Communication to the Editor

Synthesis and Evaluation of Biological Properties of 2-Amino-thiazole-4-carboxamides: Amide Linkage Analogues of Pretubulysin

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Pretubulysin is a bio-precursor of highly toxic tetrapeptide tubulysins. Although pretubulysin has a much simpler chemical structure, it has similar anti-mitotic potency. A series of 2-amino-thiazole-4-carboxamides were designed and synthesized based on the structure of cemadotin. These are all novel compounds and their structures are characterized by 1H-NMR, 13C-NMR, and high resolution (HR)MS. The antitumor activities of these compounds were screened using the methyl thiazolyl tetrazolium colorimetric (MTT) cell viability method in MCF7 (breast cancer) and NCI-H1650 (lung cancer) cells. All the synthesized compounds 6a–n showed moderate anti-proliferation activities. Compounds 6m exhibited antitumor activity with the IC50 value of 0.47 and 1.1 µM in MCF7 and NCI-H1650 cells, respectively.

Key words: tubulysin; pretubulysin; structural modification; antitumor; urea; 2-amino-thiazole-4-carboxamide

INTRODUCTION

The tubulysins are a family of anti-mitotic tetrapeptides isolated by Höfle and colleagues from Angiococcus disciformis in 2000.10 Tubulysin D, the most potent compound in the family, exhibits high cytotoxicity towards a wide range of tumor cells in the low nano- or even picomolar concentration range.11 However, synthesis and commercial supply of tubulysins present a challenge due to their complex structures.3,4 Pretubulysin (I, Fig. 1), a tubulysin precursor, shows equal anti-mitotic potency to tubulysins, but has a much simpler chemical structure.5,6 Therefore, there has been significant interest in modifying and simplifying the structure of pretubulysin, which has resulted in the synthesis of several promising analogues.7–10 Tubulysins and pretubulysin can bind to tubulin and subsequently inhibit its polymerization.7,11 These tubulin-binding agents (TBAs) were shown to be successful anti-cancer compounds.12

Cemadotin, a synthetic analogue of dolastatin, is a linear pentapeptide that inhibits cell proliferation by suppressing microtubules.13,14 Pretubulysin and cemadotin share a structural similarity, as shown in Fig. 1. Previous studies on the structure–activity relationship (SAR) of tubulysin and pretubulysin suggested that the central N-methyl-L-Valyl was the key functional group for maintaining antitumor activity.15 The methyl-L-Valyl group and the thiazole group. An amide group was used to connect the above two groups instead of the ethylene in pretubulysin.

Another notable feature of this series of compounds was that there were only two chiral centers, compared to five and six in cemadotin and pretubulysin, respectively.

RESULTS AND DISCUSSION

Chemistry The synthesis of these 2-amino-thiazole-4-carboxamides is shown in Chart 1. The commercially available compound ethyl 2-amino-4-carboxylate (3) was converted to intermediate amides 4a–c by a 3-step transformation. First, the amino of compound 3 was protected by di-tert-butyldi carbonate. Second, the ester group of 3 was hydrolyzed to the corresponding acid. Third, coupling of the acid and a variety of amines, including methylamine, benzylamine, and phenylethylamine, yielded compounds 4a–c under classic peptide coupling conditions (1-(3-dimethylaminopropyl)-3-ethylenecarbodiimide hydrochloride (EDCI), 1-hydroxybenzotriazole (HOBt), N,N-diisopropylcarbodiimide (DIPEA)). Removal of the tert-butoxycarbonyl (Boc) group in compounds 4a–c, followed by coupling with (L)-9-fluorenlyloxybenzoyl (Fmoc)-N-Me-Val,20 gave compounds 5a–c. After removal of the Fmoc group of compounds 5a–c, the resulting amines were coupled with isocyanates to give 6a–i and with isonicotinic acid to give 6j–l.

Evaluation of Biological Properties The in vitro anti-tumor activities of compounds 6a–n were screened using the methyl thiazolyl tetrazolium colorimetric (MTT) cell viability method in MCF7 (breast cancer) and NCI-H1650 (lung cancer).

