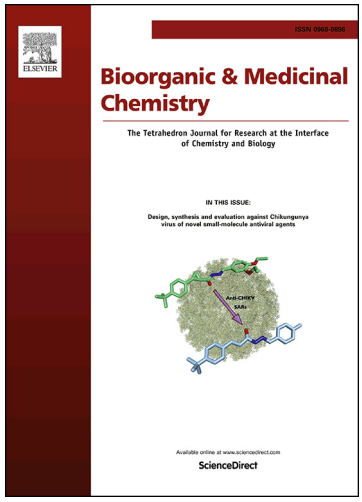

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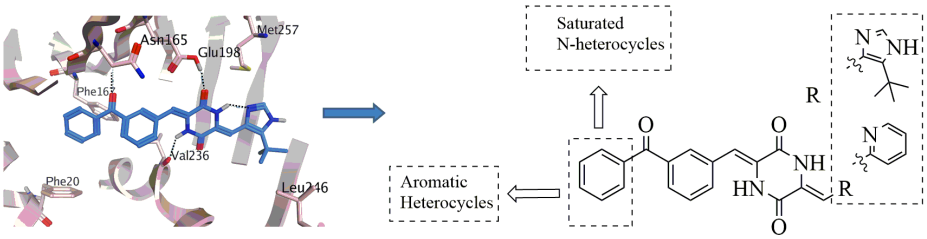

Co-crystal structure guided rational design
Novel compounds with higher activity against human BxPC-3 cell line
Interaction explained by docking studie

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ABSTRACT

Based on the co-crystal structures of tubulin with plinabulin and Compound **1** (a derivative of plinabulin), a total of 18 novel plinabulin derivatives were designed and synthesized. Their biological activities were evaluated against human pancreatic cancer BxPC-3 cell lines. Two novel Compounds **13d** and **13e** exhibited potent activities with IC_{50} at 1.56 and 1.72 nM, respectively. The tubulin polymerization assay indicated that these derivatives could inhibit microtubule polymerization. Furthermore, the interaction between tubulin and these compounds were elucidated by molecular docking. The binding modes of Compounds **13d** and **13e** were similar to the co-crystal structure of Compound **1**. H-π interaction was observed between the aromatic hydrogen of thiophene moiety with Phe20, which could enhance their binding affinities.

1. Introduction

Cancer is one of the deadliest diseases in the world. Recently, it was reported that there were 17.5 million cancer cases and 8.7 million deaths globally in 2015.[1] It is estimated that the number of new cancer cases will increase to 22 million by 2030.[2] Currently, pancreatic cancer has become the seventh leading cause of cancer-related death worldwide.[3] More than 200 thousand people die due to pancreatic cancer every year.[4] However, there are only few drugs for treatment of pancreatic cancer in general manner, such as 5-fluorouracil and gemcitabine.[4] Therefore, development of new drugs to treat pancreatic cancer has become an urgency need. Among these efforts, natural products have been used as an important source in seeking for anticancer drugs.[5,6]

The natural diketopiperzaine compound "phenylahistin" is a fungal metabolite from *Aspergillus ustus*, and has been employed to develop a potent drug candidate, plinabulin (Fig. 1).[7,8] Plinabulin is an anti-microtubule agent, which can inhibit the tubulin polymerization. Currently, the combination therapy of plinabulin and docetaxel has been studied in Clinical Phase III trials for treatment of non-small cell lung cancer.[9] However, the treatment of plinabulin has to be administered by intravenous injection because of its poor water-solubility.[9]

Compounds **1** and **2** (Fig. 1), derived from plinabulin, have been reported to displayed activities against human HT-29 cell line.[10-13] However, there were few reports about the studies of plinabulin and its derivatives on pancreatic cancer. Our previous work found that plinabulin and its deuterated derivative could inhibit the human BxPC-3 pancreatic cancer cellular proliferation, while the inhibition rates were over 85% at the concentration of 12.5 nM.[14]

Encouraged by the excellent inhibition results, in present study, Compounds **1** and **2** were used to evaluate against human BxPC-3 pancreatic cancer cell line, while the values of IC_{50} were 0.63 nM and 6.27 nM, respectively (Table 1). The results indicated that the inhibition activity of Compound **1** was higher than that of plinabulin at 4.28 nM (Table 1). To better understand its mechanism of action, the immunofluorescence staining and microtubule polymerization assays were performed. As shown in Fig. 2, Compound **1** inhibited the tubulin polymerization in a way similar to that of plinabulin (Fig. 2A); moreover, the immunofluorescence optical density (IOD) data indicated that the effect of Compound **1** was stronger than that of plinabulin (Fig. 2B).

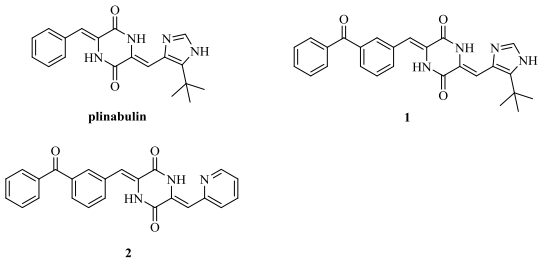

Fig. 1. Structures of plinabulin, Compounds **1** and **2**

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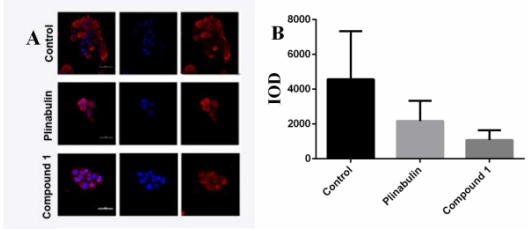


Fig. 2. Compound **1** inhibited the tubulin polymerization (A) BxPC-3 cells were treated with Compound **1** and plinabulin (5 nM) for 24 hours, and stained for β-tubulin and nucleus (4',6-diamidino-2-phenylindole (DAPI)). (B) The immunofluorescence optical density (IOD) of β-tubulin (red) was measured with Image-pro plus 6.0.

The mechanism of action and interaction between ligand and tubulin were further explored using the induced fit docking method. In the beginning, however, Compound **1** could not be docked into the co-crystal structure of tubulin-plinabulin (PDB CODE: 5C8Y). To solve this problem, the co-crystal complex of Compound **1** with tubulin (PDB CODE: 5YL4) was prepared, and then the co-crystal structure was analyzed by using X-ray crystallography (see Support information Table S1).[15]

In the X-ray crystal structure (PDB CODE: 5YL4), the conformation of Compound **1** was similar to that of plinabulin in the common part. Several hydrogen bonds were observed between NH of diketopiperazine ring and Val236 of β tubulin, between carbonyl group of dikeptopiperazine ring and Glu198 of β tubulin, between carbonyl group of benzoyl moiety and Asn165 of β tubulin, and between nitrogen atom of the imidazole group and NH of the diketopiperazine ring (Fig. 3A, blue). This described binding site on tubulin was also observed on plinabulin (Fig. 3A, off-white) except for the hydrogen bond between carbonyl group of benzoyl moiety and Asn165 of β tubulin. As shown in Fig. 3A, introduction of the benzoyl group on the structure of plinabulin led to cause rotation of the surrounding amino acid residue (Phe167), allowing sufficient space of tubulin to bind with Compound **1**. In addition, the benzoyl group of Compound **1** occupied the hydrophobic pocket and formed a π-π interaction with Phe167, which probably contributed to the binding affinity.

Based on the crystal structure, two types of derivatives were designed (Fig. 3B). Heteroaromatic groups were employed to replace the benzene ring for further structure activity relationship study, while the saturated N-heterocyclic groups were introduced to replace the benzene group for increasing its solubility.

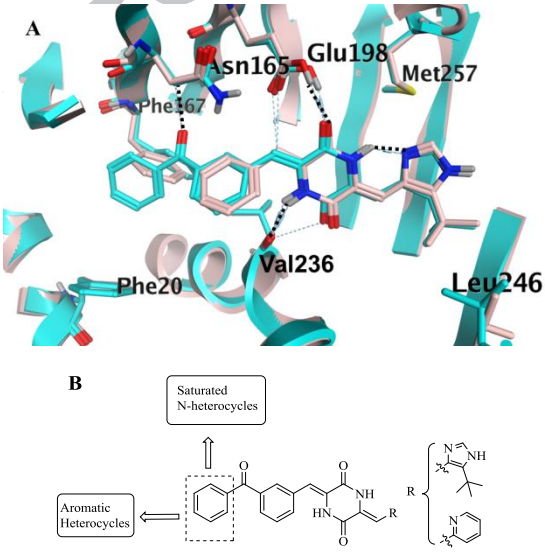


Fig. 3. (A) Co-crystal structures of tubulin with Compound **1** (green) and plinabulin (off-white). The hydrogen bonds were shown by dashed line; (B) Novel derivatives were designed based on Compound **1**

2. Results and discussion

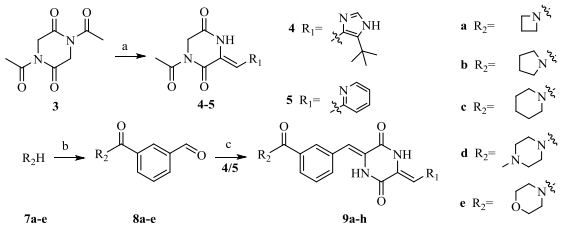
2.1 Chemistry

A total of 18 derivatives with 2,5-diketopiperazine skeleton were synthesized according two synthetic routes outlined in Schemes 1 and 2. The structures of these derivatives were summarized in Table 1.

