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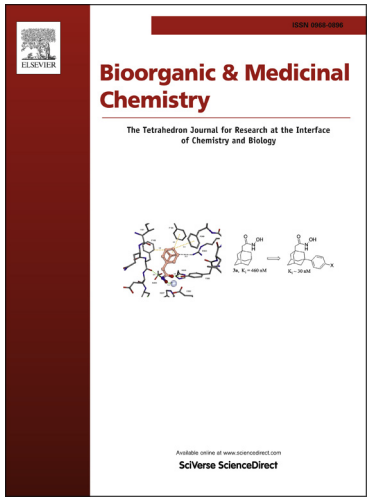

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PII: S0968-0896(13)00369-6
DOI: http://dx.doi.org/10.1016/j.bmc.2013.04.043
Reference: BMC 10783

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 14 March 2013
Revised Date: 7 April 2013
Accepted Date: 13 April 2013

Please cite this article as: Zhang, S., Luo, Y., He, L-Q., Liu, Z-J., Zhu, H-L., Synthesis, biological evaluation, and molecular docking studies of novel 1,3,4-oxadiazole derivatives possessing benzotriazole moiety as FAK inhibitors with anticancer activity, *Bioorganic & Medicinal Chemistry* (2013), doi: http://dx.doi.org/10.1016/j.bmc.2013.04.043

Synthesis, biological evaluation, and molecular docking studies of novel 1,3,4-oxadiazole derivatives possessing benzotriazole moiety as FAK inhibitors with anticancer activity

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Abstract: 1,3,4-Oxadiazole derivatives have drawn continuing interest over the years because of their varied biological activities. In order to search for novel anticancer agents, we designed and synthesized a series of new 1,3,4-oxadiazole derivatives containing benzotriazole moiety as potential focal adhesion kinase (FAK) inhibitors. All the synthesized compounds were firstly reported. Among the compounds, compound **4** shows the most potent inhibitory activity against MCF-7 and HT29 cell lines with IC_{50} values of 5.68 μg/ml and 10.21 μg/ml, respectively. Besides, all the compounds were assayed for FAK inhibitory activity using the TRAP-PCR-ELISA assay. The results showed compound **4** exhibited the most potent FAK inhibitory activity with IC_{50} values of 1.2±0.3 μM. Docking simulation by positioning compound **4** into the FAK structure active site was performed to explore the possible binding mode. Apoptosis which was analyzed by flow cytometry, demonstrated that compound **4** induced apoptosis against MCF-7 cells. Therefore, compound **4** may be a potential anticancer agent against MCF-7 cancer cell.

Keywords: benzotriazole; 1,3,4-oxadiazole derivatives; FAK; molecular docking; anticancer activity

1. Introduction

According to the statistics, cancer is the second most common cause of death after heart disease.[1] During the past decades, cancer continued to be a worldwide killer.[2] Despite the mass research was devoted and rapid progress was achieved, there is a growing demand for new therapies as usual. It is important to identify new agents and new targets for the treatment of cancer.

As we know, the invasive growth of tumor cells contains complicated progress steps, involving a variety of biological chemical factors.[3,4] Tumor cells must adhere to the extracellular matrix, and then affect cell adhesion, motility and migration by promoting the extracellular matrix signal transduction.[5-7] Focal adhesion kinase (FAK) mediated signal transduction system is one of the most important cell signal transduction pathways.[8] FAK is a non-receptor tyrosine kinase that play an important

role in cell proliferation, survival, motility, invasion, metastasis, and angiogenesis.[9] Enhanced FAK signaling may result in uncontrolled proliferation, survival or migration of cells, as observed in cancer development and progression process.[10] Given the role of FAK in tumorigenesis and metastasis, FAK may be a promising target for an anticancer drug.

The derivatives of 1,3,4-oxadiazole have high potential for biological activity and have drawn much attention during the past decades.[11-13] The wide array of biological activities include anti-diabetic,[14] anti-tubercular,[15] anti-cancer,[16] anti-fungal,[17] anti-inflammatory,[18] anti-bacterial activities.[19,20] Also, benzotriazole derivatives have been found to exhibit potential anti-mycobacterial,[21] anti-tubercular,[22] antitumor[23] and anti-inflammatory activities.[24]

Based on the above analysis, we designed and synthesized a series of new 1,3,4-oxadiazole derivatives containing benzotriazole moiety as potential antitumor agents. Biological evaluation was also carried out for screening potential FAK inhibitors of the synthesized compounds. We not only evaluated the anticancer activities and FAK inhibitory activities, but also explored the preliminary mechanism of the synthesized compounds in cell apoptosis by flow cytometry. Docking simulations were performed to investigate the inhibitor interaction with FAK and explore the binding mode of the compound at the active site.

2. Results and discussion

2.1 Chemistry

In this study, nineteen 1,3,4-oxadiazole derivatives containing benzotriazole moiety were synthesized. All of them were reported for the first time.The synthetic route of compounds **4-22** was outlined in Scheme 1.

As depicted in Scheme 1, benzotriazole was esterified with ethyl chloroacetate in acetone in the presence of potassium carbonate to give compound **1**. The compound **2** was obtained by compound **1** reacting with 85% hydrazine hydrate in methanol. Treatment of compound **2** with carbon disulfide in the presence of potassium hydroxide and anhydrous ethanol under reflux gave compound **3**.[25]The synthesis of

compounds **4-22** were accomplished by refluxing compound **3** with halogen substituted benzyl bromide in the presence of sodium hydroxide in anhydrous ethanol.[26] All of the compounds **4-22** gave satisfactory elementary analyses. ^{1}H NMR and ESI-MS spectra were consistent with the assigned structures.

2.2 Biological activity

2.2.1 Antiproliferation assay

All the synthesized compounds **4-22** were evaluated for their anticancer activity against MCF-7 (human breast cancer) and HT29 (human colorectal cancer) cell lines. Cisplatin is one of the most effective broad-spectrum anticancer drugs. Its effectiveness seems to be due to the unique properties of cisplatin, which enters cells via multiple pathways and forms multiple different DNA-platinum adducts while initiating a cellular self-defense system by activating or silencing a variety of different genes.[27] Hence, we chose cisplatin as reference drug. The results were summarized in Table 1.

As exhibited in Table 1, it was obvious that compound **4** showed best activity against MCF-7 cells with the IC_{50} value of 5.68 μg/ml, much better than reference drug *cisplatin* with the IC_{50} value of 11.20 μg/ml, however, the result of the compounds against HT29 cells seemed to be less effective. Herein, compound **4** also exhibited better activity than other compounds against HT29 cell with the IC_{50} value of 10.21 μg/ml.

