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Supporting Information

(Total of 15 pages)

for

Biological activity and interaction mechanism of the diketopiperazine derivatives as tubulin polymerization inhibitors

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Synthesis routine and characterization of DKP derivatives (Page 4-13)

Fig. S1. Spectra of the inhibitor-residue interaction for tubulin-DKP derivative complexes by α or β tubulin.

Fig. S2 Binding mode of plinabulin with tubulin.

Scheme:



Regents and conditions (a) n-BuLi, THF, -78 °C; (b) HCl (1M), THF

1,4-diacetylpiperazine-2,5-dione (2)

The mixture of glycine anhydride (15 g, 131.4 mmol) and acetic anhydride (54 g, 52.5 mmol) was stirred at reflux temperature for 7 h and the reaction was monitored using TLC. After reaction completed, solvent was removed under reduced pressure and the residue obtained was re-crystallized from ethyl acetate/diethyl ether to yield the desired product. (21.6 g, 85%)

(Z)-1-acetyl-3-((5-(tert-butyl)-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (3)

$(R_1 = 5-(tert-butyl)-1H-imidazole)$

The mixture of compound 2 (5 g, 32.85 mmol), Cs_2CO_3 (16.05 g, 49.28 mmol), aldehyde (13.02 g, 65.7 mmol) and DMF (35 mL) was stirred at room temperature under N₂ atmospher for 24 h and the

reaction was monitored using TLC. After reaction was completed, the mixture was poured into crashed ice and the solid was filtered to give product (Yield: 4.05 g, 42%)

(Z)-1-acetyl-3-((5-(tert-butyl)-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (3)

$(R_1 = 5$ -methyl-1H-imidazole)

The mixture of compound 2 (2.5 g, 16.43 mmol), Cs_2CO_3 (8.0 g, 24.65 mmol), aldehyde (6.51 g, 32.85 mmol) and DMF (18 mL) was stirred at room temperature under N₂ atmospher for 24 h and the reaction was monitored using TLC. After reaction was completed, the mixture was poured into crashed ice and the solid was filtered to give product (Yield: 2 g, 42%)

(Z)-1-acetyl-3-((5-(tert-butyl)-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (3)

(**R**₁ = pyridine)

The mixture of compound 2 (3.7 g, 18.7 mmol), Cs_2CO_3 (16.05 g, 49.28 mmol), aldehyde (1 g, 9.3 mmol) and DMF (35 mL) was stirred at room temperature under N₂ atmospher for 24 h and the reaction was monitored using TLC. After reaction was completed, the mixture was poured into crashed ice and the solid was filtered to give product (Yield: 560 g, 24%)

(Z)-1-acetyl-3-((5-(tert-butyl)-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (3)

$(\mathbf{R}_1 = \mathbf{benzene})$

The mixture of compound 2 (3.2 g, 21.06 mmol), Cs_2CO_3 (25 g, 10.29 mmol), aldehyde (13.02 g, 8.24 mmol) and DMF (22 mL) was stirred at room temperature under N₂ atmospher for 24 h and the reaction was monitored using TLC. After reaction was completed, the mixture was poured into crashed ice and the solid was filtered to give product (Yield: 2.6 g, 39%)

(Z)-3-((Z)-benzylidene)-6-((5-(tert-butyl)-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (Plinabulin)

The mixture of compound 3 (132 mg, 0.84 mmol), Cs_2CO_3 (220 g, 0.65 mmol), aldehyde (145 g, 0.65 mmol) and DMF (10 mL) was stirred at 50 °C under N₂ atmospher for 24 h and the reaction was monitored using TLC. After reaction was completed. The mixture was extracted with EtOAc. The organic layer was washed with brine and was dried over anhydrous sodium sulfate (Na₂SO₄). Then solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography give product (Yield: 86 mg, 43%) HPLC (UV=254): 99%. LC-MS: [M+H] ⁺ = 337. ¹H NMR (500 MHz, DMSO-d₆) δ 12.33 (s, 1H), 12.25 (s, 1H), 10.05 (s, 1H), 7.85 (s, 1H), 7.53 (d, J = 7.7 Hz, 2H), 7.41 (t, J = 7.6 Hz, 2H), 7.31 (t, J = 7.4 Hz, 1H), 6.85 (s, 1H), 1.38 (s, 10H).