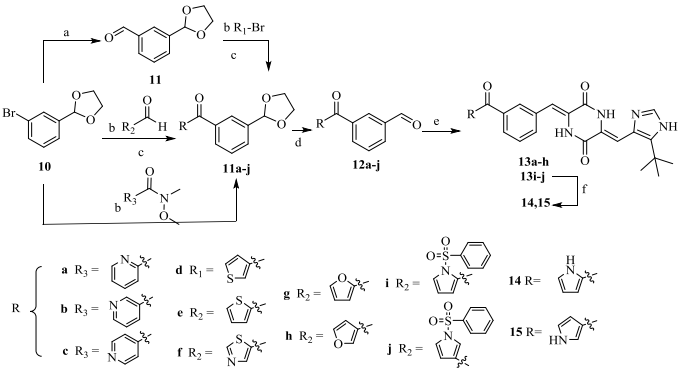
As shown in Scheme 1, N,N-diacetylpiperazine-2,5-dione **3** was reacted with 5-(tert-butyl)-1*H*-imidazole-4-carbaldehyde or 2-formylpyridine in presence of Cs_2CO_3 in N,N-dimethylformamide (DMF) under N_2 atmosphere to afford the intermediates **4** or **5**, respectively.[11,16] In parallel, the adducts **8a-e** were obtained through the carbodiimide mediated coupling reaction between 3-formylbenzoic acid **6** and a saturated N-heterocyclic group **7a-e**.[17] The final products **9a-h** were obtained via treatment of **8a-e** with intermediate **4** or **5** under Aldol condensation conditions.

Replacement of the phenyl ring of Compound **1** with heteroaromatic groups were outlined in Scheme 2. Intermediates **12a-j** were obtained by using 1-bromo-3-(1,3-dioxolan-2-yl)benzene **10** as a starting material. Compound **10** was converted to the corresponding secondary alcohol via bromo-lithium exchange reaction.[18,19] Subsequently, the un-purification secondary alcohols were oxidized by MnO_2 to afford Compounds **11d-j**.[20] Then, Compounds **11d-j** were converted to aldehydes **12d-j** via hydrochloric acid hydrolysis.[21] In other hand, Compound **10** were reacted with Weinreb amides to give Compounds **11a-c**.[22] The aldehydes **12a-c** were obtained after treatment of Compounds **11a-c** with dilute hydrochloric acid in tetrahydrofuran (THF). Finally, the substituted heteroaromatic Compounds **13a-j** were obtained through Aldol reaction between **12a-j** and the intermediate **4**. Compounds **13i-j** were subsequently hydrolyzed under basic (5 M NaOH) conditions to give **14** and **15**.[23]

Compounds **1** and **2** were synthesized following a procedure according to synthesis of Compound **13a**.



Scheme 1. Synthesis of Compounds **9a-h**. Reagents and conditions: (a) R_1CHO, Cs_2CO_3, DMF, 25 °C, 12 h; (b) 3-formylbenzoic acid **6**, EDCI, HOBT, DMF, 25 °C, 6 h; (c) **4/5**,Cs_2CO_3, DMF, 50 °C, 24 h.



Scheme 2. Synthesis of Compounds **13a-h, 14** and **15**. Reagents and conditions: (a) *n*-BuLi, THF, DMF, -78 °C; (b) *n*-BuLi, THF, -78 °C; (c) MnO_2, DCM, reflux; (d) HCl (1 M), THF; (e) Cs_2CO_3, DMF, 50 °C, 24 h. (f) 1,4-dioxane, NaOH (5 M), 25 °C, 20 h.

2.2 Water solubility

The solubility of Compounds **9a-h** and **13a-h** were assessed by High Performance Liquid Chromatography (HPLC).[24] As shown in Table 1, Compounds **9d** and **9h**, both having the hydrophilic piperazine group, had the higher solubilities compared to plinabulin and other compounds. Compound **9d** had the highest solubility at 85 μg/mL, which was over 855 times greater than that of Compound **1**.

2.3 Biological activities

The activities of the synthesized derivatives against BxPC-3 cell line were evaluated by sulforhodamine B assay (SRB).[25] In comparison with Compounds **1** and **2**, the activities of Compounds **9a-h** were reduced remarkably. The IC_{50} value of Compound **9d** was >500 nM, although its water solubility had been enhanced remarkably (Table 1). The results suggested that the introduction of hydrophilic group was unfavorable to maintain its activity. Replacement of the tert-butyl imidazole ring with pyridine ring (Compound **2**) decreased the activity. The similar result was found with Compound **9g**, while its IC_{50} was >500 nM. These results indicated that the tert-butyl imidazole ring was superior to the pyridine ring for the biological activity.

Replacement of the benzene group with pyridine moiety, Compounds **13a-c** were synthesized. In comparison with Compound **1**, their inhibitory activities decreased in potency of 12-fold, 6.4-fold and 27-fold, respectively. The lower potency of Compound **13c** with 4-pyridy moiety might be due to additional unfavorable interaction with tubulin as discussed below.

The five-member aromatic heterocyclic derivatives **13d** and **13e** were synthesized. Their inhibitory activities against BxPC-3 cell line were close to Compound **1**, while both IC_{50} were 1.56 nM and 1.72 nM, respectively. However, Compound **13f** displayed less activity, the IC_{50} value was 17.11 nM. In addition, Compounds **13g-h** exhibited moderate inhibitory activities (IC_{50} at 9.23 and 9.41 nM, respectively). In contrast, the pyrrole substituent derivatives, Compounds **14** and **15**, led to decrease in activity (both IC_{50} >100 nM). The results indicated that bioisosteric replacement of sulfur atom with –NH or oxygen atom on thiophene derivatives were detrimental to antiproliferative activity.

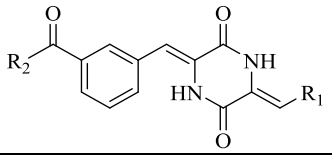
2.4 Tubulin polymerization assay

To elucidate whether the synthesized compounds bind to tubulin, the interaction between Compounds **13a-f** and tubulin were further explored by tubulin polymerization assay with concentration at 5 μM. As shown in Table 1, the inhibition rates of Compounds **13a-f** were higher than that of plinabulin. The tubulin inhibitory rates of Compounds **13a-c** were basically the same. Compounds **13d-e** exhibited higher inhibitory rates than others. However, the inhibitory rates of Compound **13f** was decreased when the CH was replaced by nitrogen atom.

2.5 Theoretical Calculations of the physical properties

Theoretical calculation of the physical properties of these synthesized compounds were completed using Qikprop software.[26] As shown in Table 1, the partition coefficient displayed by LogPo/w, and the cell permeability exhibited by PCaco. The LogPo/w plays an important role in predicting absorption of drug, while the PCaco represents the ability of access to biological membranes. When replacement of the benzene ring with saturated N-heterocyclic groups, the values of LogPo/w were reduced, in particular for Compounds **9d** and **9h**. Moreover, the PCaco of Compound **9d** was the lowest at 23.6, which the value of PCaco parameter <25 was considered as poor.[27] Therefore, introduction of the saturated N-heterocyclic groups were unfavorable to absorption of compounds, which might lead to decreased its activity. When the phenyl ring of Compound **1** substituted with heteroaromatic groups, the PCaco and LogPo/w had no significant variations except Compounds **13f**, **14** and **15**. The PCaco and LogPo/w of Compounds **13f**, **14** and **15** were less than that of Compounds **13d-e**, which presumably resulted in lower activities.

Table 1. Biological activity and water solubility of Compounds



Compounds	R_1	R_2	Solubility ng/mL	IC_{50} (nM) [a] BxPC-3	Inhibition rate [b]	LogPo/w	PCaco
plinabulin			<100	4.28 ± 0.89	18.34%	2.496	376.131
1			<100	0.63 ± 0.25	ND	3.331	142.774
2			<100	6.27 ± 1.78	ND [c]	3.033	217.412
9a			4231	>500	ND	2.769	137.396
9b			454	324.9 ± 1.00	ND	2.676	149.517

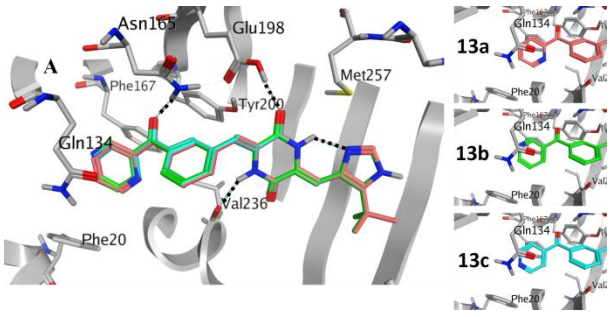
9c			<100	100.4 ± 3.70	ND	2.970	154.868
9d			85568	>500	ND	1.795	23.600
9e			1790	257 ± 11.52	ND	2.028	155.621
9f			662	>500	ND	2.508	232.603
9g			<100	>500	ND	2.734	257.655
9h			16302	>500	ND	1.503	51.087
13a			<100	7.58 ± 1.90	36.57%	2.718	91.716
13b			<100	4.05 ± 0.16	28.79%	2.430	80.300
13c			<100	23.78 ± 1.89	34.10%	2.445	79.170
13d			<100	1.56 ± 0.45	39.27%	3.257	140.940
13e			<100	1.72 ± 0.07	46.36%	3.283	149.241
13f			<100	17.11 ± 0.94	19.90%	2.335	72.301
13g			<100	9.23 ± 1.50	ND	2.665	135.082
13h			<100	9.41 ± 1.69	ND	2.628	143.971
14			ND	>100	ND	2.594	79.267
15			ND	>100	ND	2.900	68.935

[a] Values represent mean ± SD from three independent dose response curves. [b] Statistical data of inhibition of tubulin polymerization (5μM). [c] ND = not detect

2.6 Molecular docking

The interaction mechanisms of Compounds **13a-f** were investigated by molecular docking. These compounds bound similar in the pocket. There were four hydrogen bonds observed between nitrogen atom of the imidazole group and diketopiperazine ring, between oxygen atom of benzoyl group and Asn165 of β tubulin, between oxygen atom of diketopiperazine ring and Glu198 of β tubulin, and between NH of diketopiperazine ring and Val236 of β tubulin, which they were crucial for their activities. In comparison, the hydrophilic pyridine group of Compounds **13a-c** located in a hydrophobic sub-pocket (Fig. 4A), which did harm to their bioactivities, and resulted in lower activities of inhibition tubulin polymerization.