The activity of the tested compounds could be correlated to structure variations. We investigated the selectivity of the tested compounds against MCF-7 cell and HT29 cell lines, and it was clear that the tested compounds showed better activities against MCF-7 cell line than HT29 cell line. Among the tested compounds, compound **4** showed the most potent inhibitory activity against MCF-7 cell line with the IC_{50} value of 5.68 μg/ml. Structure-activity relationship (SAR) analysis indicated that compounds with electron-withdrawing group showed stronger activity than those with electron-donating group. In further study of compounds with electron-withdrawing group, it was clear that different substituent could lead to different anticancer activity,

and the potency order was F (fluorine) > Cl (chlorine) > Br (bromine) > NO_2 (nitro-group) > OCH_3 (methoxy group) > CH_3 (methyl), which showed in Table 1 with compounds **4-9** exhibiting the trend of declining inhibitory activity against MCF-7 cells. Besides, with regard to the F- substituted compounds, compound **4** has better inhibitory activity comparing with compounds **10** and **16**. Substituents in different positions also led to different anticancer activities (ortho- > meta- > para-).

2.2.2 FAK inhibitory assay

The FAK inhibitory potency of the 1,3,4-oxadiazole derivatives containing benzotriazole moiety was examined and the results were summarized in Table 2. Most of the tested compounds displayed potent FAK inhibitory activities. Among them, compound **4** displayed the best inhibitory activity with IC_{50} value of 1.2±0.3 μM comparable to reference drug *cisplatin* with IC_{50} value of 8.6±0.2 μM. The SAR analysis result of FAK inhibitory activity of the tested compounds was consistent with that of their anticancer activities. This consistency suggested that the potent anticancer activities of the synthesized compounds were likely related to their FAK inhibitory activities.

2.2.3Apoptosis assay

Apoptosis is the normal pathway for clearance of defective or aged cells in the body. It is an essential mechanism used to eliminate activated MCF-7 cells during the shut-down process of excessive immune responses and maintain proper immune homeostasis, while deficient apoptosis of activated MCF-7 cells is associated with a wide variety of immune disorders. As a representative of these 1,3,4-oxadiazole derivatives, compound **4** was studied *in vitro*. We detected the mechanism of compound **4** inhibitory activity by flow cytometry. As shown in Figure 1, we found that compound **4** could induce the apoptosis of activated MCF-7 cells in a dose-dependent manner. To explain the inhibitory activity of compound **4** against MCF-7 cells, MCF-7 cells were treated with 1, 5, 10, 20 μg/ml of compound **4** for 24h. The compound **4** increased the percentage of apoptosis by Annexin V-FITC/PI

staining in a dose-dependent manner. The percentages of cell apoptosis (14.27%, 28.09%, 46.91%, and 61.29%) are corresponding to the concentration of compound **4**. The results indicated that compound **4** induced apoptosis of MCF-7 cells.

2.3 Binding mode of compound 4 into FAK structure

Molecular docking is an application wherein molecular modeling techniques are used to predict how protein receptors interact with small molecules. In an effort to elucidate the possible mechanism by which the title compounds can induce anticancer activity in MCF-7 and HT29 cells, molecular docking of the potent inhibitor **4** into active binding site of FAK was performed to simulate a binding mode derived from FAK structure (2ETM.pdb). All docking runs were applied CDOCKER protocol of Discovery Studio 3.1.

The docking calculation of the synthesized compounds was showed in Table 3. The interaction energy between compounds **4-22** and FAK showed the corresponding results with FAK inhibitory activity. Among the synthesized compounds, compound **4** showed the lowest interaction energy. The 2D and 3D binding modes of compound **4** and FAK were depicted in Figure 2, Figure 3 and Figure 4. In the binding modes , compound **4** was nicely bound to the FAK protein catalytic subunit with two interaction bonds. Visual inspection of the pose of compound **4** into the active site revealed that compound **4** was nicely bound to FAK with its nitrogen atom of oxadiazole ring toward the amino hydrogen of CYS502, forming a H-bond interaction. Also the benzene ring formed a π-cation interaction with LYS454. The molecular docking results, along with the biological assay data, suggested that compound **4** was a potential inhibitor of FAK.

3. Conclusion

In this paper, a series of 1,3,4-oxadiazole derivatives containing benzotriazole moiety were synthesized and evaluated for their anticancer activity. The bioactivity assay results showed that compound **4** exhibited the most potent inhibitory activity for FAK with IC_{50} value of 1.2±0.3 μM, which was comparable to the positive control

cisplatin, and good activity against human breast cancer cell MCF-7 with IC_{50} value of 5.68 μg/ml, better than the reference drug *cisplatin*. Molecular docking of the most potent inhibitor **4** into binding site of FAK was performed, and the results showed compound **4** could bind well with the FAK active site. Also apoptosis assay results showed that compound **4** induced apoptosis of stimulated MCF-7 cells. The above results provided theoretical basis for further structural optimization of 1,3,4-oxadiazole derivatives as FAK inhibitors and showed that compound **4** was a potential anticancer agent.

4. Experiments

4.1 Materials and measurements

All chemicals and reagents used in current study were of analytical grade. The reactions were monitored by thin layer chromatography (TLC) on Merck pre-coated silica GF254 plates. Melting points (uncorrected) were determined on a X-4 MP apparatus (Tai ke Corp., Beijing, China). ^{1}H NMR spectra were collected on a Bruker DPX300 spectrometer at room temperature with TMS and solvent signals allotted as internal standards. Chemical shifts are reported in ppm (δ). Elemental analyses were performed on a CHN–O-Rapid instrument.

4.2 General procedure for synthesis of compound 1

Benzotriazole (50 mmol) was dissolved in dry acetone (100 ml), then added anhydrous potassium carbonate (50 mmol). After that, ethyl chloroacetate (50 mmol) was added then refluxed for 8h. The reaction was monitored by TLC. Afterwards filtered reaction solution was evaporated under reduced pressure distillation to give the crude product. The crude product is purified by column chromatography [eluent: V(petroleum ether) : V(ethyl acetate) = 9 : 1] to give white solid (compound **1**).

4.3 General procedure for synthesis of compound 2

To a solution of compound **1**(20 mmol) in methanol, 85% hydrazine hydrate (80 mmol) was added and the solution was stirred at 4 ℃ for 12h. Then the appearing

solid was filtered. The residue was recrystallized from ethanol to obtain compound **2**.

4.4 General procedure for synthesis of compound 3

To an ethanol solution of compound **2** (20 mmol) was added potassium hydroxide (20 mmol) under stirring. Then, carbon disulfide (30 mmol) was added and the mixture was refluxed for 24h. The reaction solution was evaporated under reduced pressure distillation. After removal of excess solvent, the residue was poured into ice-cold water and acidified with dilute hydrochloric acid to obtain the white solid. The crude product was filtered, washed with ice-cold water and recrystallized from ethanol to get white crystals (compound **3**).

