127.2, 126.4, 122.6, 120.3, 49.4, 40.7, 40.6, 35.9, 30.2, 29.7, 24.9, 23.5, 21.5, 21.4, 20.2, 19.8; HRMS (m/z): $[M+H]^+$ calcd for $C_{32}H_{42}ClN_5O_3S$: 611.2697; found: 611.2702.

*2-((R)-3-((S)-2-(3-(4-Methoxyphenyl)ureido)-N,4-dimethylpentanamido)-4-methylpentyl)-N-phenethylthiazole-4-carboxamide (**8i**).*

Prepared from **7c** by the same method as described in **8a**. Yellow solid, (0.08 g, 38%); mp 67.3-69.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (s, 1H, CH), 7.44-7.42 (m, 1H, NH), 7.30-7.27 (m, 3H, ArH), 7.23-7.17 (m, 4H, ArH), 7.07 (d, *J* = 6.0 Hz, 2H, ArH), 6.62 (d, *J* = 6.0 Hz, 2H, ArH), 4.91-4.88 (m, 1H, CH), 4.36-4.33 (m, 1H, CH), 3.71-3.69 (m, 1H, CH), 3.68 (s, 3H, OCH_3), 3.63-3.61 (m, 1H, CH), 3.00 (s, 3H, NCH_3), 2.91-2.87 (m, 2H, CH_2), 2.75 (dd, *J* = 12.6, 4.8 Hz, 1H, CH), 2.13-2.06 (m, 1H, CH), 1.86-1.81 (m, 2H, CH_2), 1.74-1.70 (m, 1H, CH), 1.57-1.51 (m, 1H, CH), 1.43 (t, *J* = 9.5 Hz, 1H, CH), 1.25-1.23 (m, 1H, CH), 1.02 (d, *J* = 6.5 Hz, 3H, CH_3),

0.98 (d, *J* = 8.0 Hz, 3H, CH_3), 0.92 (d, *J* = 8.0 Hz, 3H, CH_3), 0.83 (d, *J* = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.5, 170.1, 161.2, 156.4, 155.5, 149.6, 139.0, 131.9, 128.8, 128.6, 126.4, 122.5, 122.1, 113.9, 55.4, 49.13, 41.7, 40.7, 35.9, 30.2, 29.7, 24.9, 23.5, 21.8, 20.2, 19.9; HRMS (m/z): $[M+H]^+$ calcd for $C_{33}H_{45}N_5O_4S$: 607.3192; found: 607.3197.

*2-((R)-3-((S)-2-(3-(4-Chloro-3-(trifluoromethyl)phenyl)ureido)-N,4-dimethylpentanamido)-4-methylpentyl)-N-phenethylthiazole-4-carboxamide (**8j**).*

Prepared from **7c** by the same method as described in **8a**. Yellow solid, (0.07 g, 36%); mp 151.3-153.2 °C; ^1H NMR (400 MHz, $CDCl_3$): δ 7.92 (s, 1H, CH), 7.87-7.81 (m, 1H, NH), 7.37-7.34 (m, 1H, NH), 7.31-7.28 (m, 1H, ArH), 7.27-7.25 (m, 2H, ArH), 7.22-7.15 (m, 5H, ArH), 7.0 (d, *J* = 8.6 Hz, 1H, NH), 4.83-4.78 (m, 1H, CH), 4.39-4.35 (m, 1H, CH), 3.67 (d, *J* = 7.1 Hz, 1H, CH), 3.03 (s, 3H, NCH_3), 2.99-2.97 (m, 1H, CH), 2.94-2.92 (m, 1H, CH), 2.91-2.89 (m, 1H, CH), 2.87-2.85 (m, 1H, CH), 2.81-2.73 (m, 1H, CH), 2.20-2.15 (m, 1H, CH), 1.89-1.85 (m, 2H, CH_2), 1.53 (dd, *J* = 16.2, 12.5 Hz, 1H, CH), 1.49-1.41 (m, 1H, CH), 1.23 (d, *J* = 7.1 Hz, 1H, CH), 1.02 (d, *J* = 6.5 Hz, 3H, CH_3), 0.97 (d, *J* = 8.0 Hz, 3H, CH_3), 0.94 (d, *J* = 8.0 Hz, 3H, CH_3), 0.84 (d, *J* = 6.5 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.9, 169.8, 169.6, 161.2, 161.0, 155.5, 155.4, 149.7, 139.0, 138.0, 131.3, 128.8, 128.6, 126.4, 122.6 (CF_3), 49.7, 40.7, 40.6, 35.9, 30.3, 30.1, 29.1, 24.9, 23.5, 21.3, 20.1, 19.8; HRMS (m/z): $[M+H]^+$ calcd for $C_{33}H_{41}ClF_3N_5O_3S$: 679.2571; found: 679.2574.