In other hand, CH…π interactions were found between the hydrogen atom in thiophene moiety of Compounds **13d-e** and phenyl of Phe20 with distance at 3.6 Å (Fig. 4B), which were favorable to the binding affinity. In contrast, for Compound **13f**, the favorable CH…π interaction was disappeared when the CH moiety was replaced by nitrogen atom, which resulted its potency decrease in comparison with Compounds **13d-e**.



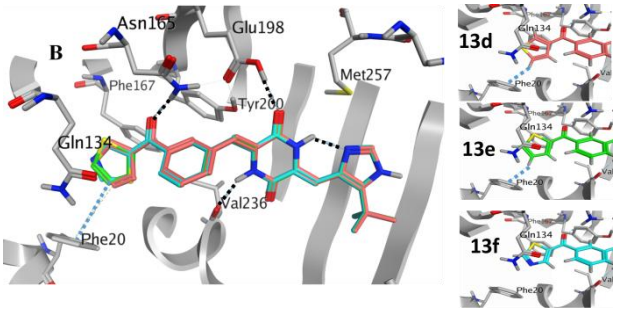


Fig. 4. Binding poses of Compounds **13a-f**. The hydrogen bonds were shown by black lines. The H-π interaction was shown by dashed lines (blue). (A) **13a-c** binding in tubulin (B) **13d-f** binding in tubulin

3. Conclusions

In conclusion, a total of 18 plinabulin derivatives were designed and synthesized based on the co-crystal structure of tubulin with Compound **1**. In general, the derivatives lost their activities when the hydrophilic groups were introduced. The interaction between tubulin and these compounds could be elucidated by docking simulation. The docking model of Compounds **13d-e** were similar to the co-crystal structure of Compound **1**, the H-π interaction formed between hydrogen atom of thiophene and phenyl of Phe20 was favorable to the binding affinity. In comparison with other synthesized compounds, two novel Compounds **13d-e** showed higher potencies against BxPC-3 cell line, further evaluating of their activities and toxicities to treat pancreatic cancer in vivo are under way.

4. Experimental section

4.1 Materials and Chemistry

Starting materials were purchased from commercial suppliers. Thin-layer chromatography (TLC) was performed on silica gel GF-254 plates (Qing-Dao Chemical Company, Qingdao, China), and the spots were visualized by UV (254 nm or 365 nm). Column chromatography was performed on silica gel (300-400 mesh, Qingdao China).

The target compounds and intermediates were characterized by nuclear magnetic resonance and mass spectroscopy. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker 400 spectrometer with tetramethylsilane (TMS) as an internal standard. The chemical shifts (δ) values were expressed in ppm: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, board singlet. Mass spectra (ESI) were recorded on a VG Autospec 3000 mass spectrometer.

The purity of Compounds **1-2**, Compounds **9a-h**, Compounds **13a-h** and Compounds **14-15** established to be ≥95% by High Performance Liquid Chromatography (HPLC) (UltiMate 3000 series 250*4.6 mm). HPLC conditions are as follows: solvent A, CH_3CN; solvent B, H_2O; flow rate of 1.0 mL/min, from 40% B to 0% B in 15 min, from 0% B to 0% B between 15 min to 20 min, from 0% B to 40% B between 20 min to 25 min.

4.1.1 General method for Synthesis of intermediate **4** and **5**

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

To a solution of 5-(tert-butyl) imidazole-4-carbaldehyde (5 g, 32.85 mmol) in DMF (35 mL) was added compound **3** (13 g, 65.59 mmol) under N_2 atmosphere. Then Cs_2CO_3 (16.05 g, 49.26 mmol) was added into the solution and the mixture was stirred at room temperature for about 24 h. After the reaction was completed, the mixture was poured into crashed ice and the solid was filtered to give intermediate (4.05 g). Solid, yield: 42%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.37 (s, 1H), 12.02 (s, 1H), 7.85 (s, 1H), 7.05 (s, 1H), 4.31 (s, 2H), 1.39 (s, 9H). MS (ESI): Calcd for $C_{14}H_{19}N_4O_3$ $[M+H]^+$ 291.15, found 291.18.

4.1.1.2 (Z)-1-acetyl-3-(pyridin-2-ylmethylene) piperazine-2,5-dione (**5**)

Solid, 24% yield. ^{1}HNMR (500 MHz, DMSO-d_6) δ 12.45 (s, 1H), 8.72 (d, *J* = 4.6 Hz, 1H), 7.92 (td, *J* = 7.7, 1.5 Hz, 1H), 7.70 (d, *J* = 7.8 Hz, 1H), 7.39 (dd, *J* = 7.0, 5.3 Hz, 1H), 6.87 (s, 1H), 4.35 (s, 2H), 2.52 (s, 3H). MS (ESI): Calcd for $C_{12}H_{12}N_3O_3$ $[M+H]^+$ 246.09, found 246.27.

4.1.1.3 General method for Synthesis of target compounds 9a-h

(3Z, 6Z)-3-(azetidine-1-carbonyl) benzylidene)-6-((5-(tert-butyl)-1H-imidazol-4-yl) methylene) piperazine-2,5-dione (**9a**)

Step A: 3-(azetidine-1-carbonyl) benzaldehyde (**8a**)

To a solution of 3-formyl-benzoic acid **6** (150 mg, 1mmol) in DMF (5 mL) was added azetidine (68 mg, 1.20 mmol) and the solution was stirred for 30 min at 25 °C. Then the solution was treated with HOBT (202 mg, 1.50mmol) and EDCI (383.4 mg, 2 mmol). The reaction was stirred at 25 °C for 4 h. After the reaction was completed, the mixture was added water and EtOAc. The organic layer was washed with 1M NaOH, 1M HCl then brine and was dried over anhydrous sodium sulfate (Na_2SO_4). Then solvent was removed under reduced pressure. The residual solid was purified by silica gel column chromatography using petroleum ether-ethyl acetate (4:1 to 2:1) to give white solid (130 mg). 69% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.05 (s, 1H), 8.13 (s, 1H), 7.97 (d, *J* = 7.6, 1H), 7.92 (d, *J* = 7.7, 1H), 7.60 (d, *J* = 7.7, 1H), 4.35 (t, *J* = 7.5, 2H), 4.26 (t, *J* = 7.7, 2H), 2.42 – 2.34 (m, 2H). MS (ESI): Calcd for $C_{11}H_{12}NO_2$ $[M+H]^+$ 190.09, found 190.18.

Step B:

To a solution of compound **4** (153 mg, 0.53 mmol) in DMF (5 mL) was added compound **8a** (130 mg, 0.69mmol) under N_2 atmosphere. Then Cs_2CO_3 (258 mg, 0.79 mmol) was added into the solution and the mixture was stirred at 50 °C for about 24 h. After the reaction was completed, the mixture was diluted with brine and extracted with EtOAc. The organic layer was wased with brine and was dried over anhydrous sodium sulfate (Na_2SO_4). Then solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using CH_2Cl_2-MeOH (50:1) to give **9a** (190 mg, 86% yield). Mp: 163.8-165.3°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 12.27 (s, 1H), 10.24 (s, 1H), 7.85 (s, 1H), 7.70 (s, 1H), 7.60 (d, *J* = 7.7 Hz, 1H), 7.53 (d, *J* = 7.7 Hz, 1H), 7.47 (t, *J* = 7.7 Hz, 1H), 6.87 (s, 1H), 6.76 (s, 1H), 4.33 (t, *J* = 7.6 Hz, 2H), 4.06 (t, *J* = 7.7 Hz, 2H), 2.31 – 2.22 (m, 2H), 1.39 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.5, 157.6, 156.2, 140.4, 134.4, 133.5, 133.4, 131.6, 130.7, 128.9, 128.2, 127.3, 127.1, 123.8, 113.1, 105.2, 52.9, 48.5, 31.9, 30.64, 15.52. HRMS (ESI): Calcd for $C_{23}H_{26}N_5O_3$ $[M+H]^+$ 420.2030, found 420.2029.

4.1.1.4 3-(pyrrolidine-1-carbonyl) benzaldehyde (**8b**)

Yield: 83%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.04 (s, 1H), 8.04 (s, 1H), 7.94 (d, *J* = 7.7 Hz, 1H), 7.80 (d, *J* = 7.6 Hz, 1H), 7.59 (t, *J* = 7.6 Hz, 1H), 3.67 (t, *J* = 7.0 Hz, 2H), 3.44 (t, *J* = 6.6 Hz, 2H), 2.02 – 1.95 (m, 2H), 1.94 – 1.87 (m, 2H). MS (ESI): Calcd for $C_{12}H_{14}NO_2$ $[M+H]^+$ 204.10, found 204.21.

4.1.1.5 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(-3-(pyrrolidine-1-carbonyl) benzylidene) piperazine-2,5-dione (**9b**)

Yield: 66%. Mp: 172.7-174.2°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.32 (s, 1H), 12.26 (s, 1H), 10.22 (s, 1H), 7.85 (s, 1H),

7.62 (s, 1H), 7.56 (d, *J* = 7.1 Hz, 1H), 7.46 (d, *J* = 7.6 Hz, 1H), 7.43 (t, *J* = 5.1 Hz, 1H), 6.86 (s, 1H), 6.76 (s, 1H), 3.48 (t, *J* = 6.7 Hz, 2H), 3.43 (t, *J* = 6.4 Hz, 2H), 1.90 – 1.84 (m, 2H), 1.85 – 1.79 (m, 2H), 1.39 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 167.9, 157.6, 156.2, 140.4, 137.5, 134.4, 133.2, 130.7, 130.5, 128.5, 127.6, 127.2, 126.5, 123.8, 113.2, 105.1, 48.9, 45.9, 31.9, 30.6, 25.9, 23.9. HRMS (ESI): Calcd for $C_{24}H_{28}N_5O_3$ $[M+H]^+$ 434.2187, found 434.2185.

4.1.1.6 3-(piperidine-1-carbonyl)benzaldehyde (**8c**)

Yield: 93%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.04 (s, 1H), 7.94 – 7.90 (m, 2H), 7.67 – 7.65 (m, 1H), 7.58 (t, *J* = 7.6 Hz, 1H), 3.73 (br s, 2H), 3.34 (br s, 2H), 1.70 – 1.63 (m, 6H). MS (ESI): Calcd for $C_{13}H_{16}NO_2$ $[M+H]^+$ 218.12, found 218.24.

