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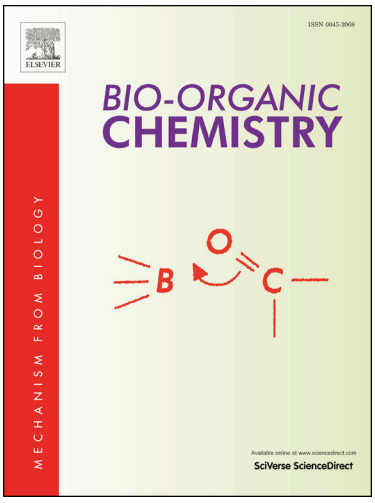

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Design, synthesis and anticancer activity of 5-aryl-4-(4-arylpiperazine-1-carbonyl)-1,2,3-thiadiazoles as microtubule-destabilizing agents

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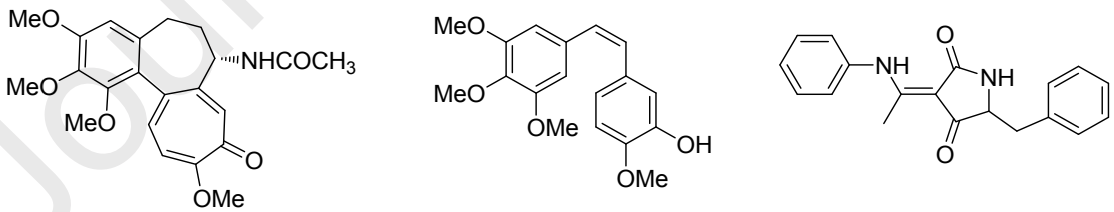
Abstract

Hereby, we report our efforts on discovery and optimization of a new series of 5-aryl-4-(4-arylpiperazine-1-carbonyl)-1,2,3-thiadiazoles as new microtubule-destabilizing agents along our previous study. Guided by docking model analysis, we introduced the 1,2,3-thiadiazole moiety containing the hydrogen-bond acceptors as B-ring of XRP44X analogues. Extensive structure modifications were performed to investigate the detailed structure and activity relationships (SARs). Some compounds exhibited potent antiproliferative activities against three human cancer cell lines (SGC-7901, A549 and HeLa). The compound **5m** exhibited the highest potency against the three cancer cell lines. The tubulin polymerization experiments indicated that compound **5m** effectively inhibited the tubulin polymerization, and immunostaining assay revealed that it significantly disrupted microtubule dynamics. Moreover, cell cycle studies revealed that compound **5m** dramatically arrested cell cycle progression at G2/M phase.

Keywords: 1,2,3-thiadiazole, antiproliferative activity, microtubule-destabilizing agent, molecular docking.

1. Introduction

Microtubules are cylindrical polymers composed of α- and β-tubulin, which are a major component of the cytoskeleton and mediate essential cellular functions including cell proliferation, trafficking, signaling and migration in eukaryotic cells. Interfering with the microtubule dynamic equilibrium, by either inhibiting tubulin polymerization or blocking microtubules disassembly, prevents proper microtubule function and ultimately leads to cell death [1-3]. Therefore, microtubules are regarded as an excellent target for development of clinically useful anticancer drugs [4,5]. Microtubule targeting agents are generally categorized into microtubule-stabilizing and microtubule-destabilizing agents [6]. Compounds such as taxol and epothilone prevent microtubule disassembly and are referred to as microtubule-stabilizing agents. On the contrary, vinca alkaloid and colchicine (**1**) interfere with the assembly of tubulin into microtubules and are classified as microtubule-destabilizing agents. In recent decades, a large number of outstanding microtubule-destabilizing agents have been reported, such as combretastatin A-4 (CA-4, **2**) and TN16 (**3**) (Fig. 1) [7,8].



Colchicine, **1** CA-4, **2** TN16, **3**

Figure 1. Chemical structures of some microtubule destabilizers.

XRP44X (**4**), a novel prominent microtubule-destabilizing agent (Wasylyk *et al.*, 2008), consists of four rings (denoted as rings A, B, C and D in Figure 2) with a carbonyl linkage between B-ring and C-ring [9]. The replacement of the B-ring of XRP44X with

a bioisosteric five-membered heterocycle nucleus yields several analogues, which have similar potency and mechanism of actions compared to XRP44X [10,11]. Based on the reported molecular modeling of XRP44X or its analogues with tubulin, the binding domain can be divided into three pockets (P-1, P-2 and P-3 in Figure 2) [10]. The carbonyl group, as a linkage, provokes an orientation for a hydrogen bond with the amide nitrogen of Alaβ317, and serves as an anchor to place XRP44X or its analogues in the proper orientation within the binding site. The A-ring fosters van der Waals interactions with the hydrophobic P-1 pocket consisting of Lysβ254 and Leuβ248, while the C- and D-ring extends deeper to strand S6 of the *N*-terminal nucleotide binding domain and interacts with the hydrophobic P-3 pocket formed by Cysβ241, Leuβ242 and Leuβ252. The B-ring in the hydrophobic P-2 pocket enhances the van der Waals interactions with it. However, we noted that some amino acid residues in P-2 pocket, such as Asnβ258 and Lysβ352, as hydrogen-bond donors might also form additional interactions with B-ring, which would facilitate the development of new XRP44X analogues.

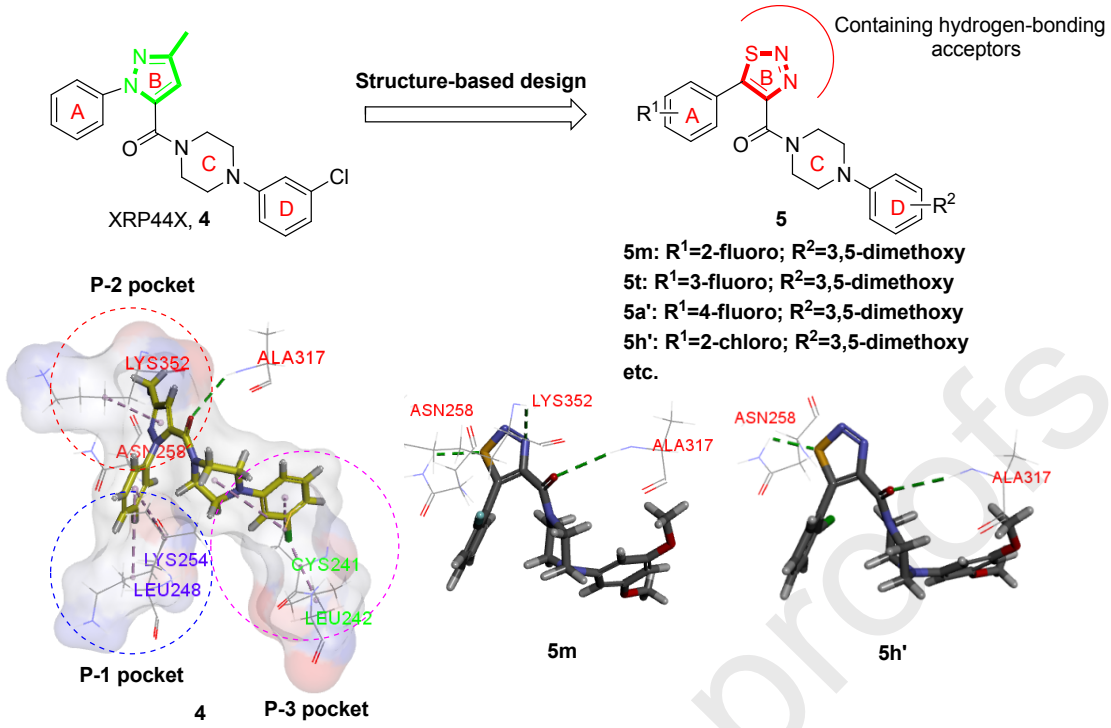


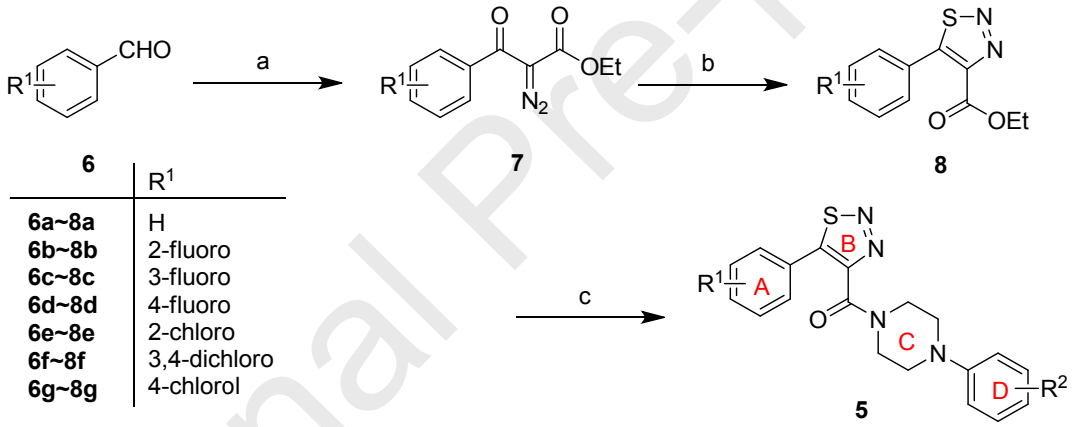
Figure 2. Design of the target compounds.

As part of our ongoing effort to develop new tubulin inhibitors, we herein attempt to replace the pyrazole moiety of XRP44X with a 1,2,3-thiadiazole, one of the significant structural fragments containing several hydrogen-bond acceptors. Based on the previous docking model, we conducted docking simulations of the designed target compounds, and the preliminary results indicated that compounds **5m** (R^1 = 2-fluoro, R^2 = 3,5-dimethoxy) and **5h'** (R^1 = 2-chloro, R^2 = 3,5-dimethoxy) might interact with tubulin in a similar way that XRP44X does. Moreover, the 1*S* of 1,2,3-thiadiazole of compounds **5m** and **5h'** and the 3*N* of 1,2,3-thiadiazole of compound **5m** might enable additional hydrogen bonding interactions with Asnβ258 and Lysβ352 in P-2 pocket. To the best of our knowledge, this new binding mode in P-2 pocket has not been explored in the design of XRP44X analogues.