*2-((R)-3-((S)-2-(3-(4-Methoxyphenyl)ureido)-N,3-dimethylbutanamido)-4-methylpentyl)-N-methylthiazole-4-carboxamide (**8k**).*

Prepared from **7a** by the same method as described in **8a**. Yellow solid, (0.11 g, 55%); mp 73.7-75.9 °C; ^1H NMR (400 MHz, $CDCl_3$): δ 7.82 (s, 1H, CH), 7.61-7.56 (m, 1H, NH), 7.36-7.27 (m, 1H, NH), 7.25-7.16 (m, 2H, ArH), 6.81-6.71 (m, 2H, ArH), 6.26-6.24 (m, 1H, NH), 5.27 (s, 1H, CH), 4.75-4.65 (m, 1H, CH), 3.71 (s, 3H, OCH_3), 3.04 (s, 3H, NCH_3), 2.96-2.95 (m, 1H, CH), 2.91-2.89 (m, 3H, CH_3), 2.76-2.57 (m, 1H, CH), 2.09 (dd, *J* = 16.4, 8.0 Hz, 1H, CH), 2.04-1.99 (m, 1H, CH), 1.71-1.68 (m, 1H, CH), 1.23 (s, 1H, CH), 1.02 (d, *J* = 4.0 Hz, 3H, CH_3), 1.00-0.96 (m, 6H, CH_3), 0.85 (d, *J* = 6.6 Hz 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.3, 170.1, 169.7, 161.9, 156.5, 156.3, 149.6, 132.0, 122.3, 114.2, 55.5, 31.2, 30.9, 30.3, 30.1, 26.0, 20.2, 19.9, 19.8,

18.0; HRMS (m/z): $[M+H]^+$ calcd for $C_{25}H_{37}N_5O_4S$: 503.2566; found: 503.2569.

*2-((R)-3-((S)-2-(3-(4-Chlorophenyl)ureido)-N,3-dimethylbutanamido)-4-methylpentyl)-N-methylthiazole-4-carboxamide (**8l**).*

Prepared from **7a** by the same method as described in **8a**. White solid, (0.13 g, 65%); mp 74.5-76.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01-7.95 (m, 1H, CH), 7.82 (s, 1H, NH), 7.37-7.35 (m, 1H, NH), 7.26 (d, *J* = 9.0 Hz, 2H, ArH), 7.12 (d, *J* = 9.0 Hz, 2H, ArH), 6.54-6.49 (m, 1H, NH), 5.27 (s, 1H, CH), 4.74-4.65 (m, 1H, CH), 3.07 (s, 3H, CH_3), 2.97-2.93 (m, 1H, CH), 2.91 (s, 3H, NCH_3), 2.78-2.74 (m, 1H, CH), 2.14-2.09 (m, 1H, CH), 2.04 (d, *J* = 7.7 Hz, 1H, CH), 1.77-1.74 (m, 1H, CH), 1.23 (s, 1H, CH), 1.04-0.99 (m, 9H, CH_3), 0.87 (d, *J* = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.5, 169.9, 161.9, 155.7, 149.6, 138.0, 128.8, 127.3, 122.4, 120.4, 55.6, 53.5, 30.9, 30.3, 30.1, 29.3, 26.0, 20.2, 19.8, 18.1; HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{34}ClN_5O_3S$: 507.2071; found: 507.2076.