4.5 General procedure for synthesis of the target compounds 4-22

To a stirred solution of compound **3** (1 mmol) and sodium hydroxide (1 mmol) in acetonitrile (30ml) was added dropwise acetonitrile containing halogen substituted compounds (1 mmol). The mixture was refluxed for 10-24h and the reaction was monitored by TLC. Afterwards the solution was cooled to room temperature and the reaction solution was evaporated under reduced pressure distillation. The residue was dissolved in ethyl acetate and the organic layer was washed with saturated brine. Then the organic phase was dried over anhydrous sodium sulfate, filtered, and removed under reduced pressure distillation. The purification of the residue by recrystallization from acetonitrile yielded the desired compounds **4-22**.

4.5.1 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(2-fluorobenzylthio)-1,3,4-oxadiazole (4)

Clear crystal: Yield 83%; mp 85-86 ℃. ^{1}HNMR (DMSO-D6, 300MHz): 4.45 (s, 2H), 6.38 (s, 2H), 7.01-7.06 (s, 1H), 7.09-7.15(s, 1H), 7.27-7.35 (m, 2H), 7.44-7.49 (m, 1H), 7.59-7.64 (m, 1H), 7.87 (d, J = 8.40Hz, 1H), 8.12 (d, J = 8.40Hz, 1H). ESI-MS: 342.36 ($C_{16}H_{13}FN_5OS$, $[M+H]^+$). Anal.Calcd for $C_{16}H_{12}FN_5OS$: C, 56.30; H, 3.54; N, 20.52%. Found: C, 56.36; H, 3.54; N, 20.53%.

4.5.2 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(2-chlorobenzylthio)-1,3,4-oxadiazole (5)

White powder: Yield 75%; mp 109-110 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.51 (s, 2H), 6.39 (s, 2H), 7.18 (t, J = 7.50Hz, 1H), 7.29 (t, J = 6.03Hz, 1H), 7.38-7.49 (m, 3H), 7.62 (t, J = 7.14Hz, 1H), 7.87 (d, J = 8.25Hz, 1H), 8.11 (d, J = 8.40Hz, 1H). ESI-MS: 358.82 ($C_{16}H_{13}ClN_5OS$, $[M+H]^+$). Anal.Calcd for $C_{16}H_{12}ClN_5OS$: C, 53.71; H, 3.38; N, 19.57%. Found: C, 53.70; H, 3.34; N, 19.54%.

4.5.3 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(2-bromobenzylthio)-1,3,4-oxadiazole (6)

Yellow powder: Yield 71%; mp 86-88 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.50 (s, 2H), 6.42 (s, 2H), 7.20-7.23 (m, 2H), 7.25-7.44 (m, 2H), 7.46-7.49 (m, 2H), 7.91 (d, J = 9.12Hz, 1H), 8.15 (d, J = 8.93Hz, 1H). ESI-MS: 403.27 ($C_{16}H_{13}BrN_5OS$, $[M+H]^+$). Anal.Calcd for $C_{16}H_{12}BrN_5OS$: C, 47.77; H, 3.01; N, 17.41%. Found: C, 47.78; H, 3.04; N, 17.36%.

4.5.4 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(2-methylbenzylthio)-1,3,4-oxadiazole (7)

Yellow crystal: Yield 80%; mp 95-97 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 2.30 (s, 3H), 4.45 (s, 2H), 6.40 (s, 2H), 6.95-7.01 (m, 1H), 7.13-7.17 (m, 3H), 7.44-7.49 (m, 1H), 7.59-7.65 (m, 1H), 7.88 (d, J = 8.43Hz, 1H), 8.12 (d, J = 8.43Hz, 1H). ESI-MS: 338.40 ($C_{17}H_{16}N_5OS$, $[M+H]^+$). Anal.Calcd for $C_{17}H_{15}N_5OS$: C, 60.52; H, 4.48; N, 20.76%. Found: C,60.51; H, 4.44; N, 20.75%.

4.5.5 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(2-methoxybenzylthio)-1,3,4-oxadiazole (8)

Yellow powder: Yield 80%; mp 55-56 ºC.^{1}HNMR (DMSO-D6, 300MHz): 3.73 (s, 3H), 4.46 (s, 2H), 6.40 (s, 2H), 6.89-7.01 (m, 1H), 7.19-7.30 (m, 3H), 7.44-7.48 (m, 1H), 7.59-7.66 (m, 1H), 7.87-7.92 (m, 1H), 8.03-8.11 (m, 1H). ESI-MS: 354.40 ($C_{17}H_{16}N_5O_2S$, $[M+H]^+$). Anal.Calcd for $C_{17}H_{15}N_5O_2S$: C, 57.78; H, 4.28; N, 19.82%.

Found: C, 57.81; H, 4.33; N, 19.83%.

4.5.6 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(2-nitrobenzylthio)-1,3,4-oxadiazole (9)

Yellow crystal: Yield 85%; mp 115-116 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.72 (s, 2H), 6.37 (s, 2H), 7.43-7.49 (s, 1H), 7.56-7.65 (m, 4H), 7.85-7.88 (m, 1H), 8.04-8.13 (m, 2H). ESI-MS: 369.37 ($C_{16}H_{13}N_6O_3S$, $[M+H]^+$). Anal.Calcd for $C_{16}H_{12}N_6O_3S$: C, 52.17; H, 3.28; N, 22.81%. Found: C, 52.13; H, 3.24; N, 22.84%.

4.5.7 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(3-fluorobenzylthio)-1,3,4-oxadiazole (10)

White powder: Yield 82%; mp 69-70 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.50 (s, 2H), 6.38 (s, 2H), 7.11-7.16 (s, 1H), 7.29-7.35(s, 1H), 7.48 (d, J= 6.62Hz, 1H), 7.59-7.64 (m, 3H), 7.84 (d, J = 8.40Hz, 1H), 8.12 (d, J = 8.40Hz, 1H). ESI-MS: 342.36 ($C_{16}H_{13}FN_5OS$, $[M+H]^+$). Anal.Calcd for $C_{16}H_{12}FN_5OS$: C, 56.30; H, 3.54; N, 20.52%. Found: C, 56.34; H, 3.52; N, 20.57%.

4.5.8 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(3-chlorobenzylthio)-1,3,4-oxadiazole (11)

Yellow powder: Yield 85%; mp 78-80 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.45 (s, 2H), 6.38 (s, 2H), 7.24-7.31 (m, 3H), 7.44-7.49 (m, 2H), 7.59-7.64 (t, J = 6.96Hz, 1H), 7.87 (d, J = 8.40Hz, 1H), 8.11 (d, J = 8.22Hz, 1H). ESI-MS: 358.82 ($C_{16}H_{13}ClN_5OS$, $[M+H]^+$). Anal.Calcd for $C_{16}H_{12}ClN_5OS$: C, 53.71; H,3.38; N, 19.57%. Found: C, 53.73; H, 3.41; N, 19.58%.