(Z)-3-((Z)-benzylidene)-6-((5-methyl-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (Compound a)

According to the procedure described for the synthesis of plinabulin. (Yield: 28 mg, 43%) HPLC (UV=254): 97%. LC-MS: $[M+H]^+ = 295$. ¹H NMR (500 MHz, DMSO-d₆) δ 11.70 (s, 1H), 11.15 (s, 1H), 9.16 (s, 1H), 7.05 (s, 1H), 6.71 (d, *J* = 7.7 Hz, 2H), 6.60 (t, *J* = 7.7 Hz, 2H), 6.51 (d, *J* = 7.4 Hz, 1H), 5.93 (s, 1H), 5.77 (s, 1H), 1.50 (s, 3H).

(Z)-3-((Z)-3-benzoylbenzylidene)-6-((5-(tert-butyl)-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (Compound b)

To a cooled (-78 °C) solution of 2.5 M n-BuLi (2 mL) in THF (3 mL) was added 1-bromo-3-(1,3dioxolan-2-yl)-benzene (4) (578 mg, 2.52 mmol) in anhydrous THF (5 mL), and the mixture was stirred for 40 min. Weinreb amides (500 mg, 3.03 mmol) was added dropwise at -78 °C. After the reaction was completed (about 2 hour), the mixture was treated with saturated NH₄Cl (aq) and extracted with EtOAc. The organic layer was washed with brine and was dried over anhydrous sodium sulfate (Na₂SO₄). Then solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography give product (Yield: 400 mg, 62%). Step b: The mixture of compound obtained from step a, (400 mg, 1.57 mmol), 1M HCl (7.8 mL), and THF (10 mL) was stirred at room temperature and the reaction was monitored using TLC. After reaction was completed, The mixture was extracted with EtOAc. The organic layer was washed with brine and was dried over anhydrous sodium sulfate (Na₂SO₄). Then solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography give product. The mixture of compound 3 (123 mg, 0.42 mmol), Cs₂CO₃ (209 mg, 0.64mmol), aldehyde (134 mg, 0.64 mmol) and DMF (5 mL) was stirred at 50 °C under N₂ atmospher for 24 h and the reaction was monitored using TLC. After reaction was completed. The mixture was extracted with EtOAc. The organic layer was washed with brine and was dried over anhydrous sodium sulfate (Na₂SO₄). Then solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography give product (Yield: 80 mg, 43%). HPLC (UV=254): 97%. LC-MS: [M+H] ⁺ = 441. ¹H NMR (500 MHz, DMSO-d₆) δ 12.33 (s, 1H), 12.28 (s, 1H), 10.34 (s, 1H), 7.86 – 7.80 (m, 4H), 7.75 (d, *J* = 7.7 Hz, 1H), 7.69 (t, *J* = 7.4 Hz, 1H), 7.64 (d, *J* = 7.7 Hz, 1H), 7.58 (t, *J* = 7.7 Hz, 3H), 6.86 (s, 1H), 6.80 (s, 1H), 1.38 (s, 8H).

(Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl)methylene)-6-((Z)-3-(4-fluorobenzoyl)benzylidene) piperazine-2,5-dione (Compound c)

According to the procedure described for the synthesis of **Compound b**. (Yield: 46 mg, 48%). HPLC (UV=254): 98%. LC-MS: [M+H] ⁺ = 459. ¹H NMR (500 MHz, DMSO-d₆) δ 12.33 (s, 1H), 12.28 (s, 1H), 10.33 (s, 1H), 7.91 (dd, *J* = 8.4, 5.7 Hz, 2H), 7.85 (s, 1H), 7.82 (s, 1H), 7.75 (d, *J* = 7.6 Hz, 1H), 7.63 (d, *J* = 7.6 Hz, 1H), 7.58 (t, *J* = 7.6 Hz, 1H), 7.40 (t, *J* = 8.7 Hz, 2H), 6.86 (s, 1H), 6.80 (s, 1H), 1.38 (s, 9H).