*2-((R)-3-((S)-2-(3-(4-Chloro-3-(trifluoromethyl)phenyl)ureido)-N,3-dimethylbutanamido)-4-methylpentyl)-N-methylthiazole-4-carboxamide (**8m**).*

Prepared from **7a** by the same method as described in **8a**. White solid, (0.09 g, 45%); mp 82.3-84.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01-7.98 (m, 1H, NH), 7.85 (s, 1H, NH), 7.68-7.66 (m, 1H, NH), 7.62-7.59 (m, 1H, ArH), 7.53-7.50 (m, 1H, ArH), 7.29-7.25 (m, 1H, ArH), 6.73-6.55 (m, 1H, NH), 4.77-4.68 (m, 1H, CH), 4.34-4.29 (m, 1H, CH), 3.06 (s, 3H, CH_3), 2.98 (d, *J* = 5.1 Hz, 1H, CH), 2.93 (s, 3H, NCH_3), 2.79 (d, *J* = 4.7 Hz, 1H, CH), 2.20 (dd, *J* = 11.0, 6.9 Hz, 1H, CH), 2.06-2.02 (m, 1H, CH), 1.68-1.65 (m, 1H, CH), 1.24 (s, 1H, CH), 1.05 (d, *J* = 6.4 Hz, 3H, CH_3), 1.02 (d, *J* = 8.0 Hz, 6H, 2XCH_3), 0.87 (d, *J* = 6.5 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.7, 175.2, 169.7, 169.4, 161.8, 155.4, 150.9, 149.6, 138.4, 131.7, 122.8 (CF_3), 122.4, 55.7, 30.8, 30.1, 29.7, 26.0, 22.7, 20.2, 19.8, 17.9, 14.1; HRMS (m/z): $[M+H]^+$ calcd for $C_{25}H_{33}ClF_3N_5O_3S$: 575.1945; found: 575.1949.

*N-Benzyl-2-((R)-3-((S)-2-(3-(4-Methoxyphenyl)ureido)-N,3-dimethylbutanamido)-4-methylpentyl)thiazole-4-carboxamide (**8n**).*

Prepared from **7b** by the same method as described in **8a**. White solid, (0.06 g, 35%); mp 75.5-77.8 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 (s, 1H, CH), 7.76-7.71 (m, 1H, NH), 7.32-7.28

(m, 5H, ArH), 7.25-7.23 (m, 1H, NH), 7.12 (d, J = 9.0 Hz, 2H, ArH), 6.70 (d, J = 9.0 Hz, 2H, ArH), 6.09-6.02 (m, 1H, NH), 4.63-4.53 (m, 1H, CH), 4.31-4.27 (m, 1H, CH), 3.74-3.72 (m, 1H, CH), 3.70 (s, 3H, OCH_3), 2.99 (s, 3H, NCH_3), 2.95-2.94 (m, 1H, CH), 2.87-2.85 (m, 1H, CH), 2.71-2.68 (m, 1H, CH), 2.07-2.00 (m, 1H, CH), 1.86-1.85 (m, 1H, CH), 1.70-1.65 (m, 1H, CH), 1.24 (s, 1H, CH), 1.01-0.93 (m, 9H, CH_3), 0.83 (d, *J* = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.1, 169.8, 161.2, 161.1, 156.4, 155.9, 149.5, 138.5, 131.8, 128.7, 127.7, 127.4, 122.8, 114.2, 55.5, 43.3, 43.2, 30.9, 30.1, 29.7, 29.4, 26.1, 20.2, 19.8, 17.9; HRMS (m/z): $[M+H]^+$ calcd for $C_{31}H_{41}N_5O_4S$: 579.2879; found: 579.2883.