4.5.9 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(3-bromobenzylthio)-1,3,4-oxadiazole (12)

Yellow powder: Yield 75%; mp 86-88 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.50 (s, 2H), 6.42 (s, 2H), 7.30-7.33 (m, 2H), 7.40-7.44 (m, 2H), 7.46-7.50 (m, 2H), 7.89 (d, J = 8.72Hz, 1H), 8.15 (d, J = 8.98Hz, 1H). ESI-MS: 403.27 ($C_{16}H_{13}BrN_5OS$,

[M+H]$^+$). Anal.Calcd for $C_{16}H_{12}BrN_5OS$: C, 47.77; H, 3.01; N, 17.41%. Found: C, 47.79; H, 3.01; N, 17.38%.

4.5.10 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(3-methylbenzylthio)-1,3,4-oxadiazole (13)

Yellow crystal: Yield 85%; mp 95-97 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 2.30 (s, 3H), 4.47 (s, 2H), 6.42 (s, 2H), 7.02-7.04 (m, 1H), 7.18-7.20 (m, 3H), 7.49-7.52 (m, 1H), 7.59-7.65 (m, 1H), 7.90 (d, J = 8.44Hz, 1H), 8.13 (d, J = 8.73Hz, 1H). ESI-MS: 338.40 ($C_{17}H_{16}N_5OS$, [M+H]$^+$). Anal.Calcd for $C_{17}H_{15}N_5OS$: C, 60.52; H, 4.48; N, 20.76%. Found: C,60.54; H, 4.49; N, 20.70%.

4.5.11 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(3-methoxybenzylthio)-1,3,4-oxadiazole (14)

Yellow powder: Yield 70%; mp 55-56 ºC.^{1}HNMR (DMSO-D6, 300MHz): 3.70 (s, 3H), 4.40 (s, 2H), 6.40 (s, 2H), 6.90-7.01 (m, 1H), 7.20-7.33 (m, 3H), 7.44-7.48 (m, 1H), 7.59-7.64 (m, 1H), 7.90-7.92 (m, 1H), 8.03-8.11 (m, 1H). ESI-MS: 354.40 ($C_{17}H_{16}N_5O_2S$, [M+H]$^+$). Anal.Calcd for $C_{17}H_{15}N_5O_2S$: C, 57.78; H, 4.28; N, 19.82%. Found: C, 57.76; H, 4.33; N, 19.84%.

4.5.12 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(3-nitrobenzylthio)-1,3,4-oxadiazole (15)

Yellow crystal: Yield 75%; mp 103-104 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.62 (s, 2H), 6.37 (s, 2H), 7.49 (t, J= 4.52Hz, 1H), 7.66-7.75 (m, 3H), 7.89-7.94 (m, 2H), 8.14-8.18 (m, 2H). ESI-MS: 369.37 ($C_{16}H_{13}N_6O_3S$, [M+H]$^+$). Anal.Calcd for $C_{16}H_{12}N_6O_3S$: C, 52.17; H, 3.28; N, 22.81%. Found: C, 52.18; H, 3.27; N, 22.82%.

4.5.13 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(4-fluorobenzylthio)-1,3,4-oxadiazole (16)

White powder: Yield 85%; mp 87-89 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.42 (s, 2H), 6.41 (s, 2H), 7.25-7.34 (m, 4H), 7.44-7.49 (m, 1H), 7.59-7.64 (m, 1H), 7.85 (d, J = 8.23Hz, 1H), 8.13 (d, J = 8.42Hz, 1H). ESI-MS: 342.36 ($C_{16}H_{13}FN_5OS$, [M+H]$^+$). Anal.Calcd for $C_{16}H_{12}FN_5OS$: C, 56.30; H, 3.54; N, 20.52%. Found: C, 56.28; H, 3.52;

N, 20.56%.

4.5.14 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(4-bromobenzylthio)-1,3,4-oxadiazole (17)

Yellow crystal: Yield 71%; mp 99-104 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.41 (s, 2H), 6.38 (s, 2H), 7.26 (d, J = 8.40Hz, 2H), 7.39-7.49 (m, 3H), 7.62 (t, J = 7.11Hz, 1H), 7.86 (d, J = 8.25Hz, 1H), 8.12 (d, J = 8.43Hz, 1H). ESI-MS: 403.27 ($C_{16}H_{13}BrN_5OS$, $[M+H]^+$). Anal.Calcd for $C_{16}H_{12}BrN_5OS$: C, 47.77;H, 3.01; N, 17.41%. Found: C, 47.71;H, 3.07; N, 17.42%.

4.5.15 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(4-methylbenzylthio)-1,3,4-oxadiazole (18)

Yellow crystal: Yield 80%; mp 96-97 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 2.22 (s, 3H), 4.38 (s, 2H), 6.39 (s, 2H), 7.00 (d, J = 7.89Hz, 2H), 7.15 (d, J = 8.07Hz, 2H), 7.44-7.50 (m, 1H), 7.60-7.65 (m, 1H), 7.87 (d, J = 8.40Hz, 1H), 8.12 (d, J = 8.22Hz, 1H). ESI-MS: 338.40 ($C_{17}H_{16}N_5OS$, $[M+H]^+$). Anal.Calcd for $C_{17}H_{15}N_5OS$: C, 60.52; H, 4.48; N, 20.76%. Found: C, 60.55; H, 4.45; N, 20.79%.

4.5.16 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(4-methoxybenzylthio)-1,3,4-oxadiazole (19)

Yellow powder: Yield 80%; mp 57-58 ºC.^{1}HNMR (DMSO-D6, 300MHz): 3.70 (s, 3H), 4.38 (s, 2H), 6.40 (s, 2H), 6.76 (d, J = 5.13Hz, 2H), 7.19-7.30 (m, 2H), 7.44-7.49 (m, 1H), 7.59-7.64 (m, 1H), 7.87-7.92 (m, 1H), 8.08-8.13 (m, 1H). ESI-MS: 354.40 ($C_{17}H_{16}N_5O_2S$, $[M+H]^+$). Anal.Calcd for $C_{17}H_{15}N_5O_2S$: C, 57.78; H, 4.28; N, 19.82%. Found: C, 57.80; H, 4.33; N, 19.85%.