4.1.1.7 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(-3-(piperidine-1-carbonyl) benzylidene) piperazine-2,5-dione (**9c**)

Yield: 74%. Mp: 188.0-190.0°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 12.27 (s, 1H), 10.20 (s, 1H), 7.85 (s, 1H), 7.55 (d, *J* = 7.8 Hz, 1H), 7.46 (t, *J* = 7.6 Hz, 2H), 7.29 (d, *J* = 7.6 Hz, 1H), 6.86 (s, 1H), 6.75 (s, 1H), 3.59 (br s, 2H), 3.34 (s, 2H), 1.74 – 1.43 (m, 6H), 1.39 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.5, 157.6, 156.2, 140.4, 136.8, 134.4, 133.5, 130.7, 130.1, 128.7, 127.3, 127.1, 126.1, 123.8, 113.2, 105.2, 32.9, 30.6, 24.1. HRMS (ESI): Calcd for $C_{25}H_{30}N_5O_3$ $[M+H]^+$ 448.2343, found 448.2342.

4.1.1.8 3-(4-methylpiperazine-1-carbonyl)benzaldehyde (**8d**)

Yield: 73%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.05 (s, 1H), 7.98 (dt, *J* = 7.4, 1.4 Hz, 1H), 7.90 (s, 1H), 7.72 (dt, *J* = 7.6, 1.5 Hz, 1H), 7.68 (t, *J* = 7.5 Hz, 1H), 3.64 (s, 2H), 3.32 (br s, 2H), 2.42 – 2.23 (m, 4H), 2.20 (s, 3H). MS (ESI): Calcd for $C_{13}H_{17}N_2O_2$ $[M+H]^+$ 233.13, found 233.21.

4.1.1.9 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(-3-(4-methylpiperazine-1-carbonyl) benzylidene) piperazine-2, 5-dione (**9d**)

Yield: 68%. Mp: 214.0-216.0°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 12.27 (s, 1H), 10.20 (s, 1H), 7.85 (s, 1H), 7.56 (d, *J* = 7.7 Hz, 1H), 7.48 – 7.45 (m, 2H), 7.30 (d, *J* = 7.5 Hz, 1H), 6.87 (s, 1H), 6.76 (s, 1H), 3.62 (s, 2H), 3.37 (s, 2H), 2.42 – 2.25 (m, 4H), 2.20 (s, 3H), 1.39 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.6, 157.6, 156.2, 140.4, 136.3, 134.4, 133.5, 130.7, 130.3, 128.8, 127.4, 127.3, 126.4, 123.8, 113.1, 105.2, 54.7, 45.6, 31.9, 30.6. HRMS (ESI): Calcd for $C_{25}H_{31}N_6O_3$ $[M+H]^+$ 463.2452, found 463.2450.

4.1.1.10 3-(morpholine-4-carbonyl)benzaldehyde (**8e**)

Yield: 59%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.05 (s, 1H), 7.95 (dt, *J* = 7.6, 1.3 Hz, 1H), 7.92 (s, 1H), 7.69 (dt, *J* = 7.6, 1.4 Hz, 1H), 7.62 (t, *J* = 7.6 Hz, 1H), 3.89 – 3.39 (m, 8H). MS (ESI): Calcd for $C_{12}H_{14}NO_3$ $[M+H]^+$ 220.10, found 220.21.

4.1.1.11 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(-3-(morpholine-4-carbonyl) benzylidene) piperazine-2,5-dione (**9e**)

Yield: 76%. Mp: 182.0-184.0°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.32 (s, 1H), 12.26 (s, 1H), 10.19 (s, 1H), 7.85 (s, 1H), 7.57 (d, *J* = 7.7 Hz, 1H), 7.52 (s, 1H), 7.47 (t, *J* = 7.7 Hz, 1H), 7.33 (d, *J* = 7.6 Hz, 1H), 6.86 (s, 1H), 6.75 (s, 1H), 3.70 – 3.40 (m, 8H), 1.39 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.7, 157.6, 156.2, 140.4, 135.9, 134.4, 133.5, 130.7, 130.4, 128.8, 127.5, 127.4, 126.5, 123.8, 113.1, 105.2, 66.1, 31.9, 30.6. HRMS (ESI): Calcd for $C_{24}H_{28}N_5O_4$ $[M+H]^+$ 450.2136, found 450.2133.

4.1.1.12 (3Z, 6Z)-3-(3-(azetidine-1-carbonyl) benzylidene)-6-(pyridin-2-ylmethylene) piperazine-2,5-dione (**9f**)

Yield: 52%. Mp: 273.8-274.4°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.60 (s, 1H), 10.57 (s, 1H), 8.74 (d, *J* = 4.3 Hz, 1H), 7.92 (td, *J* = 7.8, 1.8 Hz, 1H), 7.74 (s, 1H), 7.68 (d, *J* = 7.9 Hz, 1H), 7.63 (d, *J* = 7.7 Hz, 1H), 7.56 (d, *J* = 7.7 Hz, 1H), 7.49 (t, *J* = 7.7 Hz, 1H), 7.41 – 7.34 (m, 1H), 6.87 (s, 1H), 6.74 (s, 1H), 4.33 (t, *J* = 7.6 Hz, 2H), 4.07 (t, *J* = 7.7 Hz, 2H), 2.32 – 2.21 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.5, 156.8, 156.7, 154.6, 148.5, 137.8, 133.5, 133.1, 131.7, 131.1, 128.1, 128.5, 127.3, 126.7, 126.5, 122.6, 114.8, 107.7, 52.9, 48.5, 15.5. HRMS (ESI): Calcd for $C_{21}H_{19}N_4O_3$ $[M+H]^+$ 375.1452, found 375.1451.

4.1.1.13 (3Z, 6Z)-3-(3-(piperidine-1-carbonyl) benzylidene)-6-(pyridin-2-ylmethylene) piperazine-2,5-dione (**9g**)

Yield: 37%. Mp: 284.5-286.1°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.60 (s, 1H), 10.53 (s, 1H), 8.74 (d, *J* = 4.4 Hz, 1H), 7.91 (t, *J* = 7.1 Hz, 1H), 7.68 (d, *J* = 7.9 Hz, 1H), 7.58 (d, *J* = 7.7 Hz, 1H), 7.52 – 7.45 (m, 2H), 7.41 – 7.36 (m, 1H), 7.31 (d, *J* = 7.5 Hz, 1H), 6.86 (s, 1H), 6.73 (s, 1H), 3.59 (br s, 2H), 3.34 (s, 2H), 1.70 – 1.20 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.4, 157.1, 156.8, 154.7, 148.7, 137.9, 137.3, 133.4, 130.4, 129.7, 129.3, 127.3, 127.1, 126.7, 126.5, 122.6, 115.7, 110.1, 48.9, 43.4, 26.7, 24.7. HRMS (ESI): Calcd for $C_{23}H_{23}N_4O_3$ $[M+H]^+$ 403.1765, found 403.1763.

4.1.1.14 (3Z, 6Z)-3-(3-(4-methylpiperazine-1-carbonyl) benzylidene)-6-(pyridin-2-ylmethylene) piperazine-2,5-dione (**9h**)

Yield: 42%. Mp: 258.8-260.3°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.88 (s, 1H), 8.67 (d, *J* = 4.65 Hz, 1H), 8.22 (s, 1H), 7.74 (t, *J* = 7.7 Hz 1H), 7.51 (t, *J* = 7.6 Hz, 1H), 7.46 (d, *J* = 7.7 Hz, 1H), 7.43 (s, 1H), 7.40 (d, *J* = 7.5 Hz, 1H), 7.36 (d, *J* = 7.8 Hz, 1H), 7.25 – 7.22 (m, 1H), 7.06 (s,1H), 6.77 (s,1H), 3.81 (s, 2H), 3.47 (s, 2H), 2.50 (s, 2H), 2.38 (s, 2H), 2.33 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.3, 157.1, 156.7, 154.7, 148.7, 137.4, 137.3, 133.5, 130.3, 129.8, 129.5, 127.5, 127.4, 126.8, 126.5, 115.5, 122.6, 110.2, 55.4, 54.8, 46.2. HRMS (ESI): Calcd for $C_{23}H_{24}N_5O_3$ $[M+H]^+$ 418.1874, found 418.1872.

4.1.1.15 3-picolinoylbenzaldehyde (**12a**)

Step A: N-methoxy-N-methylpicolinamide

To a solution picolinic acid (246 mg, 2 mmol) in CH_3CN (10 mL) was added HOBT (270 mg, 2 mmol), EDCI (383.4 mg, 2 mmol) and N,O-dimethylhydroxylamine hydrochloride (293 mg, 3 mmol), NEt_3 was added dropwise to the mixture. The reaction was stirred at 25 °C under a nitrogen atmosphere. After the reaction was completed, the mixture was extracted with EtOAc. The organic layer was washed with brine and was dried over anhydrous sodium sulfate (Na_2SO_4). Then solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using petroleum ether–ethyl acetate (10:1) to give compound (235 mg, 70% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.60 (d, *J* = 4.2 Hz, 1H), 7.91 (t, *J* = 7.6 Hz, 1H), 7.58 (br s, 1H), 7.51 – 7.47 (m, 1H), 3.65 (s, 3H), 3.27 (s, 3H). MS (ESI): m/z = 167.24 $[M+H]^+$.

Step B: (3-(1, 3-dioxolan-2-yl) phenyl)(pyridin-2-yl)methanone (11a)

According to the procedure described for the synthesis of 12d (Step A) Yield: 26%. ^{1}H NMR (500 MHz,DMSO-d_6) δ 8.73 (d, *J* = 3.9 Hz, 1H), 8.08 (t, *J* = 7.7 Hz, 1H), 8.03 – 7.96 (m, 3H), 7.73 (d, *J* = 7.6 Hz, 1H), 7.70 – 7.66 (m, 1H), 7.57 (t, *J* = 7.6 Hz, 1H), 5.82 (s,1H) 4.08 – 4.02 (m, 2H), 4.01 – 3.94 (m, 2H). MS (ESI): m/z = 256.34 $[M+H]^+$.