*2-((R)-3-((S)-2-(3-(4-Methoxyphenyl)ureido)-N,3-dimethylbutanamido)-4-methylpentyl)-N-phenethylthiazole-4-carboxamide (**8o**).*

Prepared from **7c** by the same method as described in **8a**. White solid, (0.08 g, 47%); mp 156.2-158.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83 (s, 1H, CH), 7.44-7.41 (m, 1H, NH), 7.37-7.33 (m, 1H, NH), 7.29-7.26 (m, 2H, ArH), 7.23-7.20 (m, 3H, ArH), 7.12 (d, *J* = 9.0 Hz, 2H, ArH), 6.72 (d, *J* = 9.0 Hz, 2H, ArH), 6.19-6.12 (m, 1H, NH), 4.75-4.66 (m, 1H, CH), 4.33-4.29 (m, 1H, CH), 3.74-3.72 (m, 1H, CH), 3.71 (s, 3H, OCH_3), 3.63-3.60 (m, 1H, CH), 3.02 (s, 3H, NCH_3), 2.94-2.92 (m, 1H, CH), 2.90-2.87 (m, 1H, CH), 2.74 (d, *J* = 2.2 Hz, 1H, CH), 2.11-1.99 (m, 2H, CH_2), 1.87-1.86 (m, 1H, CH), 1.69 (dd, *J* = 12.9, 6.2 Hz, 1H, CH), 1.24 (s, 1H, CH), 1.02-0.96 (multiple, 9H, $3XCH_3$), 0.85 (d, *J* = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.0, 169.8, 161.2, 161.1, 156.4, 155.9, 149.6, 139.0, 131.8, 128.8, 128.3, 126.4, 122.4, 114.3, 55.5, 40.6, 35.9, 32.1, 31.2, 30.9, 30.1, 29.7, 29.3, 20.2, 19.9, 17.9; HRMS (m/z): $[M+H]^+$ calcd for $C_{32}H_{43}N_5O_4S$: 593.3036; found: 593.3039.

*2-((R)-3-((S)-2-(3-(4-Chlorophenyl)ureido)-N,3-dimethylbutanamido)-4-methylpentyl)-N-phenethylthiazole-4-carboxamide (**8p**).*

Prepared from **7c** by the same method as described in **8a**. White solid, (0.06 g, 33%); mp 81.5-83.4 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (s, 1H, CH), 7.82 (s, 1H, NH), 7.38-7.35 (m, 1H, NH), 7.31-7.28 (m, 1H, ArH), 7.25 (d, *J* = 8.8 Hz, 2H, ArH), 7.23-7.16 (m, 4H, ArH), 7.11 (d, *J* = 8.8 Hz, 2H, ArH), 6.47-6.36 (m, 1H, NH), 4.76-4.65 (m, 1H, CH), 3.69-3.60 (m, 1H, CH), 3.05 (s, 3H, NCH_3), 2.94-2.93 (m, 1H, CH), 2.88-2.86 (m, 2H, CH_2), 2.80-2.77 (m, 1H, CH),

2.75-2.73 (m, 1H, CH), 2.14-2.11 (m, 1H, CH), 2.04-2.00 (m, 1H, CH), 1.83-1.81 (m, 1H, CH), 1.72-1.68 (m, 1H, CH), 1.24 (s, 1H, CH), 1.03 (d, J = 8.0 Hz, 3H, CH_3), 1.0 (d, J = 8.0 Hz, 3H, CH_3), 0.98 (d, J = 6.6 Hz, 3H, CH_3), 0.87 (d, J = 6.6 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.3, 169.8, 169.8, 161.2, 161.1, 155.6, 149.6, 139.0, 137.9, 128.8, 128.6, 126.5, 122.5, 120.5, 55.6, 40.6, 35.9, 30.9, 30.1, 29.7, 29.2, 27.7, 20.2, 19.8, 18.0; HRMS (m/z): $[M+H]^+$ calcd for $C_{31}H_{40}ClN_5O_3S$: 597.2540; found: 597.2544.

*2-((R)-3-((S)-2-(3-(4-Chloro-3-(trifluoromethyl)phenyl)ureido)-N,3-dimethylbutanamido)-4-methylpentyl)-N-phenethylthiazole-4-carboxamide (**8q**).*