4.5.17 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(4-nitrobenzylthio)-1,3,4-oxadiazole (20)

Yellow crystal: Yield 70%; mp 105-107 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.57 (s, 2H), 6.37 (s, 2H), 7.46 (t, J = 4.48Hz, 1H), 7.58-7.69 (m, 3H), 7.84-7.89 (m, 1H),

8.07-8.16 (m, 3H). ESI-MS: 369.37 ($C_{16}H_{13}N_6O_3S$, $[M+H]^+$). Anal.Calcd for $C_{16}H_{12}N_6O_3S$: C, 52.17; H, 3.28; N, 22.81%. Found: C, 52.22; H, 3.24; N, 22.82%.

4.5.18 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(3,4-dichlorobenzylthio)-1,3,4-oxadiazole (21)

Clear crystal: Yield 81%; mp 94-95 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.46 (s, 2H), 6.38 (s, 2H), 7.31-7.33 (m, 1H), 7.40-7.48 (m, 2H), 7.55-7.62 (m, 1H), 7.63-7.69 (m, 1H), 7.85-7.89 (m, 1H), 8.08-8.12 (t, J = 5.4Hz, 1H). ESI-MS: 393.26 ($C_{16}H_{12}Cl_2N_5OS$, $[M+H]^+$). Anal.Calcd for $C_{16}H_{11}Cl_2N_5OS$: C, 48.99; H, 2.83; N, 17.85%. Found: C, 49.02; H, 2.86; N, 17.88%.

4.5.19 2-((1H-benzo[d][1,2,3]triazol-1-yl)methyl)-5-(3,4-difluorobenzylthio)-1,3,4-oxadiazole (22)

White powder: Yield 85%; mp 84-86 ºC. ^{1}HNMR (DMSO-D6, 300MHz): 4.56 (s, 2H), 6.43 (s, 2H), 7.41-7.43 (m, 1H), 7.50-7.55 (m, 2H), 7.62-7.66 (m, 1H), 7.68-7.79 (m, 1H), 7.90-7.95 (m, 1H), 8.12-8.22 (t, J = 6.80Hz, 1H). ESI-MS: 359.35 ($C_{16}H_{12}F_2N_5OS$, $[M+H]^+$). Anal.Calcd for $C_{16}H_{11}F_2N_5OS$: C, 53.48; H, 3.09; N, 19.49%. Found: C, 53.49; H, 3.10; N, 19.46%.

4.6 Antiproliferative assay

After three days of incubation, the antiproliferative activity of the prepared compounds **4-22** against MCF-7 human breast cancer cells and HT29 human colorectal cancer cells were evaluated as following protocol. Target tumor cells (MCF-7 and HT29) were grown to log phase in RPMI 1640 medium supplemented with 10% fetal bovine serum. After reaching a dilution of 3×10^4 cells mL^{-1} with the medium, 100 μL of the obtained cell suspension was added to each well of 96-well culture plates. Subsequently, incubation was performed at 37°C in 5% CO_2 atmosphere for 24 h before the cytotoxicity assessment. Tested samples at pre-set concentrations were added to six wells with 5-fluorouracil being employed as a positive reference. After 48 h exposure period, 25 μL of PBS containing 2.5 mg mL^{-1}

of MTT was added to each well. After 4 h, the medium was replaced by 150 μL DMSO to dissolve the purple formazan crystals produced. The absorbance at 570 nm of each well was measured on an ELISA plate reader. The data represented the mean of three independent experiments in triplicate. The IC_{50} value was defined as the concentration at which 50% of the cells could survive. The results were summarized in Table 1.

4.7 FAK inhibitory assay

To evaluate the effect of the compounds on FAK assembly *in vitro*, varying concentrations were preincubated with 10 μM FAK in glutamate buffer at 30 ^{o}C and then cooled to 0 ^{o}C. After addition of GTP, the mixtures were transferred to 0 ^{o}C cuvettes in a recording spectrophotometer and warmed up to 30 ^{o}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.[28,29]

4.8 Apotosis assay

MCF-7 cells were treated with various concentrations of compound **4** for 24h and then stained with both Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI). Then samples were analyzed by FACSCalibur flow cytometer.

4.9 Docking simulations

Molecular docking of compounds **4** into the three dimensional X-ray structure of FAK catalytic subunit (PDB code: 2ETM) was carried out using the Discovery Stutio (version 3.1) as implemented through the graphical user interface DiscoveryStudio CDOCKER protocol.

The three-dimensional structures of the aforementioned compounds were constructed using ChemBio 3D Ultra 11.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2008)], then they were energetically minimized by using MMFF94 with 5000 iterations and minimum RMS gradient of

0.10. The crystal structures of FAK catalytic subunit were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.-do). All bound water and ligands were eliminated from the protein and the polar hydrogen was added. The whole 2ETM was defined as a receptor and the site sphere was selected based on active centre of 2ETM according to previous report, then compounds **4** were placed during the molecular docking procedure. Types of interactions of the docked protein with ligand were analyzed after the end of molecular docking.

Acknowledgements

The work was financed by a grant (No. J1103512) from Natural Science Foundation of China.

References

1. Jemal,A.; Siegel, R.; Ward, E.; Murray, T.; Xu, J.. *CA Cancer J. Clin.* 2007, 57, 51
2. Anand, P.; Kunnumakara, A. B.; Sundaram, C.; Harikumar, K. B.; Tharakan, S. T.; Lai, O. S.; Sung, B.; Aggarwal, B. B.. *Pharmaceutical Research.* 2008, 25, 2097.
3. Seymore, L. *Cancer Treat. Rev.* 1999, 25, 301.
4. Bruce, N. A.; Gold, L. S.. *Mutation Research.* 1991, 250, 3.
5. Tamara, Kazarian; Adnan, A. Jabbar; Fei-Qui Wen; Dharmesh, A. Patel; Leonard, A. Valentino. *Clinical Experimental Metastasis.* 2003, 20, 311.
6. John, T. Price; Erik, W. Thompson. *Expert Opinion on Therapeutic Targets.* 2002, 6, 217.
7. Deroock, Ian B.; Pennington, Michael E.; Sroka, Thomas C.; Lam, Kit S.; Bowden, G. Tim; Bair, Elisabeth L.; Gress, Anne E. *Cancer Research.* 2001, 61, 3308.
8. Sonoda Y; Watanabe S; Matsumoto Y; Aizu-Yokota E; Kasahara T. *J Biol Chem.* 1999, 274, 10566.
9. Hiscox, S.; Barnfather, P.; Hayes, E.; Bramble, P.; Christensen, J.; Nicholson, R.I.; Barrett-Lee, P. *Breast Cancer Res. Treat.* 2011, 125, 659.