2. Results and discussion

2.1 Chemistry

The target compounds 5-aryl-4-(4-arylpiperazine-1-carbonyl)-1,2,3-thiadiazols (**5**) were prepared as outlined in Scheme 1. The substituted aromatic aldehydes (**6**) reacted with ethyl diazoacetate, DBU and IBX in DMSO to afford the corresponding ethyl 2-diazo-3-oxo-3-arylpropanoates (**7**) *via* a one-pot procedure in 60-90% yields [12]. Subsequently, **7** reacted with Lawesson's reagent in toluene under reflux conditions to afford the key intermediates ethyl 5-aryl-1,2,3-thiadiazole-4-carboxylates (**8**) [13]. Finally, **8** were treated with corresponding arylpiperazines to afford the target compounds **5** in the presence of trimethylaluminium in 42-70% yields [14,15].



6	R^1
6a~8a	H
6b~8b	2-fluoro
6c~8c	3-fluoro
6d~8d	4-fluoro
6e~8e	2-chloro
6f~8f	3,4-dichloro
6g~8g	4-chlorol

	R^1	R^2
5a	H	3-fluoro
5b	H	4-methoxy
5c	H	2,5-dimethoxy
5d	H	3,5-dimethoxy
5e	2-fluoro	H
5f	2-fluoro	3-fluoro
5g	2-fluoro	3-chloro
5h	2-fluoro	3-methyl
5i	2-fluoro	3-methoxy
5j	2-fluoro	3-trifluoromethyl
5k	2-fluoro	4-methoxy
5l	2-fluoro	2,5-dimethoxy
5m	2-fluoro	3,5-dimethoxy
5n	3-fluoro	H
5o	3-fluoro	3-chloro
5p	3-fluoro	3-methyl
5q	3-fluoro	3-methoxy
5r	3-fluoro	4-methoxy

	R^1	R^2
5s	3-fluoro	2,5-dimethoxy
5t	3-fluoro	3,5-dimethoxy
5u	4-fluoro	H
5v	4-fluoro	3-chloro
5w	4-fluoro	3-methyl
5x	4-fluoro	3-methoxy
5y	4-fluoro	4-methoxy
5z	4-fluoro	3,4-dimethyl
5a'	4-fluoro	3,5-dimethoxy
5b'	2-chloro	3-chloro
5c'	2-chloro	3-methyl
5d'	2-chloro	3-methoxy
5e'	2-chloro	3-trifluoromethyl
5f'	2-chloro	4-methoxy
5g'	2-chloro	2,5-dimethoxy
5h'	2-chloro	3,5-dimethoxy
5i'	3,4-dichloro	3,5-dimethoxy
5j'	4-chloro	4-methoxy
5k'	4-chloro	3,5-dimethoxy

Scheme 1. Reagents and conditions: (a) N_2CHCO_2Et, DBU, IBX, DMSO, rt., 10 h; (b) Lawesson's

reagent, toluene, reflux, 4 h; (c) arylpiperazine, $AlMe_3$ (1.0 M solution in heptane), DCM, rt., overnight.

2.2 Biological Evaluation

2.2.1 In Vitro Antiproliferative Activity

In vitro antiproliferative activities against three human cancer cell lines, including SGC-7901 (human gastric carcinoma cell line), A549 (human lung carcinoma cell line) and HeLa (Human cervical carcinoma cell line), were determined using a standard MTT assay with colchicine and CA-4 as the positive controls. As shown in Table 1, all halogenated compounds showed significant change on the activity compared with corresponding compounds without halogens. Compared with **5d**, **5m** and **5h'** showed greatly improved activity, which indicated electronegativity is crucial on the A-ring. Moreover, halogens on the A-ring exhibited an order of potency being *ortho-* > *meta-* > *para*-substituted in generally (**5g** *vs.* **5o** *vs.* **5v**, **5i** *vs.* **5q** *vs.* **5x**, **5m** *vs.* **5t** *vs.* **5a'**, etc.). It was noted that compounds (**5u** ~ **5z** and **5i'** ~ **5k'**) with a substituent on *para*-substitution of A-ring almost lost antiproliferative activity. Then, we investigated the effect of substituents on the D-ring and found that the volume and lipophilicity of substituents, rather than electronegativity, may be the important factors affecting activity (**5f** ~ **5j**, **5o** ~ **5q** and **5b'** ~ **5e'**). The compounds with OCH_3 on *meta*-substitution of the D-ring gave higher activity than those on *para*-substitution (**5i** *vs.* **5k**, **5q** *vs.* **5r**, **5d'** *vs.* **5f'**), suggesting that the substituents' positions had greater effects on activity. In addition, di-*meta*-substituted compounds showed increased activity, which could be confirmed by compounds **5l**, **5m**, **5s** and **5t**. Among the target compounds, compound **5m** (R^1 = 2-fluoro, R^2 = 3,5-dimethoxy) showed the best antiproliferative activity against the HeLa cell lines with IC_{50} value of 0.092 ± 0.005 μM.

Table 1. Antiproliferative activity of all compounds. The experiments were performed three times, and the results of representative experiments are shown.

Compound	R^1	R^2	(IC_{50} ± SD, μM)[a]		
			SGC-7901	A549	HeLa
5a	H	3-fluoro	>30	>30	>30
5b	H	4-methoxy	>30	25.30 ± 2.6	>30
5c	H	2,5-dimethoxy	7.25 ± 0.27	>30	8.97 ± 0.19
5d	H	3,5-dimethoxy	5.62 ± 0.20	4.96 ± 0.29	3.954 ± 0.21
5e	2-fluoro	H	>30	>30	>30
5f	2-fluoro	3-fluoro	27.8 ± 1.6	23.0 ± 1.4	25.6 ± 1.7
5g	2-fluoro	3-chloro	7.09 ± 0.47	8.51 ± 0.39	6.76 ± 0.31
5h	2-fluoro	3-methyl	8.20 ± 0.61	9.17 ± 0.76	10.5 ± 0.64
5i	2-fluoro	3-methoxy	3.76 ± 0.20	4.32 ± 0.14	5.60 ± 0.23
5j	2-fluoro	3-trifluoromethyl	20.1 ± 1.8	>30	8.65 ± 0.50
5k	2-fluoro	4-methoxy	>30	>30	>30
5l	2-fluoro	2,5-dimethoxy	0.92 ± 0.094	0.97 ± 0.088	0.82 ± 0.052
5m	**2-fluoro**	**3,5-dimethoxy**	**0.103 ± 0.019**	**0.276 ± 0.022**	**0.092 ± 0.005**
5n	3-fluoro	H	>30	>30	>30
5o	3-fluoro	3-chloro	15.1 ± 0.87	20.5 ± 1.19	16.6 ± 0.77
5p	3-fluoro	3-methyl	17.6 ± 0.90	14.9 ± 0.67	12.6 ± 0.68
5q	3-fluoro	3-methoxy	6.67 ± 0.24	10.2 ± 0.59	9.13 ± 0.31
5r	3-fluoro	4-methoxy	>30	>30	>30
5s	3-fluoro	2,5-dimethoxy	3.93 ± 0.24	6.67 ± 0.39	3.94 ± 0.11
5t	3-fluoro	3,5-dimethoxy	2.54 ± 0.083	3.07 ± 0.096	0.812 ± 0.018
5u	4-fluoro	H	>30	>30	>30
5v	4-fluoro	3-chloro	>30	>30	>30
5w	4-fluoro	3-methyl	>30	>30	>30
5x	4-fluoro	3-methoxy	>30	>30	>30
5y	4-fluoro	4-methoxy	28.9 ± 2.9	>30	>30
5z	4-fluoro	3,4-dimethyl	>30	>30	>30
5a'	4-fluoro	3,5-dimethoxy	13.7 ± 1.2	19.2 ± 1.4	3.48 ± 0.20
5b'	2-chloro	3-chloro	4.27 ± 0.60	>30	3.93 ± 0.13
5c'	2-chloro	3-methyl	5.11 ± 0.31	10.2 ± 0.43	4.09 ± 0.29
5d'	2-chloro	3-methoxy	7.41 ± 0.72	9.21 ± 0.80	5.72 ± 0.50
5e'	2-chloro	3-trifluoromethyl	18.6 ± 2.4	>30	28.3 ± 3.2
5f'	2-chloro	4-methoxy	>30	>30	>30
5g'	2-chloro	2,5-dimethoxy	4.23 ± 0.54	6.25 ± 0.47	3.34 ± 0.18
5h'	2-chloro	3,5-dimethoxy	0.96 ± 0.10	1.02 ± 0.32	0.89 ± 0.077
5i'	3,4-dichloro	3,5-dimethoxy	>30	>30	26.7
5j'	4-chloro	4-methoxy	>30	>30	>30
5k'	4-chloro	3,5-dimethoxy	>30	>30	>30
CA-4[b]			0.049 ± 0.004	0.080 ± 0.006	0.043 ± 0.002
Colchicine[b]			0.112 ± 0.011	0.124 ± 0.013	0.086 ± 0.008

[a] IC_{50}: the half maximal inhibitory concentration.

[b] Used as positive controls.

2.2.2 Effect on Tubulin Polymerization

In order to shed light on the biological mechanism of this series of compounds, the most potent compound **5m** was selected to examine the antitubulin activity, meanwhile CA-4 was used as positive control and paclitaxel, the first compound known to interact with and stabilize tubulin, was utilized as negative control. As shown in Fig. 3, compound **5m** effectively inhibited tubulin polymerization (IC_{50} = 36.8 μM). The experimental results showed that compound **5m** caused a dose-dependent inhibition of tubulin polymerization; In contrast, paclitaxel, a microtubule-stabilizing agent, could distinctly promote this process. Therefore, the results indicated that compound **5m** was a tubulin inhibitor.