Step C:

According to the procedure described for the synthesis of 12d (Step C) Yield: 78%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.11 (s, 1H), 8.75 (d, *J* = 4.3 Hz, 1H), 8.48 (s, 1H), 8.30 (d, *J* = 7.7 Hz, 1H), 8.19 (d, *J* = 7.5 Hz, 1H), 8.12 – 8.07 (m,2H), 7.79 (t, *J* = 7.6 Hz, 1H), 7.72 (t, *J* = 5.5 Hz, 1H). MS (ESI): Calcd for

$C_{13}H_{10}N_4O_2$ $[M+H]^+$ 212.07, found 212.26.

4.1.1.16 3-nicotinoylbenzaldehyde (**12b**)

Step A: N-methoxy-N-methylpyridine-3-carboxamide

According to the procedure described for the synthesis of 12a (Step A) Yield: 69%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.77 (d, *J* = 1.9 Hz, 1H), 8.68 (dd, *J* = 4.8, 1.6 Hz, 1H), 8.00 (dt, *J* = 7.9, 1.9 Hz, 1H), 7.49 (dd, *J* = 7.9, 4.9 Hz, 1H), 3.55 (s, 3H), 3.29 (s, 3H). MS (ESI): m/z = 167.32 $[M+H]^+$.

Step B: (3-(1, 3-dioxolan-2-yl) phenyl)(pyridin-3-yl)methanone (11b)

According to the procedure described for the synthesis of 12a (Step B) Yield: 46%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.88 (d, *J* = 2.1 Hz, 1H), 8.84 (dd, *J* = 4.8, 1.6 Hz, 1H), 8.11 (dt, *J* = 7.9, 2.0 Hz, 1H), 7.83 (s, 1H), 7.79 (t, *J* = 8.2 Hz, 2H), 7.64 – 7.59 (m, 2H), 5.85 (s, 1H), 4.08 – 4.02 (m, 2H), 4.01 – 3.95 (m, 2H). MS (ESI): m/z = 256.34 $[M+H]^+$.

Step C:

According to the procedure described for the synthesis of 12a (Step C) Yield: 62%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.12 (s, 1H), 8.93 (d, *J* = 2.0 Hz, 1H), 8.87 (dd, *J* = 4.8, 1.6 Hz, 1H), 8.27 (s, 1H), 8.22 (d, *J* = 7.6 Hz, 1H), 8.17 (dt, *J* = 7.9, 1.9 Hz, 1H), 8.11 (d, *J* = 7.7 Hz, 1H), 7.82 (t, *J* = 7.7 Hz, 1H), 7.63 (dd, *J* = 7.8, 4.8 Hz, 1H). MS (ESI): Calcd for $C_{13}H_{10}N_4O_2$ $[M+H]^+$ 212.07, found 212.32.

4.1.1.17 3-isonicotinoylbenzaldehyde (**12c**)

Step A: N-methoxy-N-methylpyridine-4-carboxamide

According to the procedure described for the synthesis of 12a (Step A) Yield: 81%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.69 (d, *J* = 1.6 Hz, 1H), 8.68 (d, *J* = 1.5 Hz, 1H), 7.52 (d, *J* = 1.6 Hz, 1H), 7.51 (d, *J* = 1.6 Hz, 1H), 3.55 (s, 3H), 3.27 (s, 3H). MS (ESI): m/z = 167.32 $[M+H]^+$.

Step B: (3-(1, 3-dioxolan-2-yl) phenyl)(pyridin-3-yl)methanone (11c)

According to the procedure described for the synthesis of 12a (Step B) Yield: 46%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.83 (d, *J* = 1.3 Hz, 1H), 8.82 (d, *J* = 1.0 Hz, 1H), 7.84 (s, 1H), 7.80 (d, *J* = 1.4 Hz, 1H), 7.78 (d, *J* = 1.3 Hz, 1H), 7.64 – 7.60 (m, 3H), 5.84 (s, 1H), 4.07 – 4.02 (m, 2H), 4.01 – 3.95 (m, 2H). MS (ESI): m/z = 256.34 $[M+H]^+$.

Step C:

According to the procedure described for the synthesis of 12a (Step C) Yield: 81%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.12 (s, 1H), 8.85 (d, *J* = 1.6 Hz, 1H), 8.84 (d, *J* = 1.6 Hz, 1H), 8.28 (d, *J* = 1.5 Hz, 1H), 8.24 (dt, *J* = 7.7, 1.2 Hz, 1H), 8.11 (dt, *J* = 7.8, 1.4 Hz, 1H), 7.83 (t, *J* = 7.7 Hz, 1H), 7.67 (d, *J* = 1.6 Hz, 1H), 7.66 (d, *J* = 1.6 Hz, 1H). MS (ESI): Calcd for $C_{13}H_{10}N_4O_2$ $[M+H]^+$ 212.07, found 212.32.

4.1.1.18 2 3-(thiophene-3-carbonyl)benzaldehyde (**12d**)

Step A: 3-(1, 3-dioxolan-2-yl)benzaldehyde (11)

To a cooled (-78 ^{o}C) solution of 2.5 M *n*-BuLi (0.92 mL) in THF (2 mL) was added 1-bromo-3-(1,3-dioxolan-2-yl)-benzene (**10**) (500 mg, 2.18 mmol) in anhydrous THF (3 mL), and the mixture was stirred for 40 min. DMF (0.5 mL) was added dropwise at -78 ^{o}C. After the reaction was completed (about 2 hour), the mixture was treated with saturated NH_4Cl (aq) and extracted with EtOAc. The organic layer was washed with brine and was dried over anhydrous sodium sulfate (Na_2SO_4). Then solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using petroleum ether–ethyl acetate (15:1) to give **11** (187 mg, 48% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.04 (s, 1H), 7.97 (s, 1H), 7.93 (d, *J* = 7.5 Hz, 1H), 7.76 (d, *J* = 7.5 Hz, 1H), 7.56 (t, *J* = 7.6 Hz, 1H), 5.84 (s, 1H), 4.10 – 4.03 (m, 2H), 4.03 – 3.95 (m, 2H).

Step B: (3-(1, 3-dioxolan-2-yl) phenyl)(thiophen-2-yl)methanone (11d)

To a cooled (-78 ^{o}C) solution of 2.5 M *n*-BuLi (0.5 mL) in THF (2 mL) was added 3-bromothiophene (206 mg, 1.26 mmol) in anhydrous THF (3 mL), and the mixture was stirred for 40 min. Compound **11** (187 mg, 1.05 mmol) in anhydrous THF (3 mL) was added dropwise at -78 ^{o}C. After the reaction was completed (about 2 h), the mixture was treated with saturated NH_4Cl (aq) and extracted with EtOAc. The organic layer was washed with brine and was dried over anhydrous sodium sulfate (Na_2SO_4). Then solvent was removed under reduced pressure to obtain oil (262 mg). To a solution of this oil (262 mg, 1 mmol) in CH_2Cl_2 (20 mL) was added MnO_2 (695 mg, 8 mmol). The mixture was heated under reflux for 8 h. Then the mixture was filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using petroleum ether–ethyl acetate (4:1) to give compound (90 mg, 33% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.22 (d, *J* = 1.4 Hz, 1H), 7.85 – 7.81 (m, 2H), 7.75 – 7.69 (m, 2H), 7.59 (t, *J* = 7.5 Hz, 1H), 7.53 – 7.51 (m, 1H), 5.85 (s, 1H), 4.09 – 4.03 (m, 2H), 4.02 – 3.94 (m, 2H). MS (ESI): m/z = 261.23 $[M+H]^+$.

Step C:

To a solution of compound (90 mg, 0.35 mmol) in THF (4 mL) was added 1M HCl (aq) (2 mL), and the mixture was stirred at room temperature. After the reaction was completed, the mixture was diluted with brine and extracted with EtOAc. The organic layer was washed with brine and was dried over anhydrous sodium sulfate (Na_2SO_4). Then solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using petroleum ether–ethyl acetate (40:1) to give white solid 12d (65 mg, 86% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.12 (s, 1H), 8.34 – 8.27 (m, 2H), 8.18 (d, *J* = 7.2 Hz, 1H), 8.11 (d, *J* = 7.6 Hz, 1H), 7.84 – 7.73 (m, 2H), 7.57 (d, *J* = 5.0 Hz, 1H). MS (ESI): Calcd for $C_{12}H_9O_2S$ $[M+H]^+$ 217.03, found 217.22.

Compounds **12e-h** were obtained from compound **10** according to the procedure described for the synthesis of **12d** (step B and step C)

4.1.1.19 3-(thiophene-2-carbonyl) benzaldehyde (**12e**)

Step A: (3-(1, 3-dioxolan-2-yl) phenyl)(thiophen-2-yl)methanone (11e)

According to the procedure described for the synthesis of 12d (Step B) Yield: 74%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.13 (dd, *J* = 4.9, 1.0 Hz, 1H), 7.88 – 7.84 (m, 2H), 7.75 – 7.72 (m, 1H), 7.70 (dd, *J* = 3.8, 1.1 Hz, 1H), 7.60 (t, *J* = 7.9 Hz, 1H), 7.31 – 7.29 (m, 1H), 5.85 (s, 1H), 4.11 – 4.03 (m, 2H), 4.03 – 3.94 (m, 2H). MS (ESI): m/z = 261.30 $[M+H]^+$.

Step B: According to the procedure described for the synthesis of 12d (Step C) Yield: 95%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.12 (s, 1H), 8.33 (s, 1H), 8.19 – 8.17 (m, 2H), 8.14 (d, *J* = 7.7 Hz, 1H), 7.82 – 7.78 (m, 2H), 7.32 (dd, *J* = 4.7, 4.0 Hz, 1H). MS (ESI): Calcd for $C_{12}H_9O_2S$ $[M+H]^+$ 217.03, found 217.39..