10. Maroesja J. van Nimwegen; Bob van de Water. *Biochemical Pharmacology.* 2007, 73, 597.

11. Dolman, S. J.; Gosselin, F.; Oshea, P. D.; Davies, I. W.. *J. Org. Chem.* 2006, 71, 9548.

12. Khan, M. T.; Choudhary, M. I.; Khan, K. M.; Rani, M.; Rahman, A. U.. *Bioorg. Med. Chem.* 2005, 13, 3385.

13. Zarghi, A.; Tabatabai, S. A.; Faizi, M,; Ahadian, A.; Navabi, P.; Zanganeh, V.; Shafiee, A.. *Bioorg. Med. Chem. Lett.* 2005, 15, 1863.

14. Hadady, Zsuzsa; Toth, Marietta; Somsak, Laszlo. *Arkivoc*, 2004, 7, 140

15. Liberman, D.; Rist, N.. *Chem. Abstr.* 1957, 51, 14137.

16. Ouyang, X.; Patnitski, E.; Pattaropong, V.; Chen, X.; He, H.; Kiselyov, A.; Velankar, A.; Kawakami, J.; Labelle, M.; Smith II, L.; Lohman, J.; Lee, S.; Malikzay, A.; Fleming, J.; Gerlak, J.; Wang, Y.; Rosler, R.; Zhou, K.; Mitelman, S.; Camara, M.. *Bioorg. Med. Chem. Lett.* 2006, 16, 1191.

17. Liu, F.; Luo, X. Q.; Song, B. A.; Bhadury, P. S.; Yang, S.; Jin, L. H.; Xue, W.; Hu, D. Y.. *Bioorg. Med. Chem*. 2008, 16, 3632.

18. Jakubkiene, V.; Burbuliene, M. M.; Udrenaite, E.; Garaliene, V.; Vainilavicius, P.. *Pharmazie.* 2002, 57, 610.

19. Khan, S. A.; Kumar, P.; Joshi, R.; Iqbal, P. F.; Saleem, K.. *Eur. J. Med. Chem.* 2008, 43, 2029.

20. Li-Rong Zhang; Zhi-Jun Liu; Hui Zhang; Jian Sun; Yin Luo; Ting-Ting Zhao; Hai-Bin Gong; Hai-Liang Zhu.. *Bioorg. Med. Chem*. 2012, 20, 3615.

21. Sanna, P.; Carta, A.; Gherardini, L.; Nikookar, M. E. R.. *Farmaco*. 2002, 57, 79.

22. Sanna, P.; Carta, A.; Gherardini, L.; Nikookar, M. E. R.. *Eur. J. Med. Chem*. 2005, 48, 2972.

23. Hande, V. D.; Vasaitis, T. S.; Njar, V. C. O.; Gedia, L. K.; Katariak, R.; Chopra, P.; Newman, D.; Farquhar, R.; Guo, Z.; Qui, Y.; Brodie, A. M. H.. *J. Med. Chem*. 2005, 48, 2972.

24. Habib, N. S.; Fahmy, S.; El-Khawas, S. M.; Aziem, T. Abdel. *Pharmazie*. 2000, 55, 900.

25. Somani, Rakesh R.; Shirodkar, Prabhakar Y.; Kadam, Vilasrao J.. *Chinese Journal of Chemistry*. 2008, 26, 1727.

26. Zheng, Q. Z.; Zhang, X. M.; Xu, Y.; Cheng, K.; Jiao, Q. C.; Zhu, H. L.. *Bioorg. Med. Chem*. 2010, 18, 7836.

27. Ding-Wu Shen, Lynn M. Pouliot, Matthew D. Hall, Michael M. Gottesman, David R. Sibley. *Pharmacological Reviews*. 2012, 64, 706.

28. Hamel, E.; Lin, C. M. *Biochem. J.* 1984, 23, 4173.

29. Hamel, E. *Cell Biochem. Biophys.* 2003, 38, 1.

Legends for figures and schemes

Table 1. Antiproliferative activity of the synthesized compounds (**4-22**)

Table 2. FAK inhibitory activity of the selected compounds (**4-22**)

Table 3. The docking calculation of the synthesized compounds (**4-22**)

Figure 1. MCF-7 cells were cultured with anticancer and various concentrations of **4** for 24h. Cells were stained by Annexin VeFITC/PI and apoptosis was analyzed by flow cytometry. The lower left quadrants showed the vital cells; the upper right quadrants shows the dead cells, containing the secondary necrotic cells and late apoptotic cells; the lower right quadrants showed the early apoptotic cells. Inhibition includes early and late apoptosis.

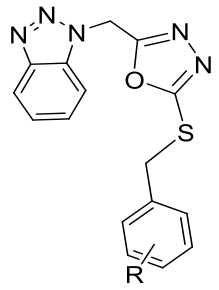
Figure 2. Ligand interaction diagram of compound **4** with FAK using Discovery Studio program with the essential amino acid residues at the binding site are tagged in circles. The purple circles show the amino acids which participate in hydrogen bonding, electrostatic or polar interactions and the green circles show the amino acids which participate in the Van der Waals interactions.

Figure 3. The 3D model structure of compound **4** binding mode with FAK: compound **4** was nicely bound to FAK with its nitrogen atom of oxadiazole ring toward the amino hydrogen of CYS502, forming H-bond interaction. Also the benzene ring formed π-cation interaction with LYS454.

Figure 4. The surface model structure of compound **4** binding mode with FAK

Scheme 1. General synthesis of compounds **4-22**. Reagents and conditions: (I) ethyl chloroacetate, acetone, K_2CO_3; reflux, 8h; (II) $NH_2NH_2{\cdot}H_2O$ (85%), methanol; 4 , 12h; (III) (i) CS_2/KOH, ethanol; reflux 24h; (ii) HCl, PH 5-6; (IV) acetonitrile, NaOH; reflux 10-24h.

Table 1.



Compounds	R	IC_{50}(μg/ml) MCF-7	HT29
4	2-F	5.68	10.21
5	2-Cl	10.75	15.27
6	2-Br	11.81	14.30
7	2-CH_3	18.89	26.81
8	2-OCH_3	16.24	25.10
9	2-NO_2	15.60	19.35
10	3-F	8.25	15.47
11	3-Cl	12.46	15.60
12	3-Br	16.30	31.20
13	3-CH_3	28.92	38.50
14	3-OCH_3	24.22	36.22
15	3-NO_2	20.80	35.10
16	4-F	8.70	17.62
17	4-Br	17.66	33.86
18	4-CH_3	30.23	42.30
19	4-OCH_3	25.82	37.62
20	4-NO_2	21.49	34.80
21	3,4-2Cl	45.16	40.24
22	3,4-2F	33.75	38.30
Cisplatin		11.20	15.83

Table 2.