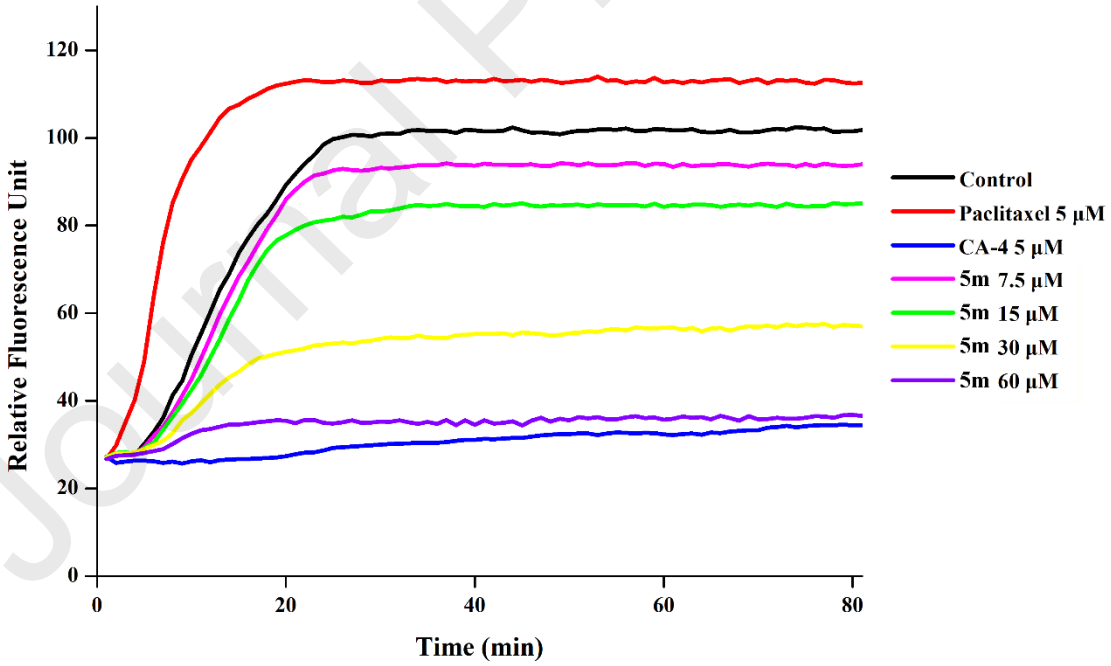


Figure 3. Effects of compound **5m** on tubulin polymerization. Tubulin had been pre-incubated for 1 min with compound **5m** at 7.5 μM, 15 μM, 30 μM and 60 μM, CA-4 at 5 μM, Paclitaxel at 5 μM or

vehicle DMSO at room temperature before GTP was added to start the tubulin polymerization reactions. The reaction was monitored at 37°C.

2.2.3 Analysis of Immunofluorescence Staining

To further investigate the impacts of compound **5m** on intracellular microtubules, HeLa cells were utilized for immunofluorescence assay to directly observe the changes in morphology of microtubules and CA-4 was utilized as reference. As illustrated in Fig. 4, the control cells displayed well-organized microtubule network throughout the cells. After treatment with compound **5m** or CA-4, (at their respective 2-fold IC_{50} concentrations, respectively) microtubules became irregular arrangement and organization, and the microtubule network showed a disruption. The study results suggested that compound **5m** disrupted cytoskeleton similarly to CA-4.

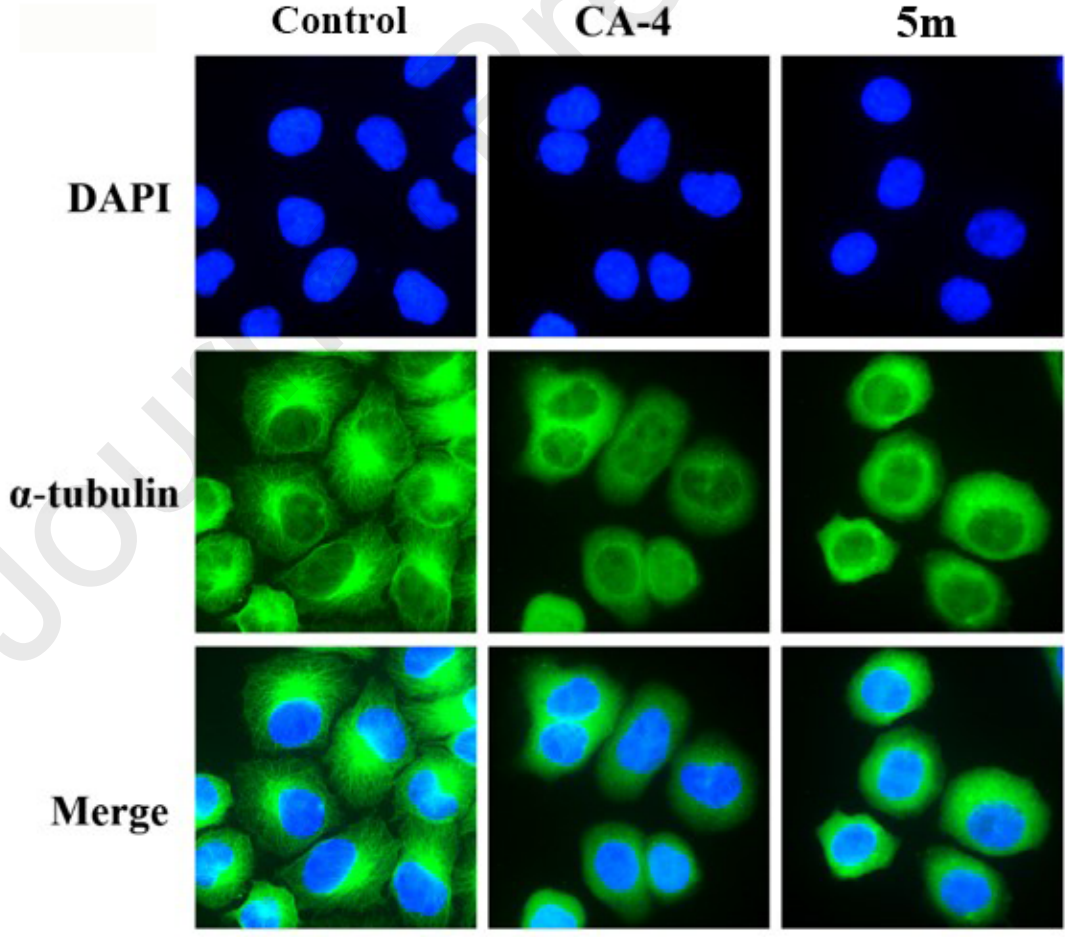


Figure 4. Effects of compound **5m** and CA-4, at their respective 2-fold IC_{50} concentrations, on the cellular microtubule network and microtubule reassemble by immunofluorescence. HeLa cells were treated with compound **5m** or CA-4 for 24 h, and then direct microscopy detection of the fixed and stained cell was performed. The cellular microtubules were stained with anti-a-tubulin-FITC specific antibodies (green). DNA was stained by 4',6-diamidino-2-phenylindole (DAPI, blue).

2.2.4 Cell Cycle Analysis

To evaluate the effects of compound **5m** on the cell mitosis, the effect of compound **5m** on the cell cycle of HeLa cells was analyzed by flow cytometry (Fig. 5) (CA-4 was also comparatively examined as a positive control). Similar as CA-4, compound **5m** arrested the cell cycle in G2/M phase significantly, increasing the percentage of cells in G2/M phase at 12 h. At the same time, polyploidy was induced increasingly by compound **5m**. The proportion of G2/M declined while the proportion of Sub-G1 increased in 24 h, 36 h, 48 h and 72 h. This indicated that part of the cells underwent mitotic catastrophe. Compound **5m** was demonstrated to clearly cause G2/M phase arrest in a time-dependent manner.

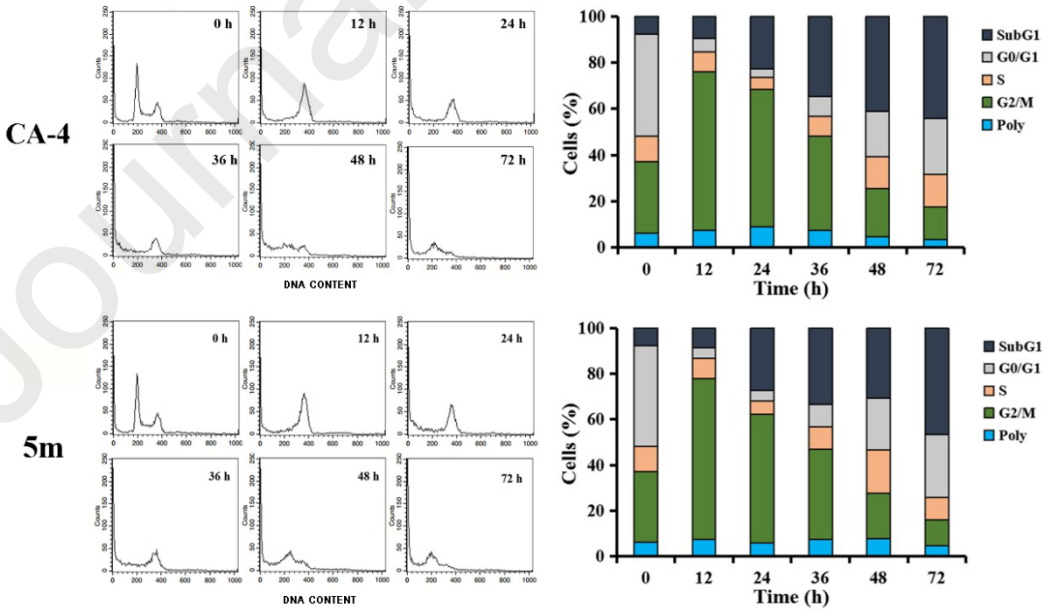


Figure 5. Effects of CA-4 and compound **5m** on cell cycle. HeLa cells lines treated with CA-4 and compound **5m**, at their respective 2-fold IC_{50} concentrations, for 0, 12, 24, 36, 48 and 72 h.

3. Conclusion

Through the detailed analysis of the binding mode of XRP44X and its analogues, we introduced 1,2,3-thiadiazole, a hydrogen-bond acceptor, as the B-ring of XRP44X analogue to design a series of 5-aryl-4-(4-arylpiperazine-1-carbonyl)-1,2,3-thiadiazole derivatives as novel tubulin inhibitors. This work reveals that the B-ring of XRP44X analogues can form additional hydrogen-bond interactions besides foster van der Waals interaction with amino acid residues in P-2 pocket. The most potent compound **5m** (R^1 = 2-fluoro, R^2 = 3,5-dimethoxy) exhibited noteworthy potency against a set of cancer cell lines (IC_{50} value, 0.092-0.276 μM) and significant inhibition of tubulin polymerization. The compound **5m** arrested most cells in the G2/M phase of the cell cycle and disrupted cellular microtubules, thus providing evidence that compound **5m** is a new kind of microtubule-destabilizing agent.

4. Experimental

4.1 Chemistry

4.1.1 Materials and methods

All of reagents and solvents were purchased from chemical company. ^{1}H NMR and ^{13}C NMR spectra were tested in $CDCl_3$ or DMSO-d_6 with TMS as the internal reference on a Bruker AVANCE 400 or 600 (^{1}H at 400 or 600 MHz, ^{13}C at 150 MHz). Mass spectra (MS) were measured on an Agilent 1100-sl mass spectrometer with an electrospray ionisation source from Agilent Co. Ltd. High resolution accurate mass determinations (HRMS) for all of the final target compounds were obtained on a Bruker Micromass Time of Flight mass spectrometer equipped with electrospray ionisation (ESI). TLC analysis was used for determining the extent of reactions under UV light (wavelength: 365 nm and 254 nm). Melting point was measured (uncorrected) on hot-stage microscope (Beijing Taike, X-4). The microwave reactions were carried out in a single

mode cavity microwave synthesizer (CEM Corporation, NC, USA).