Fig. 1. The Design of 2-Amino-thiazole-4-carboxamides

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cell lines. Taxol and pretubulysin were chosen as the control. The synthesized compounds 6a–n showed moderate antiproliferation activities against MCF7 and NCI-H1650 tumor cells. Among these compounds, 6m showed the most significant inhibitory activity (IC50 = 0.47 and 1.1 µM, respectively) against MCF7 and NCI-H1650 cells. The antiproliferation activities of the synthesized compounds are shown in Table 1.

The above biological screening results regarding the SAR of this series of compounds indicated the following: 1. Compounds with phenylethylamine (6a, d, g) in the Tup segment (R1 group) generally showed better antitumor activity than those with methylamine or benzylamine. Compounds with a phenylalanine (Phe)-OEt (an additional ester group attached to phenylethylamine) group (6m, n) also showed better antitumor activity. 2. Compounds with a urea group in the Mep segment (R2) generally showed better antitumor activity than those with an isonicotinic amide group (6j–l). Furthermore, compounds 6c and f, which contained benzamidine groups, showed similar activities to compounds 6j–l.

Although most of the compounds in this series showed antitumor activity, they were less potent than taxol and pretubulysin. In order to investigate the mechanism of the observed activity, molecular docking was carried out using the co-crystal structure of microtubules and tubulysin M (PDB code: 4ZOL).

### Chart 1.

#### Table 1. The Antitumor Activities of 2-Amino-thiazole-4-carboxamides

| Compd. | IC50 (µM) | Compd. | IC50 (µM) |
|--------|-----------|--------|-----------|
|        | MCF7      | NCI-H1650 | MCF7      | NCI-H1650 |
| 6a     | 3.2 ± 1.1 | 4.6 ± 2.5 | 6j       | 23.1 ± 7.9 | 15.3 ± 7.6 |
| 6b     | 13.5 ± 6.3 | 17.6 ± 8.2 | 6k       | 33.2 ± 11.3 | 15.7 ± 6.9 |
| 6c     | 42.5 ± 13.6 | 52.3 ± 18.7 | 6l       | 48.2 ± 13.6 | 35.7 ± 14.7 |
| 6d     | 7.6 ± 3.2  | 3.3 ± 0.9  | 6m       | 0.47 ± 0.1  | 1.1 ± 0.46  |
| 6e     | 12.3 ± 5.6 | 10.2 ± 4.5 | 6n       | 5.3 ± 2.5   | 9.6 ± 4.9   |
| 6f     | 25.3 ± 9.6 | 18.3 ± 9.8 | Taxol    | 0.03 ± 0.004 | 0.06 ± 0.01 |
| 6g     | 1.1 ± 0.6  | 2.3 ± 0.9  | 1        | <0.001      | <0.001      |
| 6h     | 3.6 ± 1.7  | 4.8 ± 2.2  | 2        | <0.001      | <0.001      |
| 6i     | 18.6 ± 8.2 | 12.5 ± 5.8 | 6j       | <0.001      | <0.001      |
| 6k     | 12.3 ± 5.6 | 10.2 ± 4.5 | 6m       | 5.3 ± 2.5   | 9.6 ± 4.9   |
| 6l     | 25.3 ± 9.6 | 18.3 ± 9.8 | Taxol    | 0.03 ± 0.004 | 0.06 ± 0.01 |
| 6m     | 0.47 ± 0.1 | 1.1 ± 0.46 | 1        | <0.001      | <0.001      |
| 6n     | 5.3 ± 2.5  | 9.6 ± 4.9  | 2        | <0.001      | <0.001      |

### CONCLUSION

A series of 2-amino-thiazole-4-carboxamides were designed and synthesized. Their antitumor activities were screened using the MTT cell viability assay. All the synthesized compounds 6a–n showed anti-proliferative activities in MCF7 and NCI-H1650 tumor cells. Compounds 6m showed relatively better antitumor activity in MCF7 and NCI-H1650 tumor cells compared to the other compounds in the series, with IC50 values of 0.47 and 1.1 µM, respectively. Although
those series of compounds were overall less potent than taxol, they may represent a promising lead for the further development of novel antitumor drugs.