Compounds	IC_{50}(μM)	Compounds	IC_{50}(μM)

4	1.2±0.3	14	7.5±0.2
5	9.5±0.1	15	7.0±0.9
6	9.8±0.2	16	9.1±0.5
7	12.1±1.3	17	23.7±2.1
8	7.1±0.2	18	33.8±1.4
9	7.6±0.5	19	8.6±0.3
10	7.1±0.3	20	9.2±0.5
11	8.3±0.7	21	28.5±0.9
12	14.2±0.4	22	33.6±0.4
13	15.8±1.1	***Cisplatin***	8.6±0.2

Table 3.

Compounds	CDOCKER INTERATION ENERGY △Gb(kcal/mol)	Compounds	CDOCKER INTERATION ENERGY △Gb(kcal/mol)
4	-37.7739	14	-35.4762
5	-33.5530	15	-35.4690
6	-33.0835	16	-33.7178
7	-32.3942	17	-31.2003
8	-35.7090	18	-29.4891
9	-35.2066	19	-33.8951
10	-35.5875	20	-33.4284
11	-33.7163	21	-30.4152
12	-31.8194	22	-30.4910
13	-31.6473		

Figure 1.

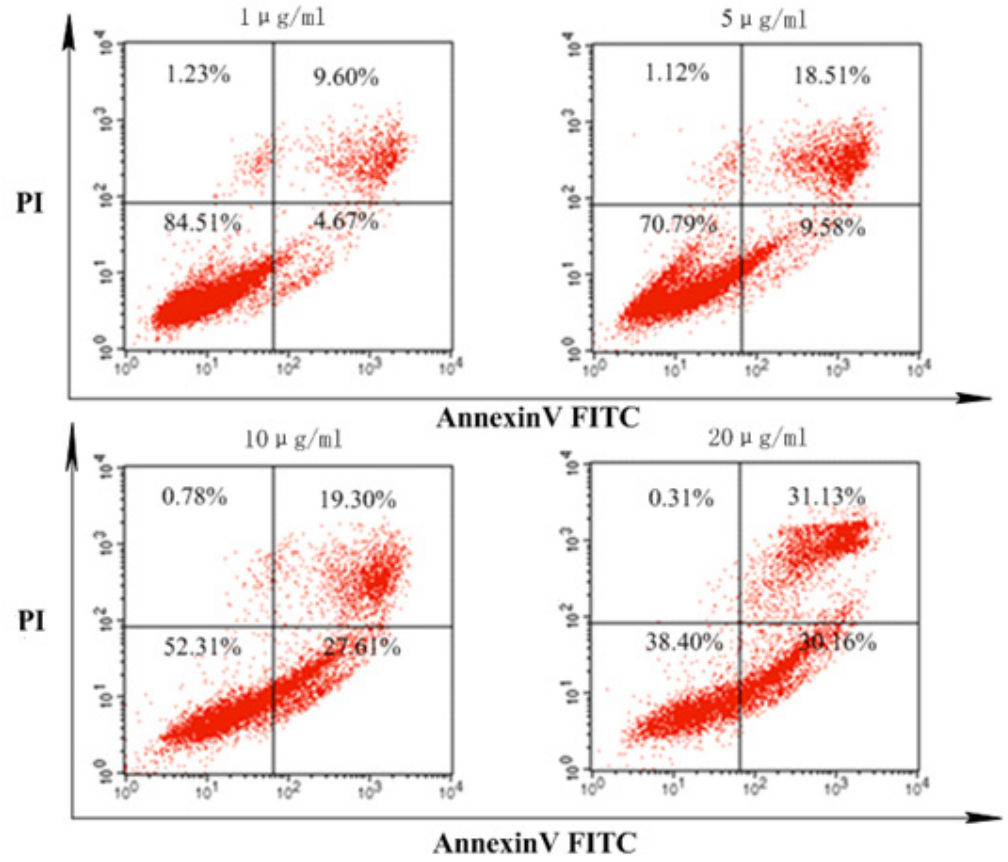


Figure 2.

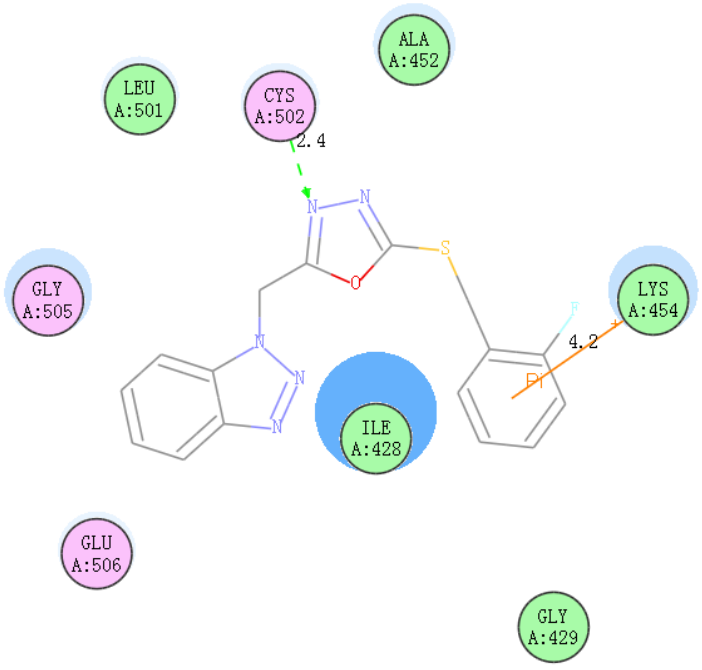


Figure 3.