4.1.2 General synthetic procedures for arylpiperazines

A solution of arylamines (1 mmol), bis(2- chloroethyl)amine hydrochloride (1.1 mmol) and K_2CO_3 (3 mmol) in *n*-BuOH were stirred at irradiated in a microwave reactor for 30 min at 150°C. The reaction mixture was cooled to room temperature and dissolved in methanol (4 mL), followed by the addition of diethyl ether (150 mL). The precipitate formed was recovered by filtration and washed with diethyl ether to obtain arylpiperazines as HCl salt. The HCl salt was used for the next reaction without further purification [16,17].

*4.1.3 General synthetic procedures for ethyl 2-diazo-3-oxo-3-arylpropanoates **7***

To a solution of ethyl diazoacetate (1.2 mmol) in DMSO at room temperature were added in succession DBU (0.1 mmol), corresponding aromatic aldehydes (1 mmol), and a solution of IBX (2 mmol) in DMSO. After being stirred for 10 h at room temperature, the reaction mixture was quenched with aqueous $NaHCO_3$ and then extracted with ethyl acetate (3 × 20 mL), the combined organic layers were copiously washed with aqueous $NaHCO_3$ and finally with water, and the mixture was then dried with Na_2SO_4, filtered, and concentrated in vacuo. The resulting crude product purified by flash column chromatography on silica gel (ethyl acetate/petroleum ether = 1:3) afforded the pure products [12].

*4.1.4 General synthetic procedures for ethyl 5-aryl-1,2,3-thiadiazole-4-carboxylates (**8**)*

A solution of various ethyl 2-diazo-3-oxo-3-arylpropanoates (*7*) (1 mmol) and Lawesson's reagent (1.2 mmol) in toluene were heated at reflux for 4 hours, then cooled to room temperature and concentrated in vacuo. The resulting oil was purified by

column chromatography (ethyl acetate/petroleum ether = 1:2) to give the pure products.

*4.1.4.1 Ethyl 5-phenyl-1,2,3-thiadiazole-4-carboxylate (**8a**)*

Yellow Solid; yield: 56%; ^{1}H NMR (600 MHz, $CDCl_3$): δ =7.51 (5H, m), 4.42 (2H, q, J = 6.9 Hz), 1.34 (3H, t, J = 7.0 Hz). ESI-MS: m/z = 235.0 $[M+H]^+$.

*4.1.4.2 Ethyl 5-(2-fluorophenyl)-1,2,3-thiadiazole-4-carboxylate (**8b**)*

Red Solid; yield: 62%; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.53 (1H, m), 7.45 (1H, m), 7.28 (1H, m), 7.23 (1H, t, J = 8.9 Hz), 4.42 (2H, q, J = 6.9 Hz), 1.31 (3H, t, J = 7.1 Hz). ESI-MS: m/z = 253.1 $[M+H]^+$.

*4.1.4.3 Ethyl 5-(3-fluorophenyl)-1,2,3-thiadiazole-4-carboxylate (**8c**)*

Light yellow Solid; yield: 50%; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.47 (1H, m), 7.30 (2H, m), 7.23 (1H, m), 4.44 (2H, q, J = 6.9 Hz), 1.36 (3H, t, J = 7.3 Hz). ESI-MS: m/z = 253.0 $[M+H]^+$.

*4.1.4.4 Ethyl 5-(4-fluorophenyl)-1,2,3-thiadiazole-4-carboxylate (**8d**)*

Yellow Solid; yield: 60%; ^{1}H NMR (600 MHz, $CDCl_3$): δ =7.55 (2H, q), 7.19 (2H, t, J = 9.5 Hz), 4.44 (2H, q, J = 7.2 Hz), 1.37 (3H, t, J = 7.1 Hz). ESI-MS: m/z = 253.1 $[M+H]^+$.

*4.1.4.5 Ethyl 5-(2-chlorophenyl)-1,2,3-thiadiazole-4-carboxylate (**8e**)*

Light yellow Solid; yield: 66%; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.54 (1H, d, J = 7.9 Hz), 7.47 (1H, m), 7.38 (2H, m), 4.35 (2H, q, J = 7.3 Hz), 1.24 (3H, t, J = 7.0 Hz). ESI-MS: m/z = 269.0 $[M+H]^+$.

*4.1.4.6 Ethyl 5-(3,4-dichlorophenyl)-1,2,3-thiadiazole-4-carboxylate (**8f**)*

Light yellow Solid; yield: 67%; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.66 (1H, d, J = 2.1 Hz), 7.57 (1H, d, J = 8.4 Hz), 7.39 (1H, dd, J = 2.1, Hz, J = 8.2 Hz), 4.45 (2H, q, J = 7.4 Hz), 1.38 (3H, t, J = 7.1 Hz). ESI-MS: m/z = 302.9 $[M+H]^+$.

*4.1.4.7 Ethyl 5-(4-chlorophenyl)-1,2,3-thiadiazole-4-carboxylate (**8g**)*

Light yellow Solid; yield: 69%; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.48 (4H, q, J = 9.3 Hz), 4.44 (2H, q, J = 7.3 Hz), 1.37 (3H, t, J = 7.1 Hz). ESI-MS: m/z = 269.0 $[M+H]^+$.

*4.1.5 General synthetic procedures for (5-aryl-1,2,3-thiadiazol-4-yl)(4-arylpiperazin-1-yl)methanones (**5**)*

To a solution of arylpiperazines (0.1 mmol) in anhydrous DCM was added trimethylaluminum (0.5 ml, 1 M in heptane). The reaction was stirred at RT under N_2 for 15 min. A solution of an appropriate ethyl 5-aryl-1,2,3-thiadiazole-4-carboxylates (**8**) (0.1 mmol) in anhydrous DCM was added and the reaction was stirred at RT under N_2 for 16 h. The reaction was quenched with 5 ml of 1 M HCl and diluted with DCM. The combined organic layer was washed with water and brine and then dried over Na_2SO_4, filtered and concentrated to yield the crude product. The crude product was purified by column chromatography (*n*-hexane/ EtOAc = 1:1) on silica gel to afford pure products.

*4.1.5.1 5-Phenyl-4-(4-(3-fluorophenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5a**)*

Yellow Solid; yield: 61%; Mp: 46.9-48.6°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.52 (2H, m), 7.41 (3H, m), 7.12 (1H, q), 6.55 (1H, dd, J = 1.9 Hz, J = 8.8 Hz), 6.50 (1H, m), 6.46 (1H, m), 3.92 (2H, t, J = 5.2 Hz), 3.33 (2H, t, J = 5.1 Hz), 3.19 (2H, t, J = 5.2 Hz), 2.87 (2H, t, J = 5.3 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 162.7 (d, J = 246.7 Hz), 160.3, 154.6, 151.3 (d, J = 10.1 Hz), 150.5, 129.9, 129.3 (d, J = 8.0 Hz), 128.5 (2C), 128.0 (2C), 125.2, 110.8 (d, J = 2.0 Hz), 105.9 (d, J = 21.2 Hz), 102.5 (d, J = 24.7 Hz), 48.0, 47.8, 45.7, 41.0; HRMS calcd for $C_{19}H_{17}FN_4NaOS$ $[M+Na]^+$ 391.1005, found 391.1009.

*4.1.5.2 5-Phenyl-4-(4-(4-methoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5b**)*

Yellow Solid; yield: 52%; Mp: 66.5-68.2°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.59 (2H, m), 7.48 (3H, m), 6.83 (4H, s), 4.00 (2H, t, J = 4.7 Hz), 3.76 (3H, s), 3.39 (2H, s),

3.12 (2H, t, J = 5.2 Hz), 2.81 (2H, s). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 160.3, 154.4, 153.5, 150.7, 143.9, 129.9, 128.5 (2C), 128.0 (2C), 125.3, 118.1 (2C), 113.5 (2C), 54.5, 50.1, 50.0, 46.1, 41.4; HRMS calcd for $C_{20}H_{20}N_4NaO_2S$ $[M+Na]^+$ 403.1205, found 403.1219.

*4.1.5.3 5-Phenyl-4-(4-(2,5-dimethoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5c**)*

Deepred Solid; yield: 52%; Mp: 36.3-37.9°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.59 (2H, m), 7.49 (3H, t, J = 6.1 Hz), 6.77 (1H, d, J = 8.1 Hz), 6.51 (1H, dd, J = 2.7 Hz, J = 8.4 Hz), 6.40 (1H, d, J = 3.0 H), 4.02 (2H, t, J = 5.1 Hz), 3.80 (3H, s), 3.75 (3H, s), 3.39 (2H, t, J = 5.1 Hz), 3.10 (2H, t, J = 5.2 Hz), 2.78 (2H, t, J = 4.9 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.4, 155.1, 154.0, 151.8, 146.5, 141.3, 130.9, 129.6 (2C), 129.0 (2C), 126.3, 111.9, 106.5, 106.0, 55.9, 55.6, 50.5, 50.2, 47.3, 42.5; HRMS calcd for $C_{21}H_{22}N_4NaO_3S$ $[M+Na]^+$ 433.1310, found 433.1309.

*4.1.5.4 5-Phenyl-4-(4-(3,5-dimethoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5d**)*

Brown Black Solid; yield: 47%; Mp: 50.7-52.2°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.58 (2H, m), 7.48 (3H, d, J = 7.1 Hz), 6.05 (1H, t, J = 2.2 Hz), 6.02 (2H, d, J = 2.2 Hz), 3.98 (2H, t, J = 5.0 Hz), 3.76 (6H, s), 3.38 (2H, t, J = 5.4 Hz), 3.24 (2H, t, J = 5.4 Hz), 2.92 (2H, t, J = 5.4 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 160.5 (2C), 160.3, 154.5, 151.6, 150.6, 129.9, 128.5 (2C), 128.0 (2C), 125.2, 94.7 (2C), 91.4, 54.3 (2C), 51.4, 49.9, 45.8, 41.2; HRMS calcd for $C_{21}H_{22}N_4NaO_3S$ $[M+Na]^+$ 433.1310, found 433.1311.