4.1.1.20 3-(thiazole-5-carbonyl)benzaldehyde (**12f**)

Step A: (3-(1, 3-dioxolan-2-yl) phenyl)(thiazol-5-yl)methanone (11f)

According to the procedure described for the synthesis of 12d (Step B) Yield: 87%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.07 (s, 1H), 8.36 (s, 1H), 8.01 (s, 1H), 7.89 (d, *J* = 7.7 Hz, 1H), 7.76 (d, *J* = 7.7 Hz, 1H), 7.55 (t, *J* = 7.7 Hz, 1H), 5.88 (s, 1H), 4.16 – 4.10 (m, 2H), 4.10 – 4.04 (m, 2H). MS (ESI): m/z = 262.28 $[M+H]^+$.

Step B: According to the procedure described for the synthesis of 12d (Step C) Yield: 89%. ^{1}H NMR (500 MHz, DMSO-d_6) δ

10.14 (s, 1H), 9.54 (s, 1H), 8.56 (s, 1H), 8.40 (t, *J* = 1.5 Hz, 1H), 8.23 – 8.18 (m, 2H), 7.82 (t, *J* = 7.7 Hz, 1H). MS (ESI): Calcd for $C_{11}H_8NO_2S$ $[M+H]^+$ 218.03, found 218.31.

4.1.1.21 3-(furan-2-carbonyl)benzaldehyde (**12g**)

Step A: (3-(1, 3-dioxolan-2-yl) phenyl)(furan-2-yl)methanone (11g)

According to the procedure described for the synthesis of 12d (Step B) Yield: 22.6%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.97 (d, *J* = 7.7 Hz, 1H), 7.74 – 7.69 (m, 2H), 7.52 (t, *J* = 7.7 Hz, 1H), 7.23 (d, *J* = 3.5 Hz, 1H), 6.62 – 6.57 (m, 1H), 5.89 (s, 1H), 4.17 – 4.10 (m, 2H), 4.10 – 4.04 (m, 2H). MS (ESI): m/z = 267.31 $[M+Na]^+$.

Step B: According to the procedure described for the synthesis of 12d (Step C) Yield: 73%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.12 (s, 1H), 8.50 (s, 1H), 8.26 (d, *J* = 7.7 Hz, 1H), 8.12 (d, *J* = 7.6 Hz, 1H), 7.75 (s, 1H), 7.70 (t, *J* = 7.7 Hz, 1H), 7.32 (d, *J* = 3.5 Hz, 1H), 6.69 – 6.60 (m, 1H). MS (ESI): Calcd for $C_{12}H_9O_3$ $[M+H]^+$ 201.06, found 201.32.

4.1.1.22 3-(furan-3-carbonyl)benzaldehyde (**12h**)

Step A: (3-(1, 3-dioxolan-2-yl) phenyl)(furan-2-yl)methanone (11h)

According to the procedure described for the synthesis of 12d (Step B) Yield: 21.4%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.97 (s, 1H), 7.92 (s, 1H), 7.85 (dd, *J* = 7.7, 0.9 Hz, 1H), 7.73 – 7.68 (m, 1H), 7.54 – 7.49 (m, 2H), 6.91 (d, *J* = 0.7 Hz, 1H), 5.87 (s, 1H), 4.16 – 4.10 (m, 2H), 4.09 – 4.03 (m, 2H). MS (ESI): m/z = 245.38 $[M+H]^+$.

Step B: According to the procedure described for the synthesis of 12d (Step C) Yield: 86%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.11 (s, 1H), 8.34 (s, 1H), 8.14 – 8.09 (m, 2H), 7.95 (s, 1H), 7.69 (t, *J* = 7.7 Hz, 1H), 7.55 (d, *J* = 1.2 Hz, 1H), 6.92 (s, 1H). MS (ESI): Calcd for $C_{12}H_9O_3$ $[M+H]^+$ 201.06, found 201.32.

4.1.1.23 3-(1-(phenylsulfonyl)-1H-pyrrole-2-carbonyl)benzaldehyde (**12i**)

Step A: 1-(phenylsulfonyl)-1H-pyrrole-2-carbaldehyde

2-formylprrole (1.5 g, 15.77 mmol), benzenesulphonyl chloride (4.18 g, 23.66 mmol) and tetra-n-butylammonium bromide (157.63 mg) in DCM (10 mL) were stirred with 30% aqueous sodium hydroxide (3.9 mL) at 15 °C for 24 h. The organic phase was separated and the aqueous phase was extracted with DCM. The combined organic layer was washed with brine and was dried over anhydrous sodium sulfate (Na_2SO_4). Then solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using petroleum ether–ethyl acetate (20:1) to give white solid 3.4 g. Yield: 91.64%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.86 (s, 1H), 8.04 (d, *J* = 7.8 Hz, 2H), 7.91 (s, 1H), 7.79 (t, *J* = 7.5 Hz, 1H), 7.67 (t, *J* = 7.9 Hz, 3H), 7.32 – 7.29 (m, 1H), 6.59 (t, *J* = 3.4 Hz, 1H). MS (ESI): m/z = 236.31 $[M+H]^+$.

Step B (11i): According to the procedure described for the synthesis of 12d (Step B) Yield: 23.31%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.11 (d, *J* = 8.0 Hz, 1H), 8.01 – 7.95 (m, 2H), 7.79 (dd, *J* = 7.4, 3.5 Hz, 2H), 7.75 – 7.61 (m, 4H), 7.56 (dd, *J* = 9.7, 5.4 Hz, 1H), 6.86 (d, *J* = 3.6 Hz, 1H), 6.54 (t, *J* = 3.3 Hz, 1H), 5.85 – 5.80 (m, 1H), 4.08 – 4.01 (m, 2H), 4.00 – 3.93 (m, 2H).

Step C: According to the procedure described for the synthesis of 12d (Step C) Yield: 81.76%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.09 (s, 1H), 8.26 (s, 1H), 8.17 (d, *J* = 7.6 Hz, 1H), 8.12 (d, *J* = 7.9 Hz, 2H), 8.10 – 8.01 (m, 2H), 7.84 – 7.67 (m, 4H), 7.00 – 6.95 (m, 1H), 6.57 (d, *J* = 2.9 Hz, 1H). MS (ESI): Calcd for $C_{18}H_{14}NO_4S$ $[M+H]^+$ 340.06, found 340.35.

4.1.1.24 3-(1-(phenylsulfonyl)-1H-pyrrole-3-carbonyl)benzaldehyde (**12j**)

Step A: 1-(phenylsulfonyl)-1H-pyrrole-3-carbaldehyde

According to the procedure described for the synthesis of 12i (Step A) Yield: 90.95%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.78 (s, 1H), 8.32 (s, 1H), 8.08 (d, *J* = 7.7 Hz, 2H), 7.81 (t, *J* = 7.5 Hz, 1H), 7.70 (t, *J* = 7.8 Hz, 2H), 7.53 (d, *J* = 2.3 Hz, 1H), 6.70 – 6.67 (m, 1H). MS (ESI): m/z = 236.25 $[M+H]^+$.

Step B (11j): According to the procedure described for the synthesis of 12d (Step B) Yield: 31.55%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.12 (d, *J* = 8.1 Hz, 2H), 7.88 (s, 1H), 7.83 – 7.78 (m, 3H), 7.74 – 7.66 (m, 3H), 7.58 (dd, *J* = 13.0, 4.7 Hz, 2H), 6.78 – 6.76 (m, 1H), 5.84 (s, 1H), 4.10 – 4.02 (m, 2H), 4.02 – 3.93 (m, 2H). MS (ESI): m/z = 384.33 $[M+H]^+$.

Step C: According to the procedure described for the synthesis of 12d (Step C) Yield: 78.22%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.11 (s, 1H), 8.26 (s, 1H), 8.19 – 8.07 (m, 4H), 8.01 (s, 1H), 7.80 (dt, *J* = 11.5, 7.5 Hz, 2H), 7.69 (t, *J* = 7.7 Hz, 2H), 7.60 – 7.57 (m, 1H), 6.83 – 6.80 (m, 1H). MS (ESI): Calcd for $C_{18}H_{14}NO_4S$ $[M+H]^+$ 340.06, found 340.30.

4.1.2 Compounds **13a-h** were obtained from Compounds **12a-h** according to the procedure described for the synthesis of **9a** (Step B)

4.1.2.1 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-picolinoylbenzylidene) piperazine-2,5-dione (**13a**)

Yield: 45%. Mp: 289.8-291.2°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.34 (s, 1H), 12.29 (s, 1H), 10.14 (s, 1H), 8.80 (d, *J* = 4.5 Hz, 1H), 8.14 (s, 1H), 8.12 – 8.05 (m, 2H), 7.90 – 7.83 (m, 2H), 7.76 (d, *J* = 7.7 Hz, 1H), 7.70 (ddd, *J* = 6.7, 4.8, 1.9 Hz, 1H), 7.58 (t, *J* = 7.7 Hz, 1H), 6.87 (s, 1H), 6.81 (s, 1H), 1.39 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.7, 157.5, 156.3, 141.6, 140.4, 137.3, 134.4, 133.3, 130.7, 130.5, 130.4, 129.5, 128.8, 128.4, 126.9, 126.5, 123.7, 115.2, 114.9, 113.7, 105.1, 39.9, 31.9, 30.6. HRMS (ESI): Calcd for $C_{25}H_{24}N_5O_3$ $[M+H]^+$ 442.1874, found 442.1869.

4.1.2.2 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-nicotinoylbenzylidene) piperazine-2,5-dione (**13b**)

Yield: 33%. Mp: 142.0-144.0°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 12.28 (s, 1H), 10.34 (s, 1H), 8.93 (s, 1H), 8.85 – 8.84 (m, 1H), 8.20 (d, *J* = 7.7 Hz, 1H), 7.86 (d, *J* = 5.6 Hz, 2H), 7.78 (d, *J* = 7.6 Hz, 1H), 7.68 (d, *J* = 7.7 Hz, 1H), 7.60 (t, *J* = 7.6 Hz, 2H), 6.86 (s, 1H), 6.81 (s, 1H), 1.38 (s, 9H). HRMS (ESI): Calcd for $C_{25}H_{24}N_5O_3$ $[M+H]^+$ 442.1874, found 442.1872.