MATERIALS AND METHODS

Chemistry  All reactions were carried out under a nitrogen atmosphere with dry solvent under anhydrous conditions, unless otherwise noted. Reagents were purchased at the highest commercial quality and used without further purification, unless otherwise noted. The NMR spectra of the intermediates and final products in deuterated solvent were recorded on a Bruker 400 or 600 MHz spectrometer. The following abbreviations were used to designate the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, h = heptet, m = multiplet, b = broad. High-resolution (HR) MS were recorded on an Agilent 6210 ESI/TOF mass spectrometer. Melting points (MP) were recorded on a Büchi B-540 melting point apparatus and are uncorrected. Flash column chromatographic separation was achieved using a silica gel from Qingdao Ocean Chemical General Procedure for Preparation of Compounds 5a–c

TFA (10 mL) was added to a solution of compound 4b (3.30 g, 9.90 mmol) in 30 mL dichloromethane at room temperature for 3 h and then concentrated in vacuo. The residue was diluted with 15 mL DCM and 8 mL N,N-diisopropylethylamine to afford the amine solution. In another flask, a solution of Fmoc-N-Me-(l)-Val (3.5 g, 9.90 mmol), EDC (2.85 g, 14.86 mmol), and HOBt (1.34 g, 9.90 mmol) in 100 mL dichloromethane was stirred at r.t. for 30 min. The above amine solution was added to the aforementioned reaction mixture and stirred for a further 6 h at r.t. Water was added to the reaction mixture and the aqueous solution was extracted with dichloromethane. The organic layers were combined, washed with water and brine, dried over sodium sulfate, filtered, and concentrated. The residue was purified using flash column chromatography to provide pure free amine intermediate. This intermediate (100 mg, 0.28 mmol) was dissolved in DCM (20 mL), then 4-methoxyphenyl isocyanate (40 mg, 0.28 mmol) was added and the reaction mixture was stirred at r.t. until TLC showed that the reaction was complete. The crude product was diluted with water (15 mL) and extracted with ethyl acetate (3 × 20 mL). The organic layers were collected, dried, filtered, and concentrated. The obtained residue was purified using flash column chromatography to provide pure free amine intermediate. This intermediate (100 mg, 0.28 mmol) was dissolved in DCM (20 mL), then 4-methoxyphenyl isocyanate (40 mg, 0.28 mmol) was added and the reaction mixture was stirred at r.t. for 3 h until TLC showed that the reaction was complete. The solid was filtered out, after which the filtrate was diluted with water and extracted with DCM (10 mL × 3). This was combined with the organic phase, dried over Na2SO4, filtered, and concentrated. The obtained residue was purified using flash column chromatography to yield product 6a (126.3 mg, yield: 89.3%) as a colorless oil. 1H-NMR (600 MHz, CDCl3) δ H 10.90 (brs, 1H), 7.58 (s, 1H), 7.15–7.26 (multiple, 8H), 6.70–6.78 (d, J = 12.8 Hz, 2H), 6.36 (s, 1H), 4.85–4.60 (m, 1H), 3.75 (s, 3H), 3.67–3.74 (m, 1H), 3.54–3.59 (m, 1H), 2.87–2.97 (multiple, 5H), 2.31–2.37 (m, 1H), 0.95–0.96 (d, J = 6.6 Hz, 3H), 0.88–0.90 (d, J = 6.6 Hz, 3H). 13C-NMR (150 MHz, CDCl3): 168.6, 160.1, 156.6, 155.3, 155.2, 143.1, 137.9, 129.2, 127.5, 127.3, 126.1, 122.9, 116.2, 54.0, 39.5, 35.1, 34.3, 25.5, 17.8, 17.6; HR MS Calcd for C32H33N4O4S [M + H]+ 569.2217, Found 569.2213.

