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Synthesis, biological evaluation, and molecular docking studies of 1,3,4-thiadiazol-2-amide derivatives as novel anticancer agents

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ABSTRACT

A series of 1,3,4-thiadiazol-2-amide derivatives (**5a–5y**) have been designed and synthesized, and their biological activities were also evaluated as potential antiproliferation and FAK inhibitors. Among all the compounds, **5h** showed the most potent activity in vitro, which inhibited the growth of MCF-7 and B16-F10 cell lines with IC_{50} values of 0.45 and 0.31 μM, respectively. Compound **5h** also exhibited significant FAK inhibitory activity (IC_{50} = 5.32 μM). Docking simulation was performed to position compound **5h** into the FAK structure active site to determine the probable binding model. The results of antiproliferative and Western-blot assay demonstrated that compound **5h** possessed good antiproliferative activity. Therefore, compound **5h** with potent FAK inhibitory activity may be a potential anticancer agent.

1. Introduction

Cancer is a class of diseases in which cell, or a group of cells display uncontrolled growth, invasion, and sometimes metastasis. During the last few decades, anticancer therapy has progressed significantly, but the management of malignancies in humans still constitutes a major challenge for contemporary medicine.[1–4]

Focal adhesion kinase (FAK) was first discovered in 1992 and was implicated in integrin signaling.[5–7] It is a 125 kDa protein tyrosine kinase recruited at an early stage to focal adhesions and is phosphorylated in response to cell attachment and mediates focal adhesion formation.[8,9] Focal adhesions are found at the cell membrane where the cytoskeleton interacts with the proteins of the extra-cellular matrix. The clustering of integrins at these sites attracts a large complex of proteins which regulate processes such as anchorage-dependent proliferation and cell migration. Signal transduction mediated by interactions between cells and the extracellular matrix (ECM) at focal adhesions is an important determinant of cell fate. Focal adhesion kinase is involved in multiple cellular functions such as cell proliferation, survival, motility, invasion, metastasis, and angiogenesis.[10] Different approaches to inhibit FAK with FAK antisense oligonucleotides,[11] dominant-negative C-terminal domain of FAK, FAK-CD or FRNK[12,13] or FAK siRNA,[14,15] caused decreased cellular viability, growth inhibition, or apoptosis. Recently, FAK was proposed to be a new potential therapeutic target in cancer.[16,17]

Phenyl-1,3,4-thiadiazoles are a class of small molecules that have received much interest in the fields of chemistry and biology due to their broad spectrum of activity. The 1,3,4-thiadiazole scaffold is an interesting building block that has been used to synthesize a variety of useful bioactive compounds. Phenyl-1,3,4-thiadiazole derivatives have been reported to be anticancer,[18] antimicrobial,[19] anti-tubercular,[20] anti-convulsant,[21] anti-inflammatory,[22] analgesic,[22] anti-anxiety, anti-depressant[23] and anti-viral[24] agents. For this type of derivatives, a different mechanism of action is assigned, depending on the type of modification of 1,3,4-thiadiazole ring.[25–27] The action connected with the apoptotic mechanisms and angiogenesis, which is a crucial step in the tumorigenesis, seems to be very promising in anticancer therapy.[28,29]

In our previous publication we described synthesis and in vitro antiproliferative activity against some human cancer cell lines of compounds of 1,3,4-thiadiazole derivatives containing 1,4-benzodioxan.[30] The significantly antiproliferative and FAK inhibitory effect of 1,3,4-thiadiazole derivatives were found.

As you know, amide derivatives were associated with broad spectrum of biological activities including antitumor properties.[31] Herein, in continuation to extend our research on antitumor compounds with FAK structure inhibitory activity, we report in the present work the synthesis and structure–activity relationships of a series of 1,3,4-thiadiazol-2-amide derivatives as antitumor

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agents. Biological evaluation indicated that some of the synthesized compounds were potent inhibitors of FAK.