4.1.2.3 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-isonicotinoylbenzylidene) piperazine-2,5-dione (**13c**)

Yield: 32%. Mp: 269.4-271.2°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 12.28 (s, 1H), 10.34 (s, 1H), 8.83 (d, *J* = 5.7 Hz, 2H), 7.88 (s, 1H), 7.85 (s, 1H), 7.80 (d, *J* = 7.7 Hz, 1H), 7.70 (d, *J* = 5.8 Hz, 3H), 7.61 (t, *J* = 7.7 Hz, 1H), 6.86 (s, 1H), 6.80 (s, 1H), 1.38 (s, 9H). HRMS (ESI): Calcd for $C_{25}H_{24}N_5O_3$ $[M+H]^+$ 442.1874, found 442.1875.

4.1.2.4 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-(thiophene-3-carbonyl) benzylidene) piperazine-2,5-dione (**13d**)

Yield: 44%. Mp: 251.0-252.9°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 12.28 (s, 1H), 10.36 (s, 1H), 8.36 – 8.35 (m, 1H), 7.90 (s, 1H), 7.85 (s, 1H), 7.76 – 7.69 (m, 3H), 7.61 – 7.56 (m, 2H), 6.86 (s, 1H), 6.81 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (125MHz, DMSO-d_6) δ 189.3, 158.1, 156.6, 140.8, 138.9, 136.2, 134.8, 134.0, 133.7, 131.1, 130.1, 129.4, 128.7, 128.5, 128.1, 128.0, 124.2, 113.3, 109.9, 105.7, 32.4, 31.1. HRMS (ESI): Calcd for $C_{24}H_{23}N_4O_3S$ $[M+H]^+$ 447.1485, found 447.1483.

4.1.2.5 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-(thiophene-2-carbonyl) benzylidene) piperazine-2,5-dione (**13e**)

Yield: 51%. Mp: 266.7-268.0°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 12.28 (s, 1H), 10.39 (s, 1H), 8.13 (d, *J* = 4.9 Hz, 1H), 7.93 (s, 1H), 7.85 (s, 1H), 7.83 (d, *J* = 3.6 Hz, 1H), 7.74 (t, *J* = 7.3 Hz, 2H), 7.59 (t, *J* = 7.7 Hz, 1H), 7.29 (t, *J* = 4.4 Hz 1H), 6.86 (s, 1H), 6.82 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 187.6, 158.2, 156.6, 143.2, 140.8, 138.3, 136.3, 136.2, 134.8, 134.1, 133.7, 131.2, 129.8, 129.4, 128.5, 128.1, 124.2, 113.2, 105.7, 32.4, 31.1. HRMS (ESI): Calcd for $C_{24}H_{23}N_4O_3S$ $[M+H]^+$ 447.1485, found 447.1483.

4.1.2.6 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-(thiazole-5-carbonyl) benzylidene) piperazine-2,5-dione (**13f**).

Yield: 57%. Mp: 284.9-286.6°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 12.29 (s, 1H), 10.39 (s, 1H), 9.51 (s, 1H), 8.58 (s, 1H), 7.98 (s, 1H), 7.85 (s, 1H), 7.79 (t, *J* = 7.0 Hz, 2H), 7.61 (t, *J* = 7.7 Hz, 1H), 6.86 (s, 1H), 6.84 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 186.8, 161.4, 157.7, 156.1, 149.6, 140.4, 138.7, 137.4, 134.4, 133.9, 133.8, 130.6, 129.5, 129.1, 128.2, 127.8, 123.8, 112.6, 105.2, 32.4, 30.6. HRMS (ESI): Calcd for $C_{23}H_{22}N_5O_3S$ $[M+H]^+$ 448.1438, found 448.1436.

4.1.2.7 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-(furan-5-carbonyl) benzylidene) piperazine-2,5-dione (**13g**)

Yield: 41%. Mp: 251.4-253.1°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 12.28 (s, 1H), 10.39 (s, 1H), 8.13 (d, *J* = 4.9 Hz, 1H), 7.93 (s, 1H), 7.85 (s, 1H), 7.83 (d, *J* = 3.6 Hz, 1H), 7.74 (t, *J* = 7.3 Hz, 2H), 7.59 (t, *J* = 7.7 Hz, 1H), 7.29 (t, *J* = 4.4 Hz 1H), 6.86 (s, 1H), 6.82 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 181.4, 157.7, 156.1, 151.3, 148.6, 140.4, 137.2, 134.4, 133.6, 133.4, 130.7, 129.5, 128.9, 128.1, 127.6, 123.7, 121.5, 112.8, 112.7, 105.2, 31.9, 30.6. HRMS (ESI): Calcd for $C_{24}H_{23}N_4O_4$ $[M+H]^+$ 431.1714, found 431.1712.

4.1.2.8 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-(furan-3-carbonyl) benzylidene) piperazine-2,5-dione (**13h**)

Yield: 27%. Mp: 242.4-243.3°C ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.33 (s, 1H), 12.28 (s, 1H), 10.38 (s, 1H), 8.50 (s, 1H), 7.95 (s, 1H), 7.90 (s, 1H), 7.85 (s, 1H), 7.75 (s,1H), 7.74 (s, 1H) 7.58 (t, *J* = 7.7 Hz, 1H), 6.95 (d, *J* = 0.7 Hz, 1H), 6.87 (s, 1H), 6.83 (s, 1H), 1.39 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 188.3, 157.7, 156.2, 149.9, 144.9, 140.4, 138.5, 134.4, 133.7, 133.4, 130.7, 129.2, 129.1, 127.8, 127.6, 125.7, 123.8, 112.9, 109.8, 105.2, 31.9, 30.6. HRMS (ESI): Calcd for $C_{24}H_{23}N_4O_4$ $[M+H]^+$, 431.1714, found 431.1713.

4.1.2.9 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-(pyrrole-5-carbonyl) benzylidene) piperazine-2,5-dione (**14**)

To a solution of compound **4** (71.37 mg, 0.25 mmol) in DMF (5 mL) was added compound 13i (100 mg, 0.29 mmol) under N_2 atmosphere. Then Cs_2CO_3 (119.73 mg, 0.37 mmol) was added into the solution and the mixture was stirred at 50 °C for about 24 h. After the reaction was completed, the mixture was diluted with brine and extracted with EtOAc. The organic layer was wased with brine and was dried over anhydrous sodium sulfate (Na_2SO_4). Then solvent was removed under reduced pressure to obtain **13i**. A solution of **13i** (73 mg, 0.13 mmol) in 2 mL of 1,4-dioxane was stired with 1 mL of 5 M NaOH at 25 °C for 20 h. The organic phase was separated and the aqueous phase was extracted with EtOAc. The combined organic layer was washed with brine and was dried over anhydrous sodium sulfate (Na_2SO_4). Then solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using CH_3OH–CH_2Cl_2 (100:1) to give yellow solid 20 mg. Yield: 19.04%. Mp: 249.6-251.2°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.26 (s, 1H), 12.07 (s, 1H), 10.38 (s, 1H), 7.91 (s, 1H), 7.84 (s, 1H), 7.70 (d, *J* = 6.8 Hz, 2H), 7.54 (t, *J* = 7.6 Hz, 1H), 7.22 (s, 1H), 6.85 (d, *J* = 9.8 Hz, 2H), 6.79 (s, 1H), 6.27 (s, 1H), 5.30 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 183.4, 157.8, 156.3, 140.4, 138.8, 134.4, 133.5, 132.4, 130.7, 130.6, 129.1, 128.7, 127.8, 126.5, 123.2, 119.5, 112.9, 110.4, 105.1, 31.9, 30.6. HRMS (ESI): Calcd for $C_{24}H_{24}N_5O_3$ $[M+H]^+$ 430.1874, found 430.1871.

4.1.2.10 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-(pyrrole-3-carbonyl) benzylidene) piperazine-2, 5-dione (**15**)

According to the procedure described for the synthesis of 14. Yield: 8.98%. Mp: 254.4-256.3°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.37 (s, 1H), 12.27 (s, 1H), 11.62 (s, 1H), 10.31 (s, 1H), 7.84 (s, 2H), 7.66 (d, *J* = 7.5 Hz, 2H), 7.53 (t, *J* = 7.7 Hz, 1H), 7.46 (s, 1H), 6.91 (s, 1H), 6.86 (s, 1H), 6.81 (s, 1H), 6.58 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 189.2, 157.7, 156.3, 140.4, 140.3, 134.4, 133.3, 132.1, 130.7, 129.0, 128.7, 127.8, 127.3, 126.4, 123.8, 123.5, 120.1, 113.3, 109.3, 105.2, 31.9, 30.6. HRMS (ESI): Calcd for $C_{24}H_{24}N_5O_3$ $[M+H]^+$ 430.1874, found 430.1871.

4.1.2.11 (3Z, 6Z)-3-((5-(tert-butyl)-1H-imidazol-4-yl) methylene)-6-(3-benzoylbenzylidene) piperazine-2,5-dione (compound **1**)

Step A: 2-(3-benzoylphenyl)-1, 3-dioxolane

According to the procedure described for the synthesis of 12a (Step B) Yield: 62%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.92 (s, 1H), 7.80 (d, *J* = 7.3 Hz, 3H), 7.72 (d, *J* = 7.6 Hz, 1H), 7.62 – 7.56 (m, 1H), 7.52 – 7.45 (m, 3H), 5.87 (s, 1H), 4.16 – 4.09 (m, 2H), 4.09 – 4.02 (m, 2H).

Step B: 3-benzoylbenzaldehyde

According to the procedure described for the synthesis of 12a (Step C) Yield: 55%, ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.09 (s, 1H), 8.28 (s, 1H), 8.12 (d, *J* = 7.7 Hz, 1H), 8.08 (d, *J* = 7.7 Hz, 1H), 7.81 (d, *J* = 7.7 Hz, 2H), 7.68 (t, *J* = 7.7 Hz, 1H), 7.63 (t, *J* = 7.4 Hz, 1H), 7.52 (t, *J* = 7.7 Hz, 2H).