*4.1.5.5 5-(2-Fluorophenyl)-4-(4-phenylpiperazine-1-carbonyl)-1,2,3-thiadiazol (**5e**)*

White Solid; yield: 57%; Mp: 62.1-63.4°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.56 (1H, m), 7.42 (1H, m), 7.20 (4H, m), 6.85 (3H, m), 3.92 (2H, t, J = 5.4 Hz), 3.94 (2H, t, J =

5.3 Hz), 3.23 (2H, t, *J* = 5.3 Hz), 3.03 (2H, t, *J* = 5.1 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 160.2, 157.8 (d, *J* = 258.9 Hz), 152.3, 149.7, 148.0 (d, *J* = 2.2 Hz), 131.7 (d, *J* = 8.6 Hz), 129.7, 128.3 (2C), 124.1 (d, *J* = 3.2 Hz), 119.7, 115.8 (2C), 115.5 (d, *J* = 22.2 Hz), 113.8 (d, *J* = 14.1 Hz), 48.7, 48.5, 46.1, 41.3; HRMS calcd for $C_{19}H_{17}FN_4NaOS$ $[M+Na]^+$ 391.1005, found 391.1012.

*4.1.5.6 5-(2-Fluorophenyl)-4-(4-(3-fluorophenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5f**)*

Yellow Solid; yield: 59%; Mp: 53.2-55.1°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.62 (1H, m), 7.49 (1H, m), 7.25 (3H, m), 6.67 (1H, dd, *J* = 1.9 Hz, *J* = 8.8 Hz), 6.59 (2H, m), 4.00 (2H, t, *J* = 5.2 Hz), 3.60 (2H, t, *J* = 5.3 Hz), 3.31 (2H, t, *J* = 5.4 Hz), 3.13 (2H, t, *J* = 5.1 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 162.7 (d, *J* = 250.8 Hz), 160.2, 157.8 (d, *J* = 254.8 Hz), 152.2, 151.4 (d, *J* = 8.6 Hz), 148.3 (d, *J* = 2.2 Hz), 131.8 (d, *J* = 7.0 Hz), 129.8, 129.3 (d, *J* = 9.1 Hz), 124.1 (d, *J* = 3.0 Hz), 115.5 (d, *J* = 20.2 Hz), 113.8 (d, *J* = 13.1 Hz), 110.8 (d, *J* = 2.3 Hz), 105.9 (d, *J* = 21.2 Hz), 102.4 (d, *J* = 23.2 Hz), 48.1, 47.8, 45.9, 41.1; HRMS calcd for $C_{19}H_{16}F_2N_4NaOS$ $[M+Na]^+$ 409.0911, found 409.0916.

*4.1.5.7 5-(2-Fluorophenyl)-4-(4-(3-chlorophenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5g**)*

Light yellow Solid; yield: 54%; Mp: 70.9-72.9°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.62 (1H, m), 7.50 (1H, m), 7.27 (2H, m), 7.19 (1H, t, *J* = 8.5 Hz), 6.88 (2H, d, *J* = 7.3 Hz), 6.79 (1H, d, *J* = 8.8 Hz), 4.01 (2H, t, *J* = 5.2 Hz), 3.60 (2H, t, *J* = 5.1 Hz), 3.31 (2H, t, *J* = 5.3 Hz), 3.13 (2H, t, *J* = 5.3 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 160.2, 157.8 (d, *J* = 254.8 Hz), 152.2, 150.8, 148.4 (d, *J* = 2.2 Hz), 134.1, 131.8 (d, *J* = 8.0 Hz), 129.8, 129.2, 124.1 (d, *J* = 3.2 Hz), 119.4, 115.6, 115.5 (d, *J* = 22.2 Hz), 113.8 (d, *J* = 14.1 Hz), 113.6, 48.2, 48.0, 45.9, 41.1; HRMS calcd for $C_{19}H_{16}ClFN_4NaOS$

$[M+Na]^+$ 425.0615, found 425.0623.

*4.1.5.8 5-(2-Fluorophenyl)-4-(4-(3-methylphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5h**)*

Light yellow Solid; yield: 58%; Mp: 34.7-36.5°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.63 (1H, m), 7.50 (1H, m), 7.28 (1H, m), 7.24 (1H, d, J = 7.6 Hz), 7.19 (1H, t, J = 7.8 Hz), 6.79 (3H, s), 4.04 (2H, s), 3.62 (2H, s), 3.31 (2H, s), 3.13 (2H, s), 2.33 (3H, s). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 160.2, 157.9 (d, J = 245.9 Hz), 152.3, 148.1, 138.1, 131.7 (d, J = 9.2 Hz), 129.7, 128.9 (d, J = 7.1 Hz), 128.1, 124.1 (d, J = 4.1 Hz), 120.9, 116.8, 115.5 (d, J = 23.5 Hz), 113.8 (d, J = 13.0 Hz), 113.1, 48.9 (2C), 46.0, 41.2, 20.7; HRMS calcd for $C_{20}H_{19}FN_4NaOS$ $[M+Na]^+$ 405.1161, found 405.1176.

*4.1.5.9 5-(2-Fluorophenyl)-4-(4-(3-methoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5i**)*

Light yellow Solid; yield: 56%; Mp: 131.5-133.4°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.62 (1H, m), 7.48 (1H, m), 7.26 (2H, m), 7.19 (1H, d, J = 8.8 Hz), 6.53 (1H, dd, J = 2.3 Hz, J = 8.1 Hz), 6.46 (2H, m), 4.00 (2H, t, J = 5.2 Hz), 3.79 (3H, s), 3.57 (2H, t, J = 5.1 Hz), 3.29 (2H, t, J = 5.3 Hz), 3.10 (2H, t, J = 5.3 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.2, 160.6, 158.9 (d, J = 251.1 Hz), 153.3, 152.1, 149.1 (d, J = 3.2 Hz), 132.8 (d, J = 10.0 Hz), 130.7, 130.0, 125.1 (d, J = 3.8 Hz), 116.5 (d, J = 21.6 Hz), 114.8 (d, J = 14.2 Hz), 109.4, 105.4, 103.3, 55.2, 49.6, 49.4, 47.1, 42.3; HRMS calcd for $C_{20}H_{19}FN_4NaO_2S$ $[M+Na]^+$ 421.1110, found 421.1125.

*4.1.5.10 5-(2-Fluorophenyl)-4-(4-(3-(trifluoromethyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5j**)*

Brown Solid; yield: 49%; Mp: 42.9-43.5°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.62 (1H, m), 7.50 (1H, m), 7.39 (1H, t, J = 8.2 Hz), 7.28 (1H, m), 7.24 (1H, m), 7.16 (1H, d, J = 7.6 Hz), 7.13 (1H, s), 7.11 (1H, d, J = 8.5 Hz), 4.04 (2H, t, J = 5.3 Hz), 3.65 (2H, t, J =

5.3 Hz), 3.36 (2H, t, *J* = 5.4 Hz), 3.19 (2H, t, *J* = 5.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.2, 158.8 (d, *J* = 245.7 Hz), 153.1, 150.8, 149.5 (d, *J* = 3.0 Hz), 132.8 (d, *J* = 9.2 Hz), 131.5, 130.9, 129.8, 125.2 (d, *J* = 3.1 Hz), 123.2, 119.7, 117.1 (d, *J* = 3.0 Hz), 116.5 (d, *J* = 21.5 Hz), 114.8 (d, *J* = 13.3 Hz), 113.1 (d, *J* = 4.0 Hz), 49.3, 49.1, 46.9, 42.2; HRMS calcd for $C_{20}H_{16}F_4N_4NaOS$ $[M+Na]^+$ 459.0879, found 459.0882.

*4.1.5.11 5-(2-Fluorophenyl)-4-(4-(4-methoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5k**)*

Brown Solid; yield: 47%; Mp: 53.7-55.2°C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 7.67 (1H, m), 7.62 (1H, m), 7.48 (1H, t, *J* = 9.6 Hz), 7.41 (1H, t, *J* = 7.9 Hz), 6.89 (2H, d, *J* = 7.9 Hz), 6.83 (2H, d, *J* = 8.4 Hz), 3.84 (2H, t, *J* = 5.6 Hz), 3.68 (3H, s), 3.41 (2H, t, *J* = 5.2 Hz), 3.07 (2H, t, *J* = 5.1 Hz), 2.79 (2H, t, *J* = 5.0 Hz). ^{13}C NMR (150 MHz, DMSO-d_6): δ = 160.8, 158.9 (d, *J* = 249.7 Hz), 153.9, 148.2, 148.2, 145.3, 133.7 (d, *J* = 9.2 Hz), 131.2, 130.1 (d, *J* = 7.1 Hz), 126.0 (d, *J* = 3.5 Hz), 118.6 (2C), 117.0 (d, *J* = 22.0 Hz), 114.8 (2C), 54.6, 50.5, 50.2, 47.0, 42.2; HRMS calcd for $C_{20}H_{19}FN_4NaO_2S$ $[M+Na]^+$ 421.1110, found 421.1121.

*4.1.5.12 5-(2-Fluorophenyl)-4-(4-(2,5-dimethoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5l**)*

Deepred Solid; yield: 51%; Mp: 46.2-7-47.1°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.64 (1H, m), 7.50 (1H, m), 7.26 (2H, m), 6.79 (1H, d, *J* = 9.5 Hz), 6.53 (1H, d, *J* = 8.8 Hz), 6.48 (1H, s), 4.03 (2H, t, *J* = 5.0 Hz), 3.82 (3H, s), 3.76 (3H, s), 3.57 (2H, t, *J* = 5.2 Hz), 3.15 (2H, t, *J* = 5.1 Hz), 2.96 (2H, t, *J* = 4.9 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.3, 158.9 (d, *J* = 245.7 Hz), 154.0, 153.4, 148.6, 146.5, 141.5, 132.7 (d, *J* = 8.7 Hz), 130.6, 125.1 (d, *J* = 3.0 Hz), 116.5 (d, *J* = 21.5 Hz), 114.9 (d, *J* = 13.3 Hz), 111.9, 106.6, 106.0, 55.9, 55.6, 50.6, 50.3, 47.4, 42.5; HRMS calcd for $C_{21}H_{21}FN_4NaO_3S$ $[M+Na]^+$ 451.1216, found 451.1221.