General Procedure for Docking of Urea Group in Compounds 6a–i

(5)-3-(4-Methoxybenzoyl)-1-methyl-1-(3-methyl-1-oxo-1-(4-phenethylenecarb Amoyl)hiazol-2-ylamino)butan-2-yl)urea (6a)

Piperidine (1.5 mL) was added to a solution of compound 5 (2.6 g, 4.46 mmol) in 50 mL N,N-dimethylformamide and the reaction mixture was stirred at r.t. until TLC showed that the reaction was complete. The crude product was diluted with water (15 mL) and extracted with ethyl acetate (3 × 20 mL). The organic layers were collected, dried, filtered, and concentrated. The obtained residue was purified using flash column chromatography to provide pure free amine intermediate. This intermediate (100 mg, 0.28 mmol) was dissolved in DCM (20 mL), then 4-methoxyphenyl isocyanate (40 mg, 0.28 mmol) was added and the reaction mixture was stirred at r.t. until TLC showed that the reaction was complete. The solid was filtered out, after which the filtrate was diluted with water and extracted with DCM (10 mL × 3). This was combined with the organic phase, dried over Na2SO4, filtered, and concentrated. The obtained residue was purified using flash column chromatography to yield product 6a (126.3 mg, yield: 89.3%) as a colorless oil. 1H-NMR (600 MHz, CDCl3) δ H 10.90 (brs, 1H), 7.58 (s, 1H), 7.15–7.26 (multiple, 8H), 6.70–6.78 (d, J = 12.8 Hz, 2H), 6.36 (s, 1H), 4.85–4.60 (m, 1H), 3.75 (s, 3H), 3.67–3.74 (m, 1H), 3.54–3.59 (m, 1H), 2.87–2.97 (multiple, 5H), 2.31–2.37 (m, 1H), 0.95–0.96 (d, J = 6.6 Hz, 3H), 0.88–0.90 (d, J = 6.6 Hz, 3H). 13C-NMR (150 MHz, CDCl3): 168.6, 160.1, 156.6, 155.3, 155.2, 143.1, 137.9, 129.2, 127.5, 127.3, 126.1, 122.9, 116.2, 54.0, 39.5, 35.1, 34.3, 25.5, 17.8, 17.6; HR MS Calcd for C32H33N4O4S [M + H]+ 569.2217, Found 569.2213.
(m, 6H). ¹³C-NMR (150 MHz, CDCl₃): 169.6, 162.0, 157.8, 156.7, 156.5, 144.6, 130.7, 127.6, 123.7, 117.4, 114.1, 55.4, 29.6, 26.7, 25.9, 19.5, 18.9; HR MS Calcd for C₂₆H₂₉ClN₅O₄S [M + H]⁺ 448.1649, Found 448.1644.

(S)-N-Benzyl-2-(2-(3-(4-methoxyphenyl)-1-methylureido)-3-methylbutanamido)-thiazole-4-carboxamide (6e)

White xate, 80%. ¹¹H-NMR (400 MHz, CDCl₃) δH 10.81 (brs, 1H), 7.63 (s, 1H), 7.43–7.46 (m, 1H), 7.27–7.34 (m, 5H), 7.13–7.14 (m, 2H), 6.74–6.76 (m, 2H), 4.66–4.71 (m, 1H), 4.48–4.57 (m, 2H), 3.74 (s, 3H), 2.83 (s, 3H), 2.27–2.34 (m, 1H), 0.89–0.92 (m, 6H). ¹³C-NMR (100 MHz, CDCl₃): 169.5, 162.7, 161.3, 157.4, 154.4, 147.7, 137.9, 131.5, 129.2, 128.7, 128.4, 120.8, 118.0, 113.8, 70.2, 70.6, 64.0, 44.0, 29.9, 26.8, 19.3; HR MS Calcd for C₂₆H₃₀ClN₅O₄S [M + H]⁺ 469.1303, Found 469.1301.