Prepared from **7c** by the same method as described in **8a**. White solid, (0.05 g, 26%); mp 63.5-65.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (s, 1H, CH), 7.82 (s, 1H, NH), 7.73-7.70 (m, 1H, NH), 7.63-7.59 (m, 1H, NH), 7.52-7.45 (m, 1H, ArH), 7.40-7.34 (m, 1H, ArH), 7.30-7.27 (m, 3H, ArH), 7.23-7.17 (m, 3H, ArH), 4.76-4.66 (m, 1H, CH), 4.29 (t, J = 6.7 Hz, 1H, CH), 3.67 (d, J = 6.9 Hz, 1H, CH), 3.02 (s, 3H, CH_3), 2.91 (d, J = 7.1 Hz, 1H, CH), 2.82-2.78 (m, 1H, CH), 2.75-2.71 (m, 1H, CH), 2.37-2.34 (m, 1H, CH), 2.19-2.14 (m, 1H, CH), 2.08-2.02 (m, 1H, CH), 1.60 (s, 1H, CH), 1.24 (s, 1H, CH), 1.04 (d, J = 6.7 Hz, 3H, CH_3), 0.98 (d, J = 6.4 Hz, 3H, CH_3), 0.88 (d, J = 4.4 Hz, 3H, CH_3), 0.85 (d, J = 6.8 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ176.6, 169.5, 168.6, 160.0, 159.4, 155.1, 154,6, 149.6, 139.8, 138.5, 131.2, 129.1, 128.6 126.9, 124.8 (CF_3), 48.9, 35.6, 30.7, 29.7, 28.6, 24.8, 23.7, 21.2, 29.2, 18.8; HRMS (m/z): $[M+H]^+$ calcd for $C_{32}H_{39}ClF_3N_5O_3S$: 665.2414; found: 665.2419.

4.2 Cell viability assay

The mitochondrial-dependent reduction of MTT to formazan was used to measure the cytotoxicity. Briefly, cells were seeded in multiple 96-well plates at the density of 4×10^4 /mL. After overnight of incubation, the cells were treated with serially diluted test compounds for 72 h. After treatment, MTT solution (5 mg/mL) was added to each well, incubation was performed in the dark, at 37°C for another 4 h. Supernatant was removed, and DMSO was then added to dissolve the MTT formazan product. Its absorbance was assessed at 570 nm using a Molecular Devices (USA) SpectraMax M5 spectrophotometer. Relative cell viability rates were calculated versus untreated controls, with 50% inhibitory concentration (IC_{50}) values calculated using Graph

Pad Prism 5 (Graph Pad Software Inc., USA).[18]

4.3 Molecular docking

The crystal structure of microtubules and tubulysin M (PDB code: 4ZOL) was downloaded from RCSB Protein Data Bank. Before the docking process, the natural co-crystalized ligand was extracted and water molecules were removed from the crystal structure, H atoms were added and side chains were fixed during protein preparation. All the steps parameters were set to the default value of 100 in the window of staged minimization. Subsequently, the protein was prepared using Biopolymer module implemented in Sybyl. Protein structure minimization was performed by applying the Tripos force field and partial atomic charges were calculated by the Gasteiger-Huckel method. The gradient of termination was defined as 0.005 kcal/(mol*A) and the maximum number of interactions were 10000.

Conflict of interest

The authors declare have no conflict of interest.