*4.1.5.13 5-(2-Fluorophenyl)-4-(4-(3,5-dimethoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5m**)*

Brown Solid; yield: 55%; Mp: 91.5-93.4°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.62 (1H, t, J = 7.4 Hz), 7.49 (1H, m), 7.25 (2H, m), 6.07 (3H, d, J = 5.7 Hz), 3.99 (2H, t, J = 5.2 Hz), 3.77 (6H, s), 3.56 (2H, t, J = 5.1 Hz), 3.28 (2H, t, J = 4.8 Hz), 3.08 (2H, t, J = 5.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.5 (2C), 160.2, 157.8 (d, J = 245.7 Hz), 152.2, 151.7, 148.0, 131.8 (d, J = 9.2 Hz), 129.7, 124.1 (d, J = 2.6 Hz), 115.5 (d, J = 21.5 Hz), 113.8 (d, J = 14.8 Hz), 94.7 (2C), 91.4, 54.3 (2C), 48.6, 48.4, 46.0, 41.2; HRMS calcd for $C_{21}H_{21}FN_4NaO_3S$ $[M+Na]^+$ 451.1216, found 451.1223.

*4.1.5.14 5-(3-Fluorophenyl)-4-(4-phenylpiperazine-1-carbonyl)-1,2,3-thiadiazol (**5n**)*

Yellow Solid; yield: 59%; Mp: 92.3-94.0°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.45 (1H, m), 7.38 (1H, d, J = 7.6 Hz), 7.34 (1H, dd, J = 2.3 Hz, J = 9.2 Hz), 7.27 (2H, t, J = 8.4 Hz), 7.19 (1H, m), 6.91 (3H, m), 4.02 (2H, t, J = 5.4 Hz), 3.46 (2H, t, J = 5.2 Hz), 3.27 (2H, t, J = 5.4 Hz), 3.02 (2H, t, J = 5.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.8 (d, J = 254.8 Hz), 159.9, 153.2 (d, J = 3.0 Hz), 151.0, 149.6, 130.2 (d, J = 10.1 Hz), 128.3 (2C), 127.1 (d, J = 7.0 Hz), 124.0 (d, J = 2.5 Hz), 119.9, 116.9 (d, J = 21.2 Hz), 115.8 (2C), 115.1 (d, J = 23.2 Hz), 48.8, 48.5, 46.0, 41.3; HRMS calcd for $C_{19}H_{17}FN_4NaOS$ $[M+Na]^+$ 391.1005, found 391.1014.

*4.1.5.15 5-(3-Fluorophenyl)-4-(4-(3-chlorophenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5o**)*

Yellow Solid; yield: 60%; Mp: 69.3-70.7°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.39 (1H, q), 7.31 (1H, d, J = 8.4 Hz), 7.27 (1H, d, J = 9.3 Hz), 7.12 (2H, m), 6.79 (2H, t), 6.69 (1H, d, J = 8.4 Hz), 3.94 (2H, t, J = 5.1 Hz), 3.40 (2H, t, J = 5.2 Hz), 3.21 (2H, t, J = 5.2 Hz), 2.98 (2H, t, J = 5.4 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.8 (d, J = 258.9 Hz), 159.9, 153.53 (d, J = 3.0 Hz), 150.9, 150.7, 134.1, 130.2 (d, J = 8.1 Hz),

129.2, 127.0, 124.0 (d, J = 3.4 Hz), 119.5, 116.9 (d, J = 22.2 Hz), 115.6, 115.2 (d, J = 24.3 Hz), 113.7, 48.3, 48.0, 45.9, 41.2; HRMS calcd for $C_{19}H_{16}ClFN_4NaOS$ $[M+Na]^+$ 425.0615, found 425.0620.

*4.1.5.16 5-(3-Fluorophenyl)-4-(4-(3-methylphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5p**)*

Yellow Solid; yield: 61%; Mp: 119.8-121.0°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.45 (1H, m), 7.38 (1H, m), 7.34 (1H, m), 7.17 (2H, m), 6.72 (3H, m), 4.01 (2H, t, J = 5.2 Hz), 3.44 (2H, t, J = 5.3 Hz), 3.25 (2H, t, J = 5.4 Hz), 3.01 (2H, t, J = 5.2 Hz), 2.31 (3H, s). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.8 (d, J = 253.8 Hz), 159.9, 153.2 (d, J = 3.1 Hz), 151.0, 149.7, 138.0, 130.2 (d, J = 8.7 Hz), 128.1, 127.1 (d, J = 8.7 Hz), 123.9 (d, J = 3.5 Hz), 120.7, 116.9 (d, J = 20.9 Hz), 116.7, 115.1 (d, J = 23.7 Hz), 112.9, 48.9, 48.6, 46.1, 41.4, 20.7; HRMS calcd for $C_{20}H_{19}FN_4NaOS$ $[M+Na]^+$ 405.1161, found 405.1176.

*4.1.5.17 5-(3-Fluorophenyl)-4-(4-(3-methoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5q**)*

Yellow Solid; yield: 64%; Mp: 123.6-124.8°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.45 (1H, m), 7.38 (1H, d, J = 8.1 Hz), 7.34 (1H, m), 7.19 (2H, m), 6.50 (1H, dd, J = 2.3 Hz, J = 8.2 Hz), 6.47 (1H, dd, J = 2.3 Hz, J = 8.1 Hz), 6.42 (1H, t), 4.00 (2H, t, J = 5.2 Hz), 3.78 (3H, s), 3.44 (2H, t, J = 5.1 Hz), 3.27 (2H, t, J = 5.2 Hz), 3.02 (2H, t, J = 5.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.8 (d, J = 251.1 Hz), 159.9, 159.6, 153.3, 153.3, 151.0, 130.2 (d, J = 8.2 Hz), 129.0, 127.1 (d, J = 8.2 Hz), 123.9 (d, J = 3.4 Hz), 117.0 (d, J = 21.3 Hz), 115.1 (d, J = 23.1 Hz), 108.4, 104.5, 102.3, 54.2, 48.7, 48.4, 46.0, 41.3; HRMS calcd for $C_{20}H_{19}FN_4NaO_2S$ $[M+Na]^+$ 421.1110, found 421.1130.

*4.1.5.18 5-(3-Fluorophenyl)-4-(4-(4-methoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5r**)*

Yellow Solid; yield: 60%; Mp: 46.3-47.6°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.56 (1H, m), 7.49 (1H, d, J = 7.4 Hz), 7.45 (1H, m), 7.30 (1H, m), 6.98 (2H, d, J = 8.4 Hz), 6.94 (2H, d, J = 9.1 Hz), 4.12 (2H, t, J = 5.2 Hz), 3.87 (3H, s), 3.55 (2H, s), 3.25 (2H, t, J = 4.7 Hz), 3.01 (2H, s). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.8 (d, J = 262.0 Hz), 159.9, 153.6, 153.2, 151.0, 143.9, 130.2 (d, J = 7.6 Hz), 127.1 (d, J = 9.2 Hz), 124.0 (d, J = 3.3 Hz), 118.2 (2C), 116.9 (d, J = 21.5 Hz), 115.1 (d, J = 23.5 Hz), 113.5 (2C), 54.5, 50.3, 50.0, 46.2, 41.8; HRMS calcd for $C_{20}H_{19}FN_4NaO_2S$ $[M+Na]^+$ 421.1110, found 421.1121.

*4.1.5.19 5-(3-Fluorophenyl)-4-(4-(2,5-dimethoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5s**)*

Brown Solid; yield: 51%; Mp: 35.2-37.1°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.46 (1H, q), 7.38 (1H, d, J = 8.1 Hz), 7.34 (1H, d, J = 9.5 Hz), 7.20 (1H, m), 6.78 (1H, d, J = 8.4 Hz), 6.52 (1H, dd, J = 2.7 H, J = 8.4 Hz), 6.4 (1H, d, J = 2.0 Hz), 4.03 (2H, t, J = 5.0 Hz), 3.81 (3H, s), 3.75 (3H, s), 3.44 (2H, t, J = 5.4 Hz), 3.13 (2H, t, J = 5.0 Hz), 2.88 (2H, t, J = 4.7 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.8 (d, J = 245.7 Hz), 160.0, 153.0, 152.9, 151.2, 145.5, 140.2, 130.2 (d, J = 8.2 Hz), 127.1 (d, J = 9.2 Hz), 123.9 (d, J = 3.0 Hz), 116.9 (d, J = 20.5 Hz), 115.1 (d, J = 23.5 Hz), 110.9, 105.5, 105.1, 54.9, 54.6, 49.7, 49.3, 46.3, 41.5; HRMS calcd for $C_{21}H_{21}FN_4NaO_3S$ $[M+Na]^+$ 451.1216, found 451.1216.

*4.1.5.20 5-(3-Fluorophenyl)-4-(4-(3,5-dimethoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5t**)*

Brown Solid; yield: 42%; Mp: 46.8-48.2°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.46 (1H, m), 7.38 (1H, m), 7.34 (1H, m), 7.20 (1H, m), 6.05 (3H, q), 3.99 (2H, t, J = 5.4 Hz), 3.76 (6H, s), 3.43 (2H, t, J = 5.4 Hz), 3.25 (2H, t, J = 5.4 Hz), 3.00 (2H, t, J = 4.7 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.8 (d, J = 257.9 Hz), 160.5 (2C), 159.9, 153.3,

151.6, 150.9, 130.2 (d, J = 8.1 Hz), 127.0 (d, J = 9.4 Hz), 123.9 (d, J = 3.5 Hz), 116.9 (d, J = 21.5 Hz), 115.1 (d, J = 23.7 Hz), 94.7 (2C), 91.5, 54.2 (2C), 48.7, 48.4, 45.9, 41.3; HRMS calcd for $C_{21}H_{21}FN_4NaO_3S$ $[M+Na]^+$ 451.1216, found 451.1211.

*4.1.5.21 5-(4-Fluorophenyl)-4-(4-phenylpiperazine-1-carbonyl)-1,2,3-thiadiazol (**5u**)*

Yellow Solid; yield: 70%; Mp: 53.5-54.8°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.61 (2H, m), 7.28 (2H, m), 7.17 (2H, t, J = 8.8 Hz), 6.91 (3H, m), 4.01 (2H, t, J = 5.3 Hz), 3.46 (2H, t, J = 5.2 Hz), 3.27 (2H, t, J = 5.1 Hz), 3.04 (2H, t, J = 5.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 163.1 (d, J = 253.9 Hz), 160.1, 153.8, 150.6, 149.6, 130.2 (d, J = 9.2 Hz) (2C), 128.3 (2C), 121.4 (d, J = 3.2 Hz), 119.9, 115.9 (d, J = 4.6 Hz) (2C), 115.7 (2C), 48.9, 48.6, 46.1, 41.3; HRMS calcd for $C_{19}H_{17}FN_4NaOS$ $[M+Na]^+$ 391.1005, found 391.1014.