(S)-3-(4-Chlorobenzoyl)-1-methyl-1-(3-methyl-1-oxo-1-(4-phenoxy carbamoyl)thiazol-2-yl)urea (6d)

White xate, 91.7%. ¹¹H-NMR (600 MHz, CDCl₃) δH 10.54 (brs, 1H), 7.62 (s, 1H), 7.21–7.29 (multiple, 10H), 6.56 (s, 1H), 4.55 (m, 1H), 3.60–3.71 (m, 2H), 2.98 (s, 3H), 2.90–2.92 (m, 2H), 2.38–2.40 (m, 1H), 0.95–0.98 (m, 6H). ¹³C-NMR (150 MHz, CDCl₃): 169.8, 164.6, 154.8, 152.8, 153.4, 139.6, 138.6, 138.3, 133.4, 129.2, 128.7, 128.4 (2), 126.1, 113.0, 54.0, 41.5, 35.5, 34.1, 27.4, 19.3; HR MS Calcd for C₂₄H₂₇ClN₅O₃S [M + H]⁺ 452.1623, Found 452.1631.

(S)-2-(2-(3-(4-Chlorophenyl)-1-methylureido)-3-methylbutanamido)-N-methylthiazol-4-carboxamide (6e)

White xate, 86.7%. ¹¹H-NMR (400 MHz, CDCl₃) δH 8.09 (s, 1H), 7.71 (s, 1H), 7.31–7.33 (m, 2H), 7.24–7.26 (m, 2H), 7.10 (brs, 1H), 4.47–4.50 (m, 1H), 3.04 (s, 3H), 2.97 (s, 3H), 2.45–2.49 (m, 1H), 1.96–1.02 (m, 6H). ¹³C-NMR (100 MHz, CDCl₃): 170.8, 169.1, 162.0, 144.7, 136.9, 133.8, 129.1, 127.3, 122.1, 113.6, 29.2, 28.7, 26.1, 17.3, 16.7; HR MS Calcd for C₂₄H₂₄Cl₂N₅O₃S [M + H]⁺ 542.1623, Found 542.1631.

(S)-N-Benzyl-2-(2-(3-(4-chlorophenyl)-1-methylureido)-3-methylbutanamido)-thiazole-4-carboxamide (6f)

White xate, 85.8%. ¹¹H-NMR (400 MHz, CDCl₃) δH 10.73 (brs, 1H), 7.62 (s, 1H), 7.38–7.41 (m, 1H), 7.28–7.35 (m, 2H), 7.15–7.25 (m, 7H), 6.55 (s, 1H), 4.64–4.69 (m, 5H), 4.54–4.56 (m, 2H), 2.87 (s, 3H), 2.29–2.35 (m, 1H), 0.91–0.94 (m, 6H). ¹³C-NMR (100 MHz, CDCl₃): 172.6, 166.9, 162.3, 152.9, 142.9, 139.3, 138.6, 130.6, 129.5, 128.6, 127.9, 127.6, 121.5, 114.2, 62.1, 44.8, 36.1, 27.1, 19.4; HR MS Calcd for C₂₄H₂₄Cl₂N₅O₃S [M + H]⁺ 500.1518, Found 500.1511.

(S)-2-(2-(3-(4-Chloro-3-(trifluoromethyl)phenyl)-1-methylureido)-3-methylbutanamido)-N-phenethylthiazole-4-carboxamide (6g)

Pale yellow xate, 77.8%. ¹¹H-NMR (400 MHz, CDCl₃) δH 8.60 (brs, 1H), 7.79 (s, 1H), 7.46–7.47 (d, J = 4 Hz, 1H), 7.25–7.29 (multiple, 5H), 7.18–7.19 (m, 1H), 4.98–5.0 (m, 1H), 3.60–3.63 (m, 2H), 3.03 (s, 3H), 2.89–2.92 (m, 2H), 2.49–2.51 (m, 1H), 1.05–1.1 (m, 6H). ¹³C-NMR (100 MHz, CDCl₃): 172.4, 165.7, 161.2, 153.8, 148.5, 146.2, 137.9, 137.2, 129.2, 127.8, 126.5, 116.6, 113.5, 62.7, 42.1, 35.0, 34.2, 27.9, 18.4; HR MS Calcd for C₂₄H₂₄Cl₂N₅O₃S [M + H]⁺ 466.1907, Found 466.1903.