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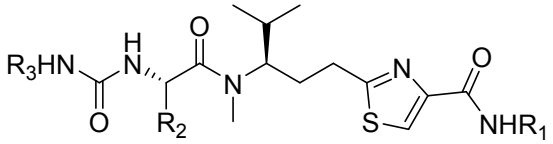
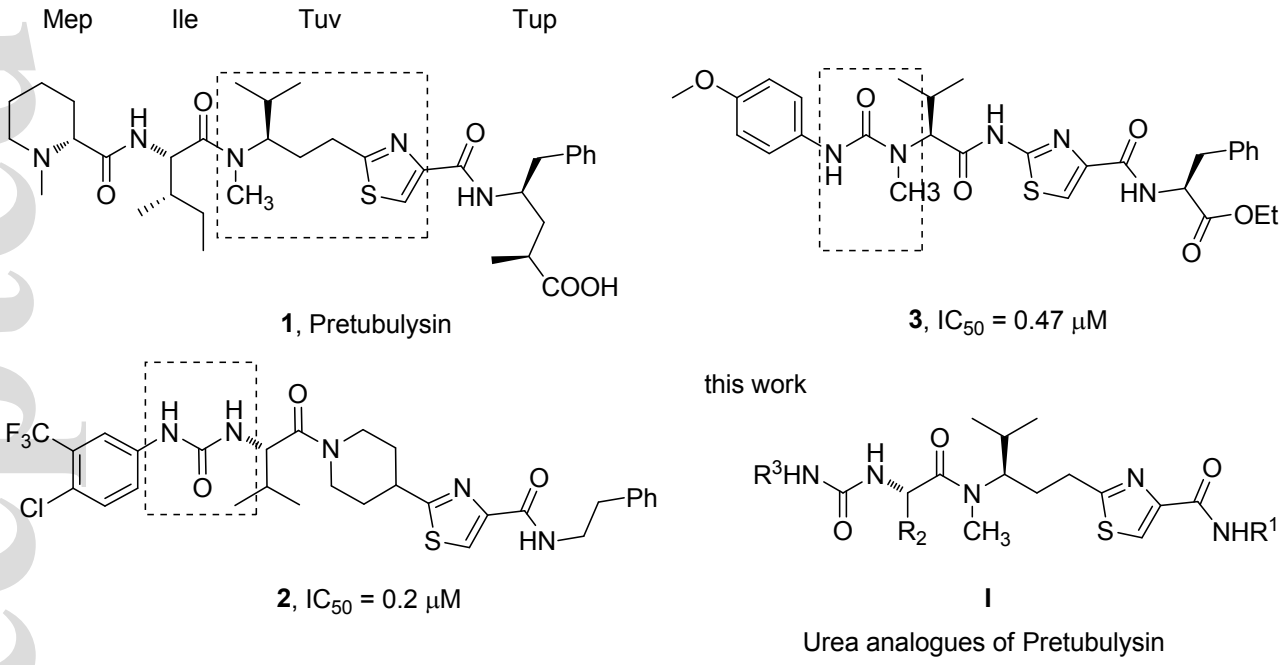
Table 1. Structures and antitumor activities of urea analogs



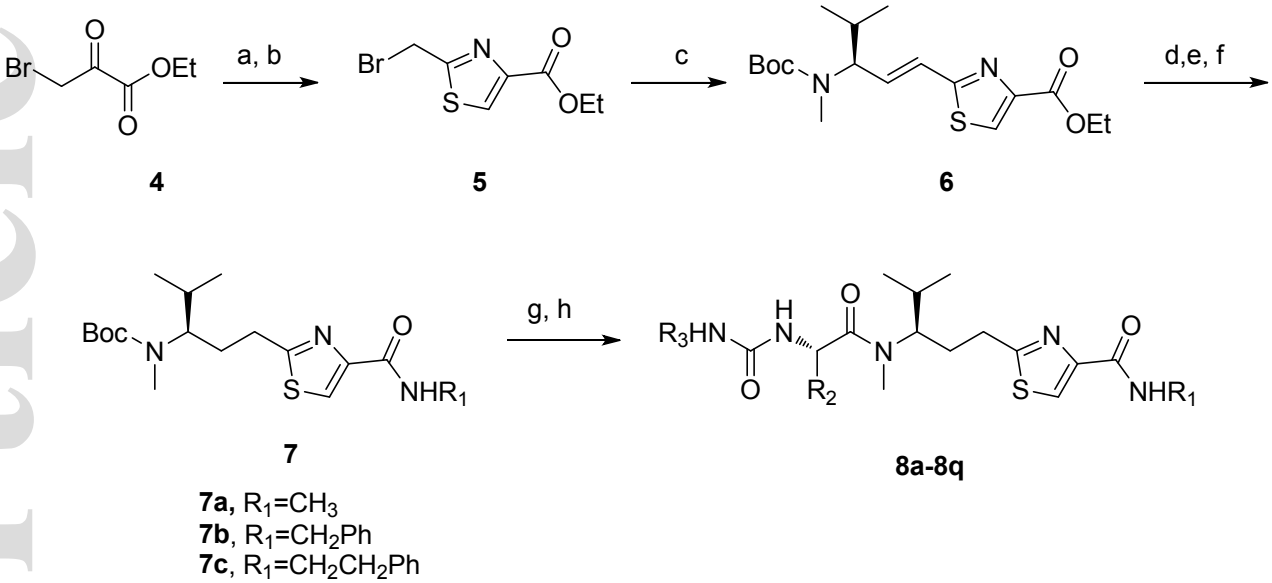
Compd.	R_1	R_2	R_3	IC_{50} (μM) (n =3, X±SD)	
				MCF-7	NCl-H157
8a	Me	$CH_2CH(CH_3)_2$	*p*-$OMeC_6H_4$	1.75±0.23	2.35±0.39
8b	Me	$CH_2CH(CH_3)_2$	*p*-ClC_6H_4	1.07±0.15	1.47±0.17
8c	Me	$CH_2CH(CH_3)_2$	3-CF_3-4-ClC_6H_3	0.05±0.009	0.09±0.01
8d	$PhCH_2$	$CH_2CH(CH_3)_2$	*p*-$OMeC_6H_4$	1.27±0.16	0.98±0.11
8e	$PhCH_2$	$CH_2CH(CH_3)_2$	*p*-ClC_6H_4	0.79±0.01	1.05±0.15
8f	$PhCH_2$	$CH_2CH(CH_3)_2$	3-CF_3-4-ClC_6H_3	0.53±0.01	0.46±0.05
8g	$PhCH_2CH_2$	$CH_2CH(CH_3)_2$	*p*-$NO_2C_6H_4$	0.35±0.009	0.46±0.02
8h	$PhCH_2CH_2$	$CH_2CH(CH_3)_2$	*p*-ClC_6H_4	0.01±0.003	0.02±0.005
8i	$PhCH_2CH_2$	$CH_2CH(CH_3)_2$	*p*-$OMeC_6H_4$	0.27±0.01	0.32±0.01
8j	$PhCH_2CH_2$	$CH_2CH(CH_3)_2$	3-CF_3-4-ClC_6H_3	0.09±0.01	0.07±0.01