Step C:

According to the procedure described for the synthesis of 9a (Step B) Yield: 43%, ^{1}H NMR (500 MHz, DMSO-d_6) 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, 9H). MS (ESI): Calcd for $C_{26}H_{25}N_4O_3$ $[M+H]^+$ 441.19, found 441.35.

4.1.2.12 (3Z, 6Z)-3-(-3-benzoylbenzylidene)-6-(pyridin-2-ylmethylene) piperazine-2,5-dione (compound **2**)

According to the procedure described for the synthesis of compound 1. Yield 37%, ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.59 (s, 1H), 10.64 (s, 1H), 8.73 (d, *J* = 4.1 Hz, 1H), 7.91 (td, *J* = 7.7, 1.8 Hz, 1H), 7.86 (s, 1H), 7.83 (d, *J* = 7.3 Hz, 2H), 7.78 (d, *J* = 7.6 Hz, 1H), 7.71 – 7.65 (m, 3H), 7.61 – 7.55 (m, 3H), 7.37 (dd, *J* = 7.0, 5.3 Hz, 1H), 6.90 (s, 1H), 6.72 (s, 1H). MS (ESI): Calcd for $C_{24}H_{18}N_3O_3$ $[M+H]^+$ 396.13, found 396.34.

4.2 Co-crystal structure

4.2.1 Crystallization, date collect and structure determination

Porcine brain tubulin (Catalog # T-238P) was purchased from Cytoskeleton, Inc. (Denver, CO, USA). Bis-Tris propane, Mes, tyrosine, DTT and β,γ-methyleneadenosine 5'-triphosphate disodium salt were purchased from Sigma (St. Louis, MO, USA). β-Mercaptoethanol was obtained from XiYa Reagent (Chengdu, China). Glycerol and antiprotease cocktail were obtained from Sangon Biotech (Shanghai, China). All of the conventional reagents, such as NaCl, $MgCl_2$ and $CaCl_2$, were supplied by

Kelun Pharmaceutical (Chengdu, China).

The complex of two tubulins with the stathmin-like domain of RB3 (RB3–SLD) and with tubulin tyrosine ligase (TTL) (the T2R–TTL complex) was produced as described. In brief, RB3–SLD was overexpressed in Escherichia coli, purified by anion-exchange chromatography (QFF; GE Healthcare Ltd, Little Chalfont, UK) and gel filtration (Superdex 75; GE-Healthcare), concentrated to 10 mg/mL and stored at -80 °C until use. The TTL construct was a kind gift from Dr. Michel O. Steinmetz (Paul Scherrer Institut, PSI, Switzerland). After overexpression in E. coli, TTL was purified by nickel-affinity chromatography followed by gel filtration (Superdex 200; GE-Healthcare). Finally, TTL in Bis-Tris propane (pH 6.5), 200 mM NaCl, 2.5 mM $MgCl_2$, 5 mM β-mercaptoethanol and 1% glycerol was concentrated to 20 mg/mL and stored at -80 °C. Porcine brain tubulin (Catalog # T-238P) was supplied at 10 mg/mL (buffer: 80 mM Pipes, pH 6.9, 2.0 mM $MgCl_2$, 0.5 mM EGTA and 1 mM GTP) and stored at -80 °C until use. The T2R–TTL complex was prepared by mixing tubulin, RB3–SLD and TTL in a 2 : 1.3:1.2 (tubulin : RB3–SLD : TTL) molar ratio, and then 1 mM β,γ-methyleneadenosine 5'-triphosphate disodium salt, 5 mM tyrosine and 10 mM DTT were added and the complex was concentrated to 20 mg/mL at 4 °C.

4.2.2 Crystallization and crystals soaking

T2R–TTL crystals were obtained at 20 °C in a buffer consisting of 6% poly(ethylene glycol) 4000, 8% glycerol, 0.1 M Mes, 30 mM $CaCl_2$ and 30 mM $MgCl_2$ (pH 6.7). Rod-like crystals grew to maximum dimensions within 1 week. Stock solutions of ligands were prepared in 100% DMSO at 5 mM (plinabulin and lexibulin) or 10 mM (tivantinib and nocodazole) concentrations. For crystal soaking, 0.1 μL of the ligand solution was added to the 2-lL crystal-containing drop for 24 h at 20 °C.

4.2.3 Data collection and structure determination

The crystals of T2R–TTL–ligand complexes were mounted in nylon loops (Hampton, Aliso Viejo, CA, USA) and flash-cooled in a cold nitrogen stream at 100 K. Diffraction data were collected on beamlines BL17U1 and BL19U1 at Shanghai Synchrotron Radiation Facility (SSRF) (Shanghai, China). Data were processed using HKL2000. The structure was determined by molecular replacement method using the T2R–TTL structure (pdb: 5FNV) as a search model. COOT and PHENIX were used to build and refine the structure. The initial model and the topology parameters of compound were generated using PRODRG. The model quality was checked with MOLPROBITY. PYMOL was used to generate the figures.

The complex structure contains stathmin-like domain of RB3 (RB3–SLD), tubulin tyrosine ligase (TTL), σ-tubulin and β-tubulin. The complex was prepared by mixing Porcine brain tubulin, purified RB3–SLD and TTL in a 2 : 1.3 : 1.2 (tubulin : RB3–SLD : TTL) molar ratio. The crystal was obtained at 20 °C in a buffer consisting of 6% poly(ethylene glycol) 4000, 8% glycerol, 0.1 M Mes, 30 mM $CaCl_2$ and 30 mM $MgCl_2$ (pH 6.7) for a week. After crystal soaking with 0.1 μL of the compound 1 solution, the complex crystal was obtained. Diffraction data were collected on beamlines BL17U1 and BL19U1 at Shanghai Synchrotron Radiation Facility (SSRF) (Shanghai, China). Data were processed using HKL2000. The structure was determined by molecular replacement method using the T2R–TTL structure (pdb: 5FNV) as a search model. COOT and PHENIX were used to build and refine the structure. The initial model and the topology parameters of compound 1 were generated using PRODRG. The model quality was checked with MOLPROBITY. Details of the data collection and refinement statistics were summarized in Table S1

4.3 Water solubility

Test compounds were saturated in distilled water and the saturated solutions were shaken for 24 h at 37 ^{o}C. First, we established a standard curve of each tested compound by dissolution of the compound in MeOH-H_2O (6:4) at rt at various concentrations. After 24 h, the solutions were filtered through a centrifugal filter (0.45 μm). The filtrate was injected into HPLC to analyze the water solubility.

4.4 Biology

4.4.1 Anticancer activities

Human cancer cell line purchased from American Type Cell Culture Collection (ATCC, USA). Cells were maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin-streptomycin (100 U/mL-100 g/mL) and 2 mM glutamine at 37 °C in a humidified atmosphere (CO_2 5% -95% air). Cells (5×10^3 per well) were seeded in 96-well plates for 24 h. All test derivatives were dissolved in 100% cell culture grade DMSO. After incubation cells were treated with test compounds for 72 h. Subsequently, cells were fixed with 50% TCA. Cell viability was assessed by using sulforhodamine B assay. The absorbance at 540 nm was measured on a microplate reader (Perkin-Elmer, USA).

4.4.2 Tubulin polymerization assay in vitro

The fluorescence-based in vitro tubulin polymerization assay was performed using the Tubulin Polymerization Assay Kit (BK011P, Cytoskeleton, USA) according to the manual. The conditions are 2 mg/ml tubulin in 80 mM PIPES PH 6.9, 2.0 mM $MgCl_2$, 0.5 mM EGTA, 1.0 mM GTP and 15% glycerol. All test derivatives were dissolved in 100% cell culture grade DMSO. First, 96-well plate was incubated with 5 μL of inhibitors in the same concentrations (5 μM) at 37 °C for 1 min. Then 45 μL of the tubulin reaction mix was added. Immediately, the increase in fluorescence was monitored by excitation at 360 nm and emission at 450 nm in a multimode reader (SpectraMax® I3, Molecular Devices, USA).

4.4.3 Immunofluorescence Staining.

BxPC-3 cells were seeded in 6-well plate (with coverslips plated) at density of 5×10^4 cells. After overnight adherence, they were exposed to 1 at 5 nM, for 24 h, respectively. Cells were fixed with cold MeOH at −20 °C for 15 min, washed three times with PBS (G002, Servicebio, China), and blocked with 3% PBS plus 0.1% Triton X-100 for 30 min at 37 °C. Microtubules were detected by incubation with a monoclonal anti-β-tubulin (Servicebio, China) at 37 °C for 1 h. Then, the cells were washed with PBS three times and incubated with a FITC-conjugated antimouse IgG antibody. Nuclei were stained with DAPI (G1012, Servicebio, China). The coverslips were visualized under fluorescence microscope (Nikon Eclipse C1, Nikon, Japan).

4.5 Molecular modeling

Target compounds were docking into the co-crystal structure of tubulin-compound **1**. The protein domain subnits C, D and water molecules were removed using MOE. The X-ray crystallographic structure was retrieved from the protein data bank (PDB ID: 5C8Y, 5YL4) at a resolution of 2.59 Å. The three-dimensional structures of the small molecules were generated in Molecular Operating Environment System (MOE) 2016.10 (Chemical Computing Group, Montreal, Canada). A subsequent energy minimization was carried out using Amber10: EHT force field and R-field solvation. The protein structures were prepared using QuickPrep module of MOE, and the energy was minimized through General method at 0.1 kcal/mol/Å2 RMS Gradient. Initial placement pose was performed with Alpha Triangle placement method. The docking poses were scored

using London ΔG scoring function with five parameters, such as rotational and translational entropy, ligand flexibility, hydrogen bonding, metal ligations, and desolvation energy. At least 20 poses for each compound were retained, and ranked via GBVI/WSA ΔG scoring function.

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Highlight

1) Co-crystal structure guided rational design

2) Novel compounds with higher activity against human BxPC-3 cell line

3) Interaction explained by molecular docking studies