(S)-N-Methyl-2-(2-(3-methylisonicotinamido)butanamido)-N-phenethylthia Zole-4-carboxamide (6j)

Pale yellow xate, 71.7%. ¹¹H-NMR (400 MHz, CDCl₃) δH 10.31 (brs, 1H), 7.72 (s, 1H), 7.28–7.31 (m, 2H), 7.11 (brs, 1H), 6.57–6.58 (m, 8H), 4.76–4.77(mH), 3.60–3.63 (m, 2H), 3.04 (s, 3H), 2.97 (s, 3H), 2.43–2.49 (m, 1H), 0.93–1.02 (m, 6H). ¹³C-NMR (100 MHz, CDCl₃): 172.1, 167.9, 162.5, 161.2, 149.0, 144.8, 144.5, 121.9, 114.9, 60.2, 33.6, 27.1, 26.3, 18.7; HR MS Calcd for C₂₄H₂₄Cl₂N₅O₃S [M + H]⁺ 376.1438, Found 376.1433.

(S)-N-Benzyl-2-(2-(3-methylisonicotinamido)butanamido)-thiazole-4-carboxamide (6i)

White xate, 76.9%. ¹¹H-NMR (400 MHz, CDCl₃) δH 9.9
(brs, 1H), 7.79 (s, 1H), 8.71–8.73 (m, 2H), 7.79 (s, 1H), 7.31–7.34 (multiple, 8H), 4.67–4.70 (m, 1H), 4.61–4.64 (m, 2H), 2.91 (s, 3H), 2.52–2.58 (m, 1H), 1.06–1.08 (m, 6H).

1H-NMR (400 MHz, CDCl 3): 171.8, 170.7, 162.0, 160.3, 155.5 (q, \( J = 4.8 \) Hz), 3.69 (s, 3H), 3.30 (q, \( J = 4.8 \) Hz), 1.18 (t, \( J = 4.4 \) Hz, 3H), 2.91 (s, 3H), 2.52–2.58 (m, 1H), 1.06–1.08 (m, 6H).

13C-NMR (100 MHz, CDCl 3): 171.9, 168.8, 162.8, 161.8, 159.7, 135.9, 124.9, 117.4, 115.5, 113.7, 67.4, 65.8, 56.8, 53.1, 38.9, 29.1, 25.7, 18.1, 16.2; HRMS Calcd for C_{29}H_{36}N_{5}O_{6}S [M + H]^{+} 582.2389, Found 582.2391.

(5)-Ethyl 2-((S)-(3-Methyl-2-(1-methyl-3-(pyridin-4-yl)ureido)-3- methylbutanamido)thiazole-4-carboxamido)-3-phenylpropanoate (6m)

Pale yellow wax, 76.3%. 1H-NMR (400 MHz, CDCl 3), \( \delta \)H 9.9 (br s, 1H), 7.79 (s, 1H), 8.71–8.73 (m, 2H), 7.79 (s, 1H), 7.31–7.34 (multiple, 8H), 4.67–4.70 (m, 1H), 4.61–4.64 (m, 2H), 2.91 (s, 3H), 2.52–2.58 (m, 1H), 1.06–1.08 (m, 6H).

13C-NMR (100 MHz, CDCl 3): 171.9, 170.5, 163.7, 161.4, 158.3, 155.2, 145.1, 136.4, 132.1, 128.4, 127.5, 124.8, 119.7, 124.8, 119.7, 115.7, 113.2, 67.4, 65.8, 56.8, 53.1, 38.9, 29.1, 25.7, 18.1, 16.2; HRMS Calcd for C_{29}H_{36}N_{5}O_{6}S [M + H]^{+} 582.2289, Found 582.2283.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

Experimental procedures and spectral data (1H-NMR, 13C-NMR, HR MS), and copies of 1H-NMR and 13C-NMR for all compounds described in the paper. The online version of this article contains supplementary materials.

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