8k	Me	CH_3CHCH_3	*p*-$OMeC_6H_4$	2.31±0.57	4.31±0.87
8l	Me	CH_3CHCH_3	*p*-ClC_6H_4	1.59±0.19	2.34±0.31
8m	Me	CH_3CHCH_3	3-CF_3-4-ClC_6H_3	0.76±00.6	0.62±0.15
8n	$PhCH_2$	CH_3CHCH_3	*p*-$OMeC_6H_4$	1.02±0.15	2.05±0.37
8o	$PhCH_2CH_2$	CH_3CHCH_3	*p*-$OMeC_6H_4$	0.97±0.11	0.69±0.01
8p	$PhCH_2CH_2$	CH_3CHCH_3	*p*-ClC_6H_4	0.38±0.02	0.12±0.005
8q	$PhCH_2CH_2$	CH_3CHCH_3	3-CF_3-4-ClC_6H_3	0.11±0.03	0.09±0.01
1	/	/	/	0.001	0.001
Taxol	/	/	/	0.01	0.01

Fig. 1: The design of pretubulysin analogs

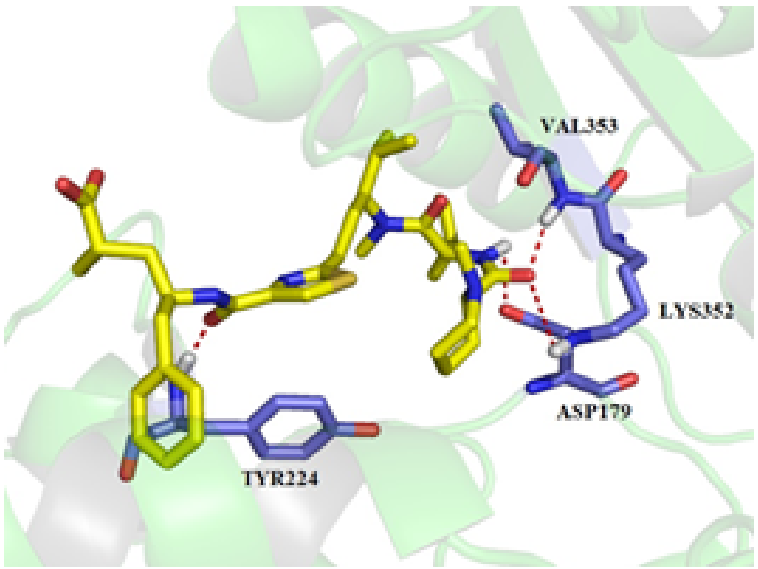


Scheme 1. Synthesis of compounds 8a-8q.