4.1.5.22 5-(4-Fluorophenyl)-4-(4-(3-chlorophenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (5v)

Yellow Solid; yield: 59%; Mp: 37.1-39.1°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.61 (2H, m), 7.18 (3H, t, J = 7.6 Hz), 6.86 (2H, t), 6.76 (1H, d, J = 9.0 Hz), 4.00 (2H, t, J = 5.0 Hz), 3.47 (2H, t, J = 5.3 Hz), 3.28 (2H, t, J = 5.2 Hz), 3.06 (2H, t, J = 5.1 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 163.1 (d, J = 242.7 Hz), 160.1, 154.1, 150.7, 150.5, 134.1, 130.2 (d, J = 9.1 Hz) (2C), 129.2, 121.4 (d, J = 4.5 Hz), 119.5, 115.9, 115.7 (d, J = 10.1 Hz) (2C), 113.6, 48.4, 48.0, 45.9, 41.2; HRMS calcd for $C_{19}H_{16}ClFN_4NaOS$ $[M+Na]^+$ 425.0615, found 425.0629.

*4.1.5.23 5-(4-Fluorophenyl)-4-(4-(3-methylphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5w**)*

Yellow Solid; yield: 61%; Mp: 112.6-114.1°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.61 (2H, m), 7.16 (3H, q), 6.72 (3H, m), 4.00 (2H, t, J = 5.3 Hz), 3.44 (2H, t, J = 5.2 Hz), 3.25 (2H, t, J = 5.1 Hz), 3.02 (2H, t, J = 5.3 Hz), 2.31 (3H, s). ^{13}C NMR (150 MHz,

$CDCl_3$): δ = 163.1 (d, J = 255.9 Hz), 160.1, 153.7, 150.7, 149.7, 138.0, 130.2 (d, J = 8.2 Hz) (2C), 128.1, 121.4 (d, J = 3.8 Hz), 120.8, 116.7, 115.8 (d, J = 21.9 Hz) (2C), 113.0, 48.9, 48.6, 46.1, 41.4, 20.7; HRMS calcd for $C_{20}H_{19}FN_4NaOS$ $[M+Na]^+$ 405.1161, found 405.1183.

*4.1.5.24 5-(4-Fluorophenyl)-4-(4-(3-methoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5x**)*

Yellow Solid; yield: 65%; Mp: 81.6-82.7°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.61 (2H, m), 7.18 (3H, m), 6.50 (1H, dd, J = 2.3 Hz, J = 8.2 Hz), 6.47 (1H, dd, J = 2.3 Hz, J = 8.4 Hz), 6.42 (1H, m), 4.00 (2H, t, J = 5.2 Hz), 3.78 (3H, s), 3.45 (2H, t, J = 5.3 Hz), 3.27 (2H, t, J = 5.4 Hz), 3.03 (2H, t, J = 5.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 163.1 (d, J = 251.1 Hz), 160.1, 159.6, 153.8, 151.0, 150.6, 130.2 (d, J = 8.9 Hz) (2C), 129.0, 121.4 (d, J = 3.2 Hz), 115.8 (d, J = 22.5 Hz) (2C), 108.5, 104.5, 102.3, 54.2, 48.7, 48.41, 46.0, 41.3; HRMS calcd for $C_{20}H_{19}FN_4NaO_2S$ $[M+Na]^+$ 421.1110, found 421.1121.

*4.1.5.25 5-(4-Fluorophenyl)-4-(4-(4-methoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5y**)*

Yellow Solid; yield: 67%; Mp: 62.3-63.7°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.61 (2H, m), 7.18 (2H, t, J = 8.8 Hz), 6.85 (4H, m), 4.00 (2H, s), 3.77 (3H, s), 3.45 (2H, s), 3.14 (2H, s), 2.91 (2H, s). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 163.1 (d, J = 253.9 Hz), 160.1, 153.7, 153.6, 150.7, 143.9, 130.2 (d, J = 10.2 Hz) (2C), 121.4 (d, J = 3.6 Hz), 118.1 (2C), 115.8 (d, J = 22.5 Hz) (2C), 116.5 (2C), 54.5, 50.3, 50.0, 46.2, 41.5; HRMS calcd for $C_{20}H_{19}FN_4NaO_2S$ $[M+Na]^+$ 421.1110, found 421.1119.

*4.1.5.26 5-(4-Fluorophenyl)-4-(4-(3,4-dimethylphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5z**)*

Yellow Solid; yield: 66%; Mp: 82.9-84.0°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.61

(2H, m), 7.17 (2H, t, *J* = 8.5 Hz), 7.03 (1H, d, *J* = 8.5 Hz), 6.70 (2H, d, *J* = 43.7 Hz), 4.01 (2H, s), 3.45 (2H, s), 3.21 (2H, s), 2.98 (2H, s), 2.23 (3H, s), 2.19 (3H, s). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 164.1 (d, *J* = 261.6 Hz), 161.6, 154.8, 151.7, 148.9, 137.5, 131.2 (d, *J* = 8.9 Hz) (2C), 130.3 (2C), 122.4 (d, *J* = 3.8 Hz), 119.0, 116.8 (d, *J* = 20.7 Hz) (2C), 114.7, 50.5, 50.3, 47.1, 42.4, 20.2, 18.9; HRMS calcd for $C_{21}H_{21}FN_4NaOS$ $[M+Na]^+$ 419.1318, found 419.1327.

*4.1.5.27 5-(4-Fluorophenyl)-4-(4-(3,5-dimethoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5a'**)*

Light brown Solid; yield: 54%; Mp: 55.5-57.2°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.53 (2H, m), 7.10 (2H, t, *J* = 8.8 Hz), 5.97 (3H, m), 3.91 (2H, t, *J* = 5.1 Hz), 3.69 (6H, s), 3.36 (2H, t, *J* = 5.0 Hz), 3.18 (2H, t, *J* = 4.7 Hz), 2.94 (2H, t, *J* = 5.0 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 163.1 (d, *J* = 250.2 Hz), 160.5 (2C), 160.1, 153.8, 151.6, 150.6, 130.2 (d, *J* = 8.5 Hz) (2C), 121.4 (d, *J* = 5.2 Hz), 115.8 (d, *J* = 22.2 Hz) (2C), 94.7 (2C), 91.4, 54.3 (2C), 48.7, 48.4, 45.9, 41.3; HRMS calcd for $C_{21}H_{21}FN_4NaO_3S$ $[M+Na]^+$ 451.1216, found 451.1218.

*4.1.5.28 5-(2-Chlorophenyl)-4-(4-(3-chlorophenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5b'**)*

Brown Solid; yield: 63%; Mp: 96.3-97.4°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.53 (2H, m), 7.44 (1H, m), 7.37 (1H, m), 7.18 (1H, d, *J* = 8.0 Hz), 6.86 (2H, d, *J* = 8.5 Hz), 6.77 (1H, t), 3.91 (2H, t, *J* = 5.1 Hz), 3.64 (2H, t, *J* = 5.1 Hz), 3.23 (2H, t, *J* = 5.1 Hz), 3.08 (2H, t, *J* = 5.1 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 159.5, 153.2, 152.2, 150.8, 134.1, 131.8 (2C), 130.7, 129.3, 129.2, 126.4, 124.7, 119.3, 115.5, 113.6, 48.3, 47.9, 45.9, 41.1; HRMS calcd for $C_{19}H_{16}Cl_2N_4NaOS$ $[M+Na]^+$ 441.0320, found 441.0324.

*4.1.5.29 5-(2-Chlorophenyl)-4-(4-(3-methylphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5c'**)*

Deepred Solid; yield: 61%; Mp: 89.2-90.7°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.52 (2H, m), 7.43 (1H, m), 7.38 (1H, m), 7.16 (1H, t, J = 7.6 Hz), 6.72 (3H, m), 3.91 (2H, t, J = 5.3 Hz), 3.61 (2H, t, J = 5.3 Hz), 3.20 (2H, t, J = 5.2 Hz), 3.04 (2H, t, J = 5.2 Hz), 2.32 (3H, s). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 159.5, 153.3, 151.9, 149.8, 138.0, 131.8, 130.7, 130.6, 129.3, 128.1, 126.4, 124.7, 120.6, 116.7, 112.9, 48.8, 48.5, 46.1, 41.3, 20.7; HRMS calcd for $C_{20}H_{19}ClN_4NaOS$ $[M+Na]^+$ 421.0866, found 421.0891.

*4.1.5.30 5-(2-Chlorophenyl)-4-(4-(3-methoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5d'**)*

Red Solid; yield: 54%; Mp: 60.5-62.3°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.52 (2H, m), 7.43 (1H, m), 7.38 (1H, m), 7.18 (1H, d, J = 8.5 Hz), 6.51 (1H, d, J = 7.6 Hz), 6.46 (1H, d, J = 8.5 Hz), 6.44 (1H, s), 3.91 (2H, t, J = 5.3 Hz), 3.79 (3H, s), 3.62 (2H, t, J = 5.2 Hz), 3.21 (2H, t, J = 5.1 Hz), 3.05 (2H, t, J = 5.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 159.6, 159.5, 153.3, 151.9, 151.0, 131.8, 130.7 (2C), 129.3, 129.0, 126.4, 124.7, 108.4, 104.4, 102.3, 54.2, 48.7, 48.3, 46.0, 41.2; HRMS calcd for $C_{20}H_{19}ClN_4NaO_2S$ $[M+Na]^+$ 437.0815, found 437.0837

*4.1.5.31 5-(2-Chlorophenyl)-4-(4-(3-(trifluoromethylphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5e'**)*

Light yellow Solid; yield: 58%; Mp: 42.9-42.6°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.53 (2H, m), 7.44 (1H, m), 7.38 (2H, m), 7.14 (1H, d, J = 8.2 Hz), 7.09 (1H, s), 7.05 (1H, d, J = 9.4 Hz), 3.94 (2H, t, J = 5.1 Hz), 3.67 (2H, t, J = 5.3 Hz), 3.28 (2H, t, J = 5.2 Hz), 3.14 (2H, t, J = 5.4 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 160.5, 154.2, 153.3, 150.9, 132.8, 131.7, 131.7, 131.5, 130.3, 129.8, 127.4, 125.7, 125.0, 119.5, 116.9 (d, J = 4.0 Hz), 113.0 (d, J = 3.5 Hz), 49.3, 49.1, 46.9, 42.2; HRMS calcd for $C_{20}H_{16}ClF_3N_4NaOS$ $[M+Na]^+$ 475.0583, found 475.0613.