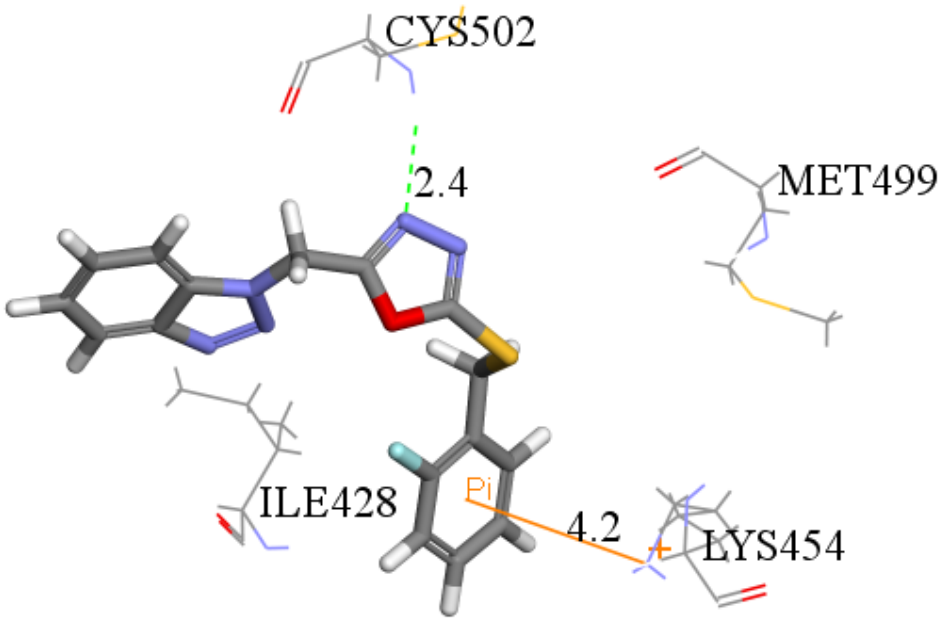
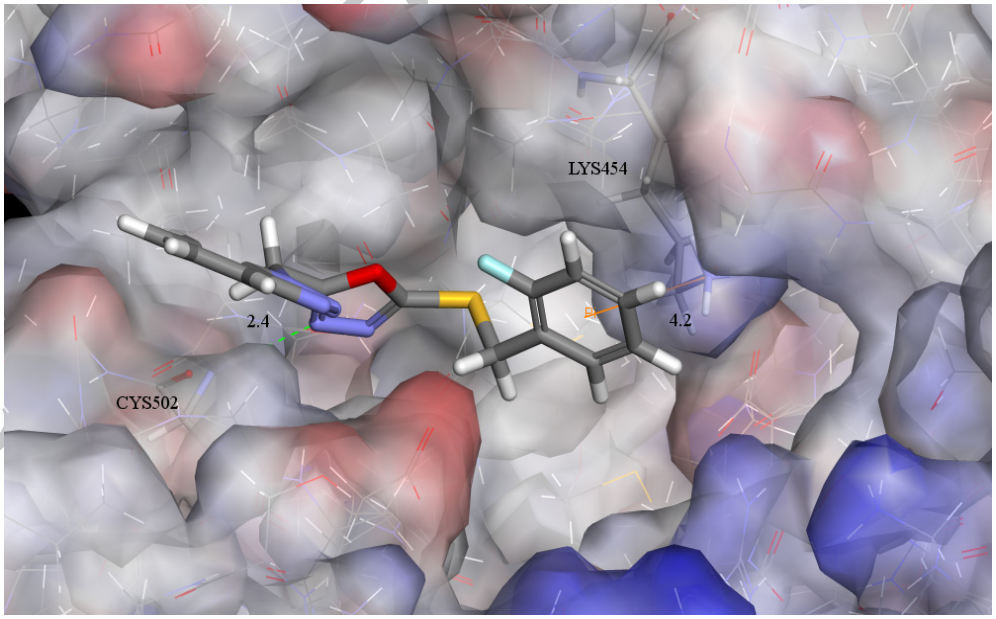
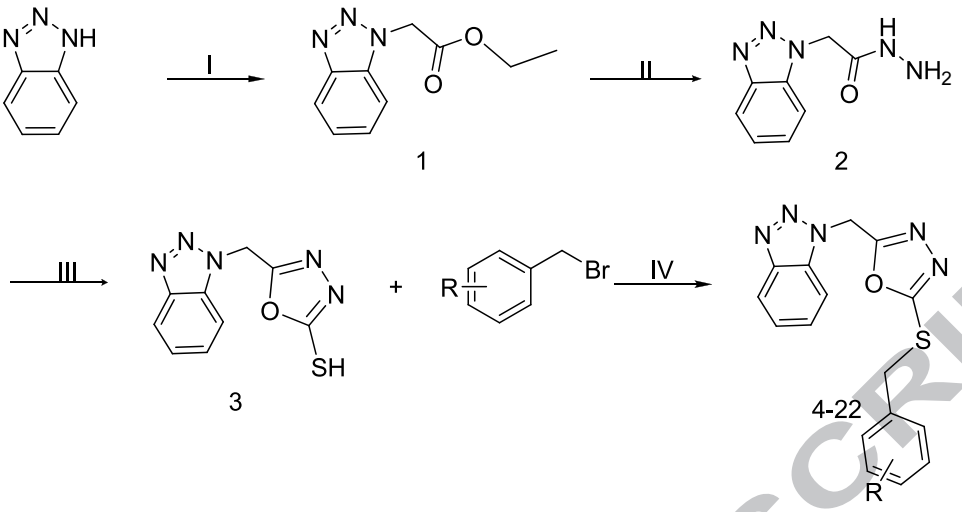


Figure 4.



Scheme 1.



Synthesis, biological evaluation, and molecular docking studies of novel 1,3,4-oxadiazole derivatives possessing benzotriazole moiety as FAK inhibitors with anticancer activity

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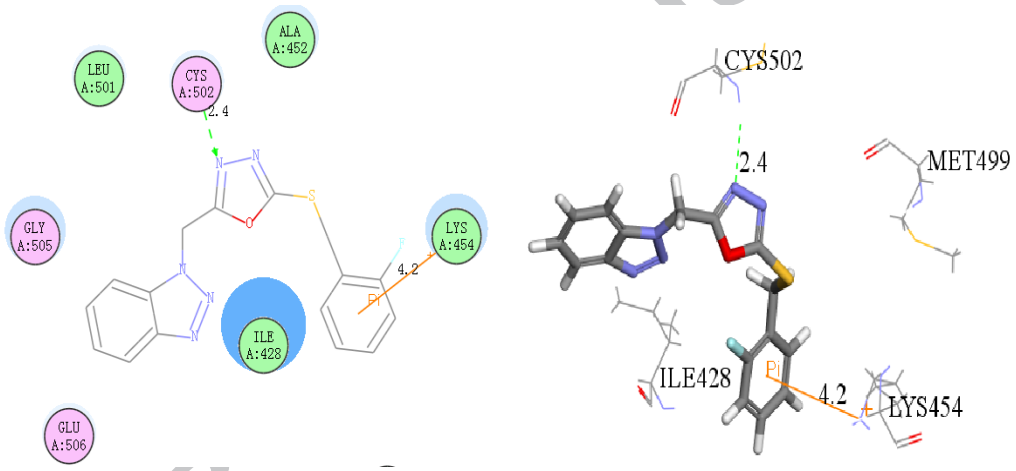

A series of 1,3,4-oxadiazole derivatives have been designed and synthesized, and their biological activities were also evaluated for FAK inhibitory activity. Compound **4** possessed the most potent enzyme inhibition activities (IC_{50} = 1.2±0.3 μM for FAK) and anticancer activities (IC_{50} = 5.68 μg/ml for MCF-7 and IC_{50} = 10.21 μg/ml for HT29). Structure-activity relationship was also analysed to provide more pharmacophore understanding that could be used to design new agents with more potent FAK inhibitory activity. Docking simulation was performed to explore the binding model of compound **4** with FAK.