2. Results and discussion

2.1. Chemistry

The synthetic route of the 1,3,4-thiadiazol-2-amide derivatives **5a–5y** is outlined in Scheme 1. These compounds were synthesized from 2-amino-1,3,4-thiadiazoles **3** and different substituted carboxylic acids **4**. Firstly, the different substituted benzoic acid **1** were treated with thiosemicarbazide **2** in presence of phosphoryl chloride yielded 2-amino-1,3,4-thiadiazoles. Secondly, the coupling reaction between the obtained different substituted 2-amino-1,3,4-thiadiazoles and different substituted carboxylic acids was performed by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCl) and *N*-hydroxybenzotriazole (HOBt) in anhydrous methylene dichloride, and refluxed to give the desired compounds **5a–5y** (Table 1). Among these compounds, **5c–5k** and **5m** are reported for the first time. All of the synthetic compounds gave satisfactory elementary analytical and spectroscopic data. ^{1}H NMR and ESI-MS spectra were consistent with the assigned structures.

2.2. Bioactivity

To test the anticancer activities of the synthesized compounds, we evaluated antiproliferative activities of compounds **5a–5y** against MCF-7 and B16-F10 cells. The results were summarized in Table 2. As illustrated in Table 2, the active analogs showed a distinctive potential pattern of selectivity as well as broad-spectrum in antitumor activity. Among them, compound **5h** displayed the most potent inhibitory activity (IC_{50} = 0.45 μM for MCF-7 and IC_{50} = 0.31 μM for B16-F10), comparable to the positive control Staurosporine (IC_{50} = 3.07 μM for MCF-7 and IC_{50} = 2.82 μM for B16-F10, respectively).

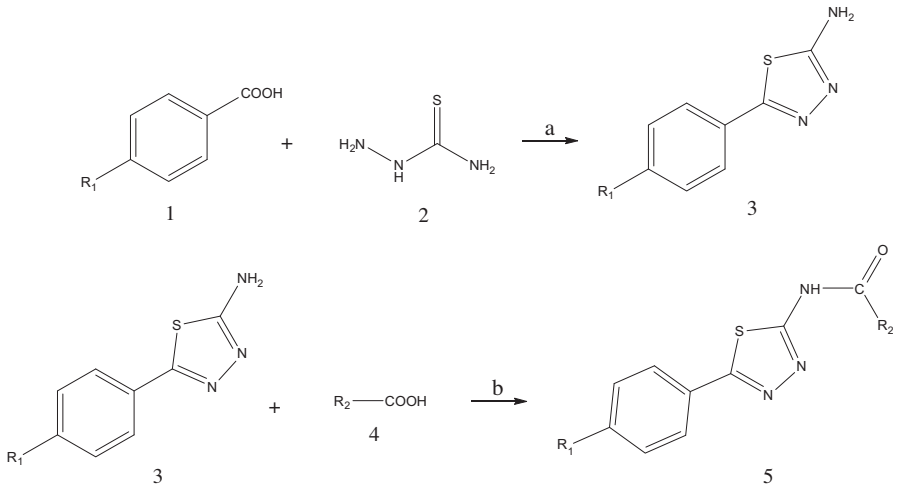
The activity of the tested compounds could be correlated to structure variation and modifications. By investigating the variation in selectivity of the tested compounds over the two cell lines, it was revealed that different acids substitutes led to different antitumor activity, and the potency order was salicylic acid > benzoic acid > nicotinic acid > phenylacetic acid. Regarding these compounds (**5a**–**5k**) with salicylic acid substituents, Structure–activity relationships in these compounds demonstrated that compounds with *para* electron-withdrawing substituents (**5d, 5g**) showed more potent activities than those with electron-donating substituents (**5j, 5k**) in the A-ring. A comparison of the *para* substituents on the A-ring demonstrated that an electron-withdrawing group (**5d, 5g**) have slightly improved antiproliferative activity, whereas Me (**5j**) and OMe (**5k**) group substituent had minimal effects compared with **5a**, which has no substituent. In the case of constant A ring substituents, compounds with substituted 5-Cl on salicylic acid moiety showed better antitumor activity than compounds with substituted 5-Br.

Among these compounds (**5o–5w**) with benzoic acid substituents, compounds with *para* electron-donating substituents (**5r, 5s**) showed more potent activities than those with electron-withdrawing substituents (**5p, 5q**) in the A-ring. A comparison of the *para* substituents on the A-ring demonstrated that an electron-donating group (**5r, 5s**) have slightly improved antiproliferative activity, whereas F (**5p**) and Cl (**5q**) substituent had minimal effects compared with **5o**. In the case of constant A ring substituents, change of substituents on benzoic acid moiety could also affect the activities of these compounds. Among the compounds **5t–5w**, these compounds (**5v, 5w**) with *para* electron-donating substituted showed stronger anticancer activity and the strength order was OMe > Me, followed that **5t** and **5u** led to a noteworthy poor activity.

