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Design, synthesis, biological evaluation and molecular modeling of novel 1,3,4-oxadiazole derivatives based on Vanillic acid as potential immunosuppressive agents

Jian-Feng Tang [a,†], Xian-Hai Lv [c,†], Xiao-Liang Wang [a], Jian Sun [a], Yan-Bin Zhang [a], Yu-Shun Yang [a], Hai-Bin Gong [b,*], Hai-Liang Zhu [a,*]

[a] State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, PR China
[b] Xuzhou Central Hospital, Xuzhou 221009, PR China
[c] College of Science, Anhui Agricultural University, Hefei 230036, PR China

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ABSTRACT

In present study, a series of novel 1,3,4-oxadiazole derivatives have been designed, synthesized and purified. All of these compounds are reported for the first time, the chemical structures of these compounds were confirmed by means of ^{1}H NMR, ESI-MS and elemental analyses. Besides, we evaluated their immunosuppressive activity. Most of these synthesized compounds were proved to have potent immunosuppressive activity and low toxicity. Among them, the bioassay results demonstrated that compounds **5c**, **5n**, **5p**, **5o**, 6f and **6g** exhibited immunosuppressive activities with IC_{50} concentration range from 1.25 μM to 7.60 μM against the T cells, and the IC_{50} of positive control (csa) is 2.12 μM. Moreover, all the title compounds were assayed for PI3K/AKT signaling pathway inhibition using the ELISA assay. We examined the compounds with potent inhibitory activities against IL-1, IL-6 and IL-10 released in ConA-simulated mouse lymph node cells. The results showed compounds **5o** and **6f** displayed the most potential biological activity against T cells (IC_{50} = 1.25 μM and 4.75 μM for T cells). The preliminary mechanism of compound **5o** inhibition effects was also detected by flow cytometry (FCM). The results of apoptosis and ELISA assay demonstrated that the immunosuppressive activity of compounds **5o** and **6f** against T cells may be mediated by the inhibition of PI3Kγ/AKT signaling pathway. Molecular docking was performed to position compounds **5o** and **6f** into PI3Kγ binding site in order to indicate the potential target.

1. Introduction

Autoimmune diseases represent a major cause of morbidity and mortality. More than 70 autoimmune diseases have been described, and, although many of these diseases are quite rare, the collective prevalence of autoimmune diseases is high. The major autoimmune diseases include rheumatoid arthritis, psoriasis, Crohn's disease, systemic lupus erythematosus, and multiple sclerosis.[1–3] Immunosuppressant is an important class of clinical drugs for an array of medical processes, including transplant rejection and the treatment of autoimmune diseases. Although immunosuppressive drugs have been used for the treatment of autoimmune diseases in clinic, their side effects including liver toxicity, nephro toxicity, infection, cardiovascular toxicity and others cannot be neglected.[4–8] Therefore, there is a clinical need for new and less toxic therapeutic agents with new mechanisms of action.

Phosphoinositide 3-kinases (PI3Kγ) are lipid kinases that use the g-phosphate of ATP to 3-phosphorylate phosphoinositides on the inositol moiety. Their lipid products, the 3-phosphorylated phosphoinositides, are ubiquitous intracellular messengers that carry signals relevant to a huge range of cell functions in both health and disease.[9–12] According to their molecular structure, cellular regulation and in vivo