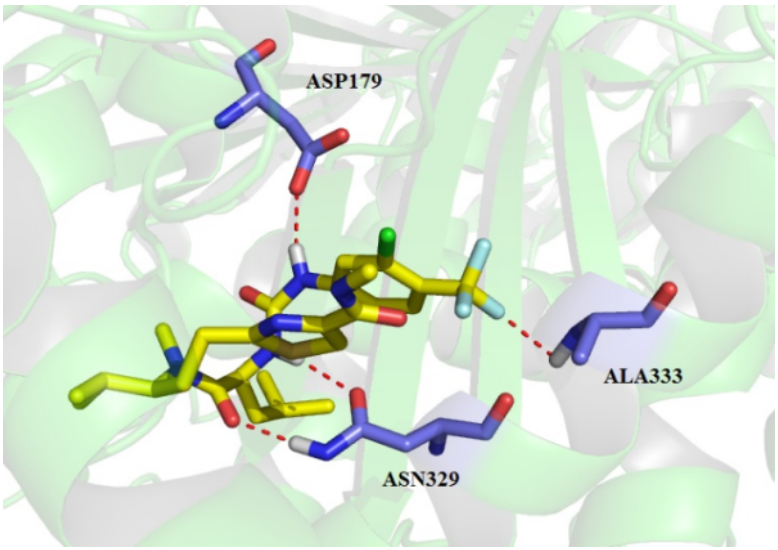


Scheme 1. Reagents and conditions: (a). thioacetamide, EtOH, r.t. 82%; (b). NBS, BPO, CCl_4, reflux, 64%; (c). 1). triphenylphosphine, Tol, reflux, 75%; 2). *t*-BuOK, (*S*)-*N*-tert-butoxycarbonyl-*N*-methylvalinal, DCM, r.t. 69%; (d). H_2, Pd/C, MeOH, r.t. 98%; (e). LiOH, THF/H_2O, r.t. 92%; (f). EDC, HOBt, R_1NH_2, r.t.74-85%; (g). TFA, DCM, r.t. 89%; (h). EDC, HOBt, DCM, $R_3NHCONHCH(R_2)CO_2H$, r.t. 63-75%.

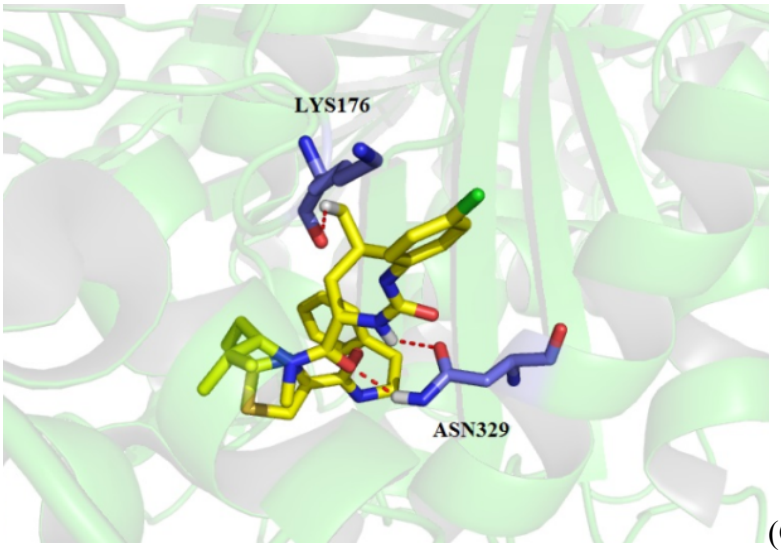
Fig. 2. Predicted binding mode of pretubulysin (yellow, A), **8c** (yellow, B), and **8h** (yellow, C) with tubulin. Dashed red lines indicate the H-bond interaction.



（A）



（B）



(C)