To examine whether the compounds inhibit FAK, we tested their FAK inhibitory activity against MCF-7 cell line. The results were summarized in Table 2. Most of the tested compounds displayed potent FAK inhibitory. Among them, compounds **5h** showed the most potent inhibitory with IC_{50} of 5.32 μM (the positive control Staurosporine with an IC_{50} of 11.32 μM for FAK). The results of FAK inhibitory activity of the tested compounds were corresponding to the Structure–activity relationships (SAR) of their antitumor activities. This demonstrated that the potent antitumor activities of the synthetic compounds were probably correlated to their FAK inhibitory activities.

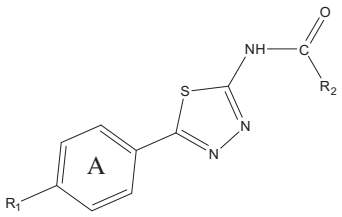
In an effort to study the preliminary mechanism of the compound with potent inhibitory activity, the Western-blot experiment was performed to assay the effect of compound **5h**. The Western-blot results were summarized in Figure 1. The result indicated that compound **5h** possessed good antiproliferative activity is probably correlated to its excellent FAK inhibitory activity.

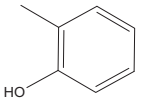
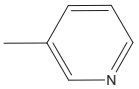
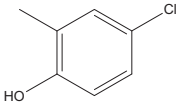
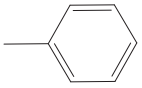
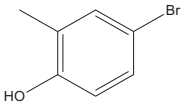
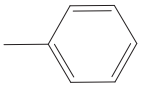
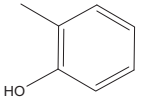
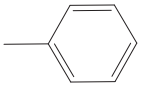
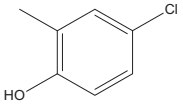
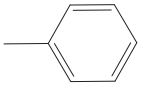
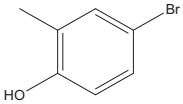
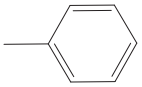
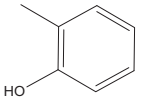
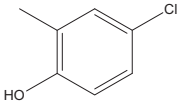
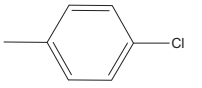
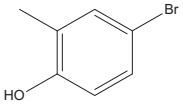
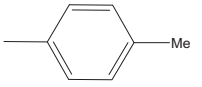
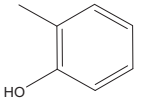
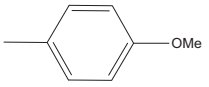
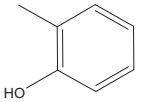
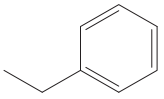
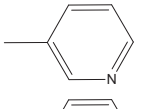
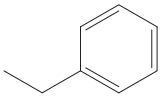
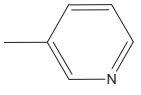
To gain better understanding on the potency of the studied compounds and guide further SAR studies, we proceeded to examine the interaction of compound **5h** with FAK crystal structure (2ETM pdb). The molecular docking was performed by inserting compound **5h** into ATP binding site of FAK. All docking runs



Scheme 1. General synthesis of 1,3,4-thiadiazol-2-amide derivatives (**5a**–**5y**). Reagents and conditions: (a) $POCl_3$, reflux, 8 h, 50%NaOH; (b) EDCl, HOBt, dichloromethane, reflux, 8 h.

Table 1
Structure of compounds **5a**–**5y**



Compound	R_1	R_2	Compound	R_1	R_2
5a	H		**5n**	OCH_3	
5b	H		**5o**	H	
5c	H		**5p**	F	
5d	F		**5q**	Cl	
5e	F		**5r**	CH_3	
5f	F		**5s**	OCH_3	
5g	Cl		**5t**	H	
5h	Cl		**5u**	H	
5i	Cl		**5v**	H	
5j	CH3		**5w**	H	
5k	OCH3		**5x**	Cl	
5l	H		**5y**	Br	
5m	CH3				

were applied LigandFit Dock protocol of Discovery Studio 3.1. The binding modes of compound **5h** and FAK were depicted in Figure 2. All the amino acid residues which had interactions with FAK were exhibited in Figure 3. In the binding mode, compound **5h** is potently bound to the ATP binding site of FAK via hydrophobic interactions and binding is stabilized by one hydrogen bond, one π–cation interaction and one π–Sigma interaction. The hydrogen atom of the hydroxyl group on salicylic acid moiety formed one

Table 2
Inhibition (IC_{50}) of MCF-7 and B16-F10 cells proliferation and inhibition of FAK by compounds **5a–5y**

Compound	IC_{50} ± SD (μM)		
	MCF-7[a]	B16-F10[a]	FAK[b]
5a	2.76 ± 0.21	2.02 ± 0.17	9.36 ± 0.86
5b	1.12 ± 0.09	1.14 ± 0.11	6.89 ± 0.85
5c	1.28 ± 0.12	1.28 ± 0.13	7.25 ± 0.81
5d	1.21 ± 0.07	1.16 ± 0.08	7.02 ± 0.87
5e	0.75 ± 0.07	0.68 ± 0.03	6.13 ± 0.72
5f	0.86 ± 0.06	1.02 ± 0.06	6.41 ± 0.59
5g	1.02 ± 0.08	1.15 ± 0.10	6.79 ± 0.83
5h	0.45 ± 0.03	0.31 ± 0.04	5.32 ± 0.69
5i	0.67 ± 0.04	0.56 ± 0.04	5.85 ± 0.65
5j	2.36 ± 0.12	1.97 ± 0.11	8.54 ± 0.79
5k	2.17 ± 0.10	1.39 ± 0.09	7.47 ± 0.74
5l	8.22 ± 0.45	6.18 ± 0.47	18.59 ± 1.07
5m	4.52 ± 0.23	3.88 ± 0.19	12.37 ± 0.85
5n	2.99 ± 0.18	2.36 ± 0.15	9.75 ± 0.91
5o	5.87 ± 0.26	5.15 ± 0.50	16.18 ± 1.13
5p	5.35 ± 0.27	4.75 ± 0.42	15.49 ± 0.93
5q	4.85 ± 0.33	4.06 ± 0.38	12.68 ± 0.87
5r	3.57 ± 0.18	3.52 ± 0.32	11.72 ± 0.96
5s	1.55 ± 0.09	1.58 ± 0.11	7.71 ± 0.52
5t	4.85 ± 0.38	4.36 ± 0.45	13.97 ± 0.94
5u	4.49 ± 0.25	4.65 ± 0.31	11.87 ± 0.88
5v	3.28 ± 0.23	2.76 ± 0.26	9.84 ± 0.55
5w	1.83 ± 0.13	1.75 ± 0.13	7.89 ± 0.48
5x	6.47 ± 0.41	8.23 ± 0.66	19.67 ± 1.09
5y	7.52 ± 0.46	9.06 ± 0.62	21.52 ± 1.25
Staurosporine	3.07 ± 0.13	2.82 ± 0.22	11.32 ± 0.85

[a] Inhibition of the growth of tumor cell lines.
[b] Inhibition of FAK.

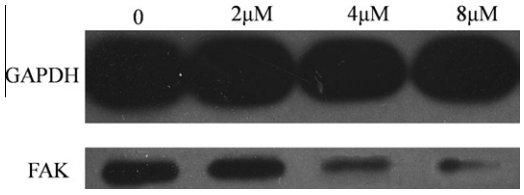


Figure 1. Compound **5h** was examined by Western blotting. Data are representative of three independent experiments.

hydrogen bond with the carboxylic oxygen of Glu A:506 (bond length: 0.952 Å; bond angle: 162.5°). The enzyme surface model was showed in Figure 2b, which revealed that the molecule was well embedded in the active pocket. This molecular docking result, along with the biological assay data, suggested that compound **5h** was a potential inhibitor of FAK.

3. Conclusion

In this study, a series of 1,3,4-thiadiazol-2-amide derivatives have been synthesized and evaluated for their antitumor activities. These compounds exhibited potent antiproliferative activities against MCF-7 and B16-F10 cells and FAK inhibitory activities. Among all of the compounds, **5h** showed the most potent inhibition activity which inhibited the growth of MCF-7 and B16-F10 cell lines with IC_{50} values of 0.45 and 0.31 μM, respectively and inhibited the FAK with IC_{50} of 5.32 μM. Molecular docking was further performed to study the inhibitor-FAK protein interactions. After analysis of the binding model of compound **5h** with FAK, it was found that several interactions with the protein residues in the ATP binding site might play a crucial role in its FAK inhibitory activities and antiproliferative activities. Western-blot results also showed that compound **5h** possessed good antiproliferative activity is probably correlated to its excellent FAK inhibitory activity. The information of this work might be helpful for the design and synthesis of FAK inhibitors with stronger activities.

4. Experiments

4.1. Materials and measurements

All chemicals and reagents used in the current study were of analytical grade. All the ^{1}H NMR spectra were recorded on a Bruker DPX300 model Spectrometer in DMSO-d_6 and chemical shifts were reported in ppm (δ). ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. Elemental analyses were performed on a CHN-O-Rapid instrument. TLC was performed on the glassbacked silica gel sheets (Silica Gel 60 GF254) and visualized in UV light (254 nm). Column chromatography was performed using silica gel (200–300 mesh) eluting with ethyl acetate and petroleum ether.

4.2. General procedure for synthesis of 2-amino-1,3,4-thiadiazoles

A stirring mixture of substituted benzoic acid (10 mmol), thiosemicarbazide (10 mmol) and $POCl_3$ (10 mL) was heated at 75–80 °C for 6 h, The reaction was monitored by TLC. After cooling down to room temperature, water was added. The reaction

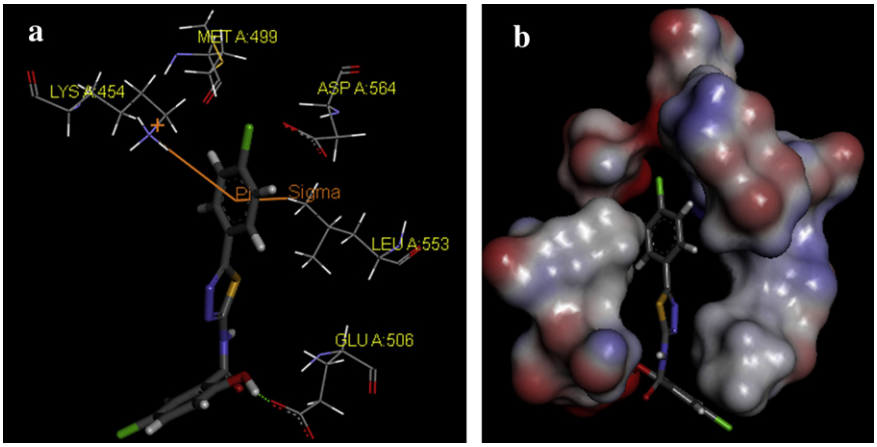


Figure 2. (a) Compound **5h** (colored by atom: carbons: gray; nitrogen: blue; oxygen: red; sulfur: yellow; Chlorine: green) is bond into FAK (entry 2ETM in the Protein Data Bank). The dotted lines show the hydrogen bond and the yellow line show the π–cation interaction and π–Sigma interaction. Glu A:506 is the mean of Glu 506 in A chain. (b) The surface model structure of compound **5h** binding model with FAK complex.

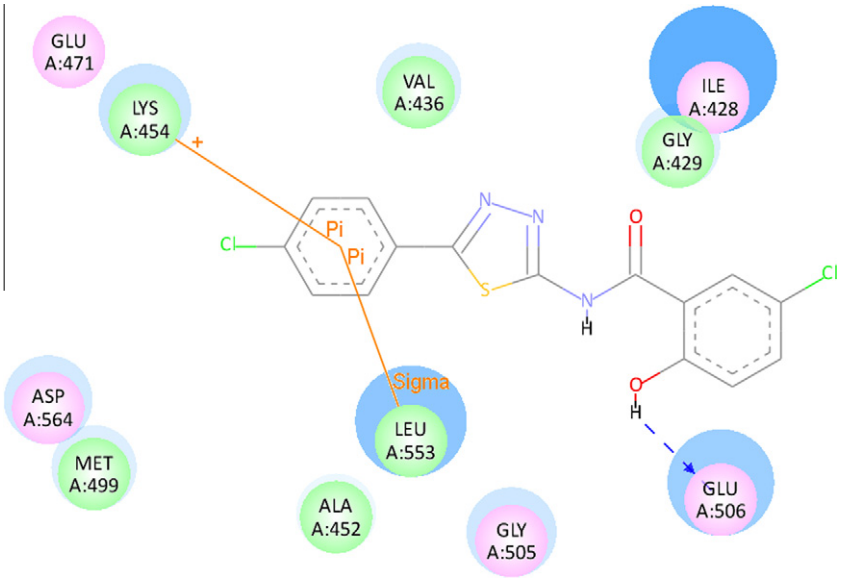


Figure 3. 2D Ligand interaction diagram of compound **5h** with FAK using Discovery Studio program with the essential amino acid residues at the binding site are tagged in circles. The purple circles show the amino acids which participate in hydrogen bonding, electrostatic or polar interactions and the green circles show the amino acids which participate in the Van der Waals interactions.

mixture was refluxed for 1 h. After cooling, the mixture was basified to pH 8–9 by the dropwise addition of 50% NaOH solution under ice bath. The precipitate was filtered and recrystallized from ethanol to gain compounds **3**.

4.3. General procedure for synthesis of target compounds 5a–5y

Compounds **5a–5y** were synthesized by coupling substituted 2-amino-1,3,4-thi- adiazoles with substituted carboxylic acids, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCl) and *N*-hydroxybenzotriazole (HOBt) as condensing agent. The mixture was refluxed in anhydrous CH_2Cl_2 for 8–10 h. The products were extracted with ethyl acetate. The extract was washed successively with 10% HCl, saturated $NaHCO_3$ and water, respectively, then dried over anhydrous Na_2SO_4, filtered and evaporated. The residue was purified by column chromatography using petroleum ether and ethyl acetate (3:1).

4.3.1. 5-Bromo-2-hydroxy-*N*-(5-phenyl-1,3,4-thiadiazol-2-yl)benzamide(5c)

White solid, yield 81%, mp: 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 7.01 (d, J = 8.8 Hz, 1H), 7.56 (t, J = 2.7 Hz, 3H), 7.62–7.65 (m, 1H), 7.97–7.99 (m, 2H), 8.04 (d, J = 2.4 Hz, 1H), 11.70 (s, 1H), 12.32 (s, 1H). ESI-MS: 377.2 ($C_{15}H_{11}BrN_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{15}H_{10}BrN_3O_2S$: C, 47.89; H, 2.68; N, 11.17. Found: C, 47.82; H, 2.69; N, 11.23.

4.3.2. *N*-(5-(4-Fluorophenyl)-1,3,4-thiadiazol-2-yl)-2-hydroxybenzamide(5d)

White solid, yield 63%, mp: 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 6.99–7.08 (m, 2H), 7.37–7.43 (m, 2H), 7.48–7.54 (m, 1H), 7.97–8.07 (m, 3H), 11.74 (s, 1H), 12.27 (s, 1H). ESI-MS: 316.3 ($C_{15}H_{11}FN_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{15}H_{10}FN_3O_2S$: C, 57.14; H, 3.20; N, 13.33. Found: C, 57.06; H, 3.31; N, 13.28.

4.3.3. 5-Chloro-*N*-(5-(4-fluorophenyl)-1,3,4-thiadiazol-2-yl)-2-hydroxybenzamide(5e)

White solid, yield 66%, mp: 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 7.06 (d, J = 8.7 Hz, 1H), 7.40 (t, J = 8.5 Hz, 2H), 7.52 (d, J = 8.5 Hz, 1H), 7.91 (s, 1H), 8.04 (t, J = 6.7 Hz, 2H), 11.73 (s, 1H), 12.26 (s, 1H). ESI-MS: 350.7 ($C_{15}H_{10}ClFN_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{15}H_9ClFN_3O_2S$: C, 51.51; H, 2.59; N, 12.01. Found: C, 51.44; H, 2.61; N, 12.07.

4.3.4. 5-Bromo-*N*-(5-(4-fluorophenyl)-1,3,4-thiadiazol-2-yl)-2-hydroxybenzamide(5f)

White solid, yield 79%, mp: 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 7.01 (d, J = 8.8 Hz, 1H), 7.40 (d, J = 8.7 Hz, 2H), 7.61–7.65 (m, 1H), 8.02–8.07 (m, 3H), 11.64 (s, 1H), 12.35 (s, 1H). ESI-MS: 395.2 ($C_{15}H_{10}BrFN_3O_2S$ $[M+H]^+$). Anal. Calcd for $C_{15}H_9BrFN_3O_2S$: C, 45.70; H, 2.30; N, 10.66. Found: C, 45.61; H, 2.32; N, 10.61.

4.3.5. *N*-(5-(4-Chlorophenyl)-1,3,4-thiadiazol-2-yl)-2-hydroxybenzamide(5g)

White solid, yield 66%, mp: 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 6.99–7.07 (m, 2H), 7.48–7.54 (m, 1H), 7.62 (t, J = 4.3 Hz, 2H), 7.97–8.02 (m, 3H), 11.69 (s, 1H), 12.28 (s, 1H). ESI-MS: 332.8 ($C_{15}H_{11}ClN_3O_2S$ $[M+H]^+$). Anal. Calcd for $C_{15}H_{10}ClN_3O_2S$: C, 54.30; H, 3.04; N, 12.67. Found: C, 54.21; H, 3.06; N, 12.62.

4.3.6. 5-Chloro-*N*-(5-(4-chlorophenyl)-1,3,4-thiadiazol-2-yl)-2-hydroxybenzamide(5h)

White solid, yield 68%, mp: 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 7.06 (d, J = 9.0 Hz, 1H), 7.52 (d, J = 6.2 Hz, 1H), 7.63 (d, J = 8.6 Hz, 2H), 7.91 (d, J = 2.1 Hz, 1H), 8.00 (d, J = 8.2 Hz, 2H), 11.70 (s, 1H), 12.31 (s, 1H). ESI-MS: 367.2 ($C_{15}H_{10}C_{l2}N_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{15}H_9C_{l2}N_3O_2S$: C, 49.19; H, 2.48; N, 11.47. Found: C, 49.24; H, 2.46; N, 11.53.

4.3.7. 5-Bromo-*N*-(5-(4-chlorophenyl)-1,3,4-thiadiazol-2-yl)-2-hydroxybenzamide(5i)

White solid, yield 76%, mp: 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 6.98 (d, J = 8.8 Hz, 1H), 7.59–7.63 (m, 3H), 7.97–8.02 (m, 3H), 11.65 (s, 1H), 12.34 (s, 1H). ESI-MS: 411.7 ($C_{15}H_{10}BrN_3O_2S$ $[M+H]^+$). Anal. Calcd for $C_{15}H_9BrN_3O_2S$: C, 43.87; H, 2.21; N, 10.23. Found: C, 43.78; H, 2.23; N, 10.28.

4.3.8. 2-Hydroxy-*N*-(5-(*p*-tolyl)-1,3,4-thiadiazol-2-yl)benzamide (5j)

White powders, yield 81%, mp: 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 2.39 (s, 3H), 6.99–7.07 (m, 2H), 7.37 (d, J = 7.86 Hz, 2H),

7.51 (t, J = 7.14 Hz, 1H), 7.87 (d, J = 8.07 Hz, 2H), 7.99 (d, J = 6.60 Hz, 1H), 11.71 (s, 1H), 12.30 (s, 1H). ESI-MS: 312.4 ($C_{16}H_{14}N_3O_2S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}N_3O_2S$: C, 61.72; H, 4.21; N, 13.50. Found: C, 61.76; H, 4.22; N, 13.54.

4.3.9. 2-Hydroxy-*N*-(5-(4-methoxyphenyl)-1,3,4-thiadiazol-2-yl) benzamide(5k)

White powders, yield 70%, mp: 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 3.84 (s, 3H), 6.98–7.06 (m, 2H), 7.10 (d, J = 8.04 Hz, 2H), 7.50 (t, J = 7.68 Hz, 1H), 7.91 (d, J = 8.04 Hz, 2H), 7.99 (d, J = 7.86 Hz, 1H), 12.24 (s, 2H). ESI-MS: 328.4 ($C_{16}H_{14}N_3O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}N_3O_3S$: C, 58.70; H, 4.00; N, 12.84. Found: C, 58.63; H, 3.98; N, 12.86.

4.3.10. *N*-(5-(*p*-Tolyl)-1,3,4-thiadiazol-2-yl)nicotinamide(5m)

White powders, yield 85%, mp: 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 2.39 (s, 3H), 7.37 (d, J = 7.68 Hz, 2H), 7.61 (t, J = 6.12 Hz, 1H), 7.88 (d, J = 7.32 Hz, 2H), 8.46 (d, J = 7.86 Hz, 1H), 8.82 (d, J = 4.11 Hz, 1H), 9.25 (s, 1H), 13.31 (s, 1H). ESI-MS: 297.3 ($C_{15}H_{13}N_4OS$, $[M+H]^+$). Anal. Calcd for $C_{15}H_{12}N_4OS$: C, 60.79; H, 4.08; N, 18.91. Found: C, 60.73; H, 4.10; N, 18.95.

4.4. Antiproliferation assay

The antiproliferative activities of the prepared compounds against MCF-7 and B16-F10 cells were evaluated as described elsewhere with some modifications.[32] Target tumor cell lines were grown to log phase in RPMI 1640 medium supplemented with 10% fetal bovine serum. After diluting to 2×10^4 cells mL^{-1}with the complete medium, 100 μL of the obtained cell suspension was added to each well of 96-well culture plates. The subsequent incubation was permitted at 37 °C, 5% CO_2 atmosphere for 24 h before the cytotoxicity assessments. Tested samples at pre-set concentrations were added to six wells with Staurosporine co-assayed as positive references. After 48 h exposure period, 40 μL of PBS containing 2.5 mg TmL^{-1} of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) was added to each well. Four hours later, 100 μL extraction solution (10% SDS-5% isobutyl alcohol-0.01 M HCl) was added. After an overnight incubation at 37 °C, the optical density was measured at a wavelength of 570 nm on an ELISA microplate reader. In all experiments three replicate wells were used for each drug concentration. Each assay was carried out for at least three times. The results were summarized in Table 2.

4.5. FAK inhibitory assay

Bovine brain FAK was purified as described previously.[33] To evaluate the effect of the compounds on FAK assembly in vitro,[34] varying concentrations were preincubated with 10 μM FAK in glutamate buffer at 30 °C and then cooled to 0 °C. After addition of GTP, the mixtures were transferred to 0 °C cuvettes in a recording spectrophotometer and warmed up to 30 °C and the assembly of FAK was observed turbid metrically. The IC_{50} was defined as the compound concentration that inhibited the extent of assembly by 50% after 20 min incubation.

4.6. Western-blot analysis

After incubation, cells were washed with PBS and lysed in lysis buffer (30 mm Tris, pH 7.5, 150 mm NaCl, 1 mm phenylmethylsulfonyl fluoride, 1 mm Na_3VO_4, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). After centrifugation at 12,000g for 5 min, the protein content of the supernatant was determined by a BCATM protein assay kit (Pierce, Rockford, IL, USA). The protein samples were separated by 10% SDS–PAGE and subsequently electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% nonfat milk for 2 h at room temperature. The blocked membrane was probed with the indicated primary antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase (HRP)-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

4.7. Docking simulations

The three-dimensional X-ray structure of tubulin (PDB code: 2ETM) was chosen as the template for the modeling study of compound **5h** bound to FAK. The crystal structure was obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do).

The molecular docking procedure was performed by using LigandFit protocol within Discovery Studio 3.1. For ligand preparation, the 3D structures of **5h** was generated and minimized using Discovery Studio 3.1. For protein preparation, the hydrogen atoms were added, and the water and impurities were removed. The whole FAK was defined as a receptor and the site sphere was selected based on the ligand binding location of ATP, then the ATP molecule was removed and **5h** was placed during the molecular docking procedure. Types of interactions of the docked protein with ligand were analyzed after the end of molecular docking.

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