(Z)-3-((Z)-3-benzoylbenzylidene)-6-((5-methyl-1H-imidazol-4-yl)methylene)piperazine-2,5dione (Compound d)

According to the procedure described for the synthesis of **Compound b**. (Yield: 62 mg, 46%). HPLC (UV=254): 98%. LC-MS: $[M+H]^+ = 399$. ¹H NMR (500 MHz, DMSO-d₆) δ 11.98 (s, 1H), 7.86 (d, *J* = 8.0 Hz, 2H), 7.81 (s, 2H), 7.78 (d, *J* = 7.6 Hz, 1H), 7.70 (t, *J* = 7.4 Hz, 1H), 7.63 (d, *J* = 7.7 Hz, 1H), 7.61 – 7.56 (m, 3H), 6.78 (s, 1H), 6.58 (s, 1H), 2.32 (s, 3H).

(Z)-3-((Z)-3-benzoylbenzylidene)-6-(pyridin-2-ylmethylene)piperazine-2,5-dione (Compound e)

According to the procedure described for the synthesis of **Compound b**. (Yield: 30 mg, 37%). HPLC (UV=254): 97%. LC-MS: [M+H] ⁺ = 396. ¹H NMR (500 MHz, DMSO-d₆) δ 12.60 (s, 1H), 12.60 (s, 1H), 10.65 (s, 1H), 8.73 (d, *J* = 4.4 Hz, 1H), 7.91 (td, *J* = 7.8, 1.6 Hz, 1H), 7.87 (s, 1H), 7.83 (d, *J* = 7.3 Hz, 2H), 7.79 (d, *J* = 7.7 Hz, 1H), 7.73 – 7.65 (m, 3H), 7.59 (dd, *J* = 13.8, 7.5 Hz, 3H), 7.38 (dd, *J* = 7.0, 5.3 Hz, 1H), 6.90 (s, 1H), 6.73 (s, 1H).

(3-((Z)-3-benzoylbenzylidene)-6-((Z)-benzylidene)piperazine-2,5-dione) (Compound f)

According to the procedure described for the synthesis of Compound b. (Yield: 12 mg, 29%). HPLC (UV=254): 98%. LC-MS: [M+H] ⁺ = 395 ¹H NMR (500 MHz, DMSO-d₆) δ 9.61 (d, *J* = 82.9 Hz, 2H), 7.04 (s, 1H), 7.01 (d, *J* = 7.5 Hz, 2H), 6.96 (d, *J* = 7.3 Hz, 2H), 6.78 (dd, *J* = 7.6, 4.4 Hz, 3H), 6.73 (d, *J* = 7.5 Hz, 3H), 6.60 (t, *J* = 7.6 Hz, 3H), 6.51 (t, *J* = 7.3 Hz, 1H), 6.02 (s, 1H), 5.96 (s, 1H).

(Z)-3-((Z)-benzylidene)-6-((5-(tert-butyl)-1H-imidazol-4-yl)methylene)piperazine-2,5-dione

(Plinabulin)



(Z)-3-((Z)-benzylidene)-6-((5-methyl-1H-imidazol-4-yl)methylene)piperazine-2,5-dione

(Compound a)



(Z)-3-((Z)-3-benzoylbenzylidene)-6-((5-(tert-butyl)-1H-imidazol-4-yl)methylene)piperazine-

PROTON_01 MBRI-0-8 $<^{12.3268}_{12.2809}$ -10.33661.3804 -10000 -**9**000 -8000 11 ſ // 7000 6000 -5000 -4000 -3000 -2000 -1000 -0 $\overset{1.00}{\times}\overset{1}{\times}$ 1.18-≖ 4.27 1.15 1.12 3.28 3.28 1.02 9.74--.4 11 13 12 10 9 3 2 1 -1 -2 6 fl (ppm) 4 0 7 5 8

2,5-dione (Compound b)

(Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl)methylene)-6-((Z)-3-(4-fluorobenzoyl)benzylidene)



piperazine-2,5-dione (Compound c)

(Z)-3-((Z)-3-benzoylbenzylidene)-6-((5-methyl-1H-imidazol-4-yl)methylene)piperazine-2,5-

dione (Compound d)



(Z)-3-((Z)-3-benzoylbenzylidene)-6-(pyridin-2-ylmethylene)piperazine-2,5-dione (Compound e)









Fig. S1. Spectra of the inhibitor–residue interaction for the tubulin-DKP derivative complexes by α

or β subunit.



Fig. S2. Binding mode of plinabulin with tubulin.