4.1.5.32 5-(2-Chlorophenyl)-4-(4-(4-methoxyphenyl)piperazine-1-carbonyl)-1,2,3-

*thiadiazol (**5f'**)*

Brown Solid; yield: 43%; Mp: 57.9-59.1°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.53 (2H, m), 7.44 (1H, m), 7.39 (1H, m), 6.85 (4H, d, *J* = 8.4 Hz), 3.93 (2H, s), 3.78 (3H, s), 3.62 (2H, s), 3.10 (2H, s), 2.94 (2H, s). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 159.5, 153.6, 153.3, 151.8, 144.0, 131.8, 130.7, 130.7, 129.3, 126.4, 124.7, 118.1 (2C), 113.5 (2C), 54.5, 50.3, 49.9, 46.2, 41.3; HRMS calcd for $C_{20}H_{19}ClN_4NaO_2S$ $[M+Na]^+$ 437.0815, found 437.0817.

*4.1.5.33 5-(2-Chlorophenyl)-4-(4-(2,5-dimethoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5g'**)*

Brown Solid; yield: 66%; Mp: 96.8-97.9°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.53 (2H, m), 7.44 (1H, m), 7.39 (1H, m), 6.78 (1H, d, *J* = 8.8 Hz), 6.52 (1H, dd, *J* = 3.0 Hz, *J* = 8.8 Hz), 6.44 (1H, d, *J* = 3.0 H), 3.94 (2H, t, *J* = 5.1 Hz), 3.81 (3H, s), 3.76 (3H, s), 3.60 (2H, t, *J* = 5.1 Hz), 3.06 (2H, t, *J* = 5.1 Hz), 2.90 (2H, t, *J* = 4.8 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 159.6, 153.4, 153.0, 151.4, 145.5, 140.4, 131.9, 130.7 (2C), 129.3, 126.4, 124.8, 110.9, 105.5, 105.9, 54.9, 54.6, 49.7, 49.3, 46.3, 41.5; HRMS calcd for $C_{21}H_{21}ClN_4NaO_3S$ $[M+Na]^+$ 467.0921, found 467.0926.

*4.1.5.34 5-(2-Chlorophenyl)-4-(4-(3,5-dimethoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5h'**)*

Brown Solid; yield: 68%; Mp: 44.7-46.2°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.52 (2H, m), 7.43 (1H, m), 7.38 (1H, m), 6.05 (3H, s), 3.90 (2H, t, *J* = 5.2 Hz), 3.77 (6H, s), 3.61 (2H, t, *J* = 5.0 Hz), 3.20 (2H, t, *J* = 4.9 Hz), 3.04 (2H, t, *J* = 5.0 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 160.5 (2C), 159.5, 153.3, 151.9, 151.7, 131.8, 130.7, 130.7, 129.3, 126.4, 124.7, 94.7 (2C), 91.4, 54.3 (2C), 48.6, 48.3, 46.0, 41.2; HRMS calcd for $C_{21}H_{21}ClN_4NaO_3S$ $[M+Na]^+$ 467.0921, found 467.0922.

4.1.5.35 5-(3,4-Dichlorophenyl)-4-(4-(3,5-dimethoxyphenyl)piperazine-1-carbonyl)-

*1,2,3-thiadiazol (**5i'**)*

Brown Solid; yield: 68%; Mp: 87.7-89.2°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.71 (1H, d, J = 2.1 Hz), 7.55 (1H, d, J = 8.4 Hz), 7.47 (1H, dd, J = 2.3 Hz, J = 8.1 Hz), 6.06 (3H, s), 3.99 (2H, t, J = 5.0 Hz), 3.77 (6H, s), 3.49 (2H, t, J = 5.2 Hz), 3.28 (2H, t, J = 4.9 Hz), 3.09 (2H, t, J = 5.0 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 161.5 (2C), 160.6, 153.7, 152.6, 152.1, 135.5, 133.8, 131.4, 130.9, 128.2, 126.1, 95.8 (2C), 92.6, 55.3 (2C), 49.8, 49.5, 47.1, 42.4; HRMS calcd for $C_{21}H_{20}Cl_2N_4NaO_3S$ $[M+Na]^+$ 501.0531, found 501.0548.

*4.1.5.36 5-(4-Chlorophenyl)-4-(4-(4-methoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol **(5j')***

Yellow Solid; yield: 63%; Mp: 43.1-45.0°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.55 (2H, d, J = 7.5 Hz), 7.46 (2H, d, J = 8.3 Hz), 6.85 (4H, t, J = 8.8 Hz), 4.02 (2H, s), 3.77 (3H, s), 3.65 (2H, s), 3.16 (2H, s), 2.94 (2H, s). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 160.0, 153.6, 153.6, 150.8, 143.8, 136.3, 129.4 (2C), 128.8 (2C), 123.7, 118.3 (2C), 113.6 (2C), 54.5, 50.2, 50.2, 46.2, 41.5; HRMS calcd for $C_{20}H_{19}ClN_4NaO_2S$ $[M+Na]^+$ 437.0815, found 437.0824.

*4.1.5.37 5-(4-Chlorophenyl)-4-(4-(3,5-dimethoxyphenyl)piperazine-1-carbonyl)-1,2,3-thiadiazol (**5k'**)*

Brown Solid; yield: 63%; Mp: 43.5-45.2°C; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.55 (2H, d, J = 7.8 Hz), 7.45 (2H, d, J = 8.4 Hz), 6.06 (3H, s), 3.99 (2H, t, J = 5.1 Hz), 3.77 (6H, s), 3.45 (2H, t, J = 5.2 Hz), 3.27 (2H, t, J = 4.8 Hz), 3.05 (2H, t, J = 5.1 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 160.5 (2C), 160.0, 153.8, 151.5, 150.7, 136.3, 129.3 (2C), 128.8 (2C), 123.7, 94.8 (2C), 91.6, 54.3 (2C), 48.8, 48.5, 45.9, 41.3; HRMS calcd for $C_{21}H_{21}ClN_4NaO_3S$ $[M+Na]^+$ 467.0921, found 467.0930.

4.2 Biological evaluation

4.2.1 Cell culture

The human gastric adenocarcinoma SGC-7901 cells, lung adenocarcinoma A549 cells and cervical carcinoma HeLa cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL streptomycin and 100 U/mL penicillin at 37°C in a humidified atmosphere containing 5% CO_2. All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA).

4.2.2 In vitro antiproliferative activity

The antiproliferative activities of target compounds **5**, CA-4 and colchicine were determined by a standard MTT assay. In brief, cells were seeded into 96-well plates at a density of 1-3×10^4/well (depending on the cell growth rate). 24 h later, cells were incubated with various concentrations of the test compounds for 72 h. Then the drug-containing medium was removed and replaced with 100 mL of fresh medium containing 5 mg/mL MTT solution. After 4 h of incubation, the medium with MTT was removed, and 100 mL of dimethyl sulphoxide (DMSO) was added to each well. The plates were gently agitated until the purple formazan crystals were dissolved, and the absorbance was measured at 570 nm by a microplate spectrophotometer (MK3, Thermo, Germany). The data were calculated and plotted as the per cent viability compared to the control. The 50% inhibitory concentration (IC_{50}) was defined as the concentration that reduced the absorbance of the untreated wells by 50% of the vehicle in the MTT assay [18].

4.2.3 Effect on tubulin polymerization

The effects of **5m** and CA-4 on the polymerization of tubulin were determined by employing a fluorescence-based tubulin polymerization assay kit (BK011P, Cytoskeleton, USA) according to the manufacturer's protocol. The tubulin reaction mix contained 2 mg/mL porcine brain tubulin (> 99% pure), 2 mM $MgCl_2$, 0.5 mM EGTA,

1 mM GTP, and 15% glycerol. First, 96-well plate was incubated with 5 mL of inhibitors in various concentrations at 37°C for 1 min. Then 50 mL of the tubulin reaction mix was added. The samples were mixed well, and tubulin assembly was monitored (emission wavelength of 420 nm; excitation wavelength pf 360 nm) at 1 min intervals for 90 min at 37°C using a plate reader (FASCalibur, BD Biosciences, USA). The IC_{50} values were calculated after 20 min using SPSS software [19].

4.2.4 Analysis of immunofluorescence staining

The HeLa cells were seeded in 24-well plate (with coverslips plated) at density of 1×10^4 cells. After overnight adherence, they were exposed to **5m** or CA-4 respectively, for 24 h. The coverslips were fixed in ice-cold methanol/acetic acid (3:1) for 10 min and blocked with 3% bovine serum albumin for 20 min at room temperature. The primary α-tubulin antibody (Santa Cruz, CA) was diluted (1:100) with 2% BSA in PBS and incubated overnight at 4°C. The cells were washed with PBS to remove unbound primary antibody, and the cells were then incubated with FITC-conjugated antimouse secondary antibody and diluted (1:1000) with 2% BSA in PBS for 3 h at 37°C. The cells were washed with PBS to remove unbound secondary antibody, the nuclei were stained with 4,6-diamino-2-phenolindol dihydrochloride (DAPI) and immunofluorescence was then detected using a fluorescence microscope (Olympus, Tokyo, Japan) [19].

4.2.5 Cell cycle analysis

HeLa cells (8×10^4 cells) were incubated with various concentrations of CA-4, **5m** or 0.05% DMSO for the indicated times. The cells were collected by centrifugation, washed with PBS and fixed in ice-cold 70% ethanol. The fixed cells were harvested by centrifugation and treated with RNase A at 37°C for 30 min, and incubated with PI

solution (Solarbio, China) at 4°C for 15 min. The samples were then analysed by FACScan flow cytometry (BectonDickinson, Franklin Lakes, NJ, USA). The experiments were repeated at least three times [20].

4.3 Molecular modelling

The molecular modelling studies were performed with Accelrys Discovery Studio 3.0. The crystal structure of tubulin complexed (PDB: 3HKC) was retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb). After extracting the ligand, hydrogen atoms were added to the crystal. Charges were added to biopolymer by CHARMm force field. Finally, **5m** and XRP44X were docked into the binding site using the CDOCKER protocol with the default settings [11].

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Conflicts of Interest

The authors declare no conflict of interest.

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Highlights

- Structure-based drug design of novel XRP44X-analogs as potent microtubule-destabilizing agents.
- Docking study suggested enhanced binding of XRP44X-analogs to tubulin.
- Select XRP44X-analogs were synthesized and tested.
- Select novel XRP44X-analogs exhibited strong antiproliferative activities.
- The most potent compound was selected to examine the antitubulin activity and evaluate the effects on intracellular microtubules and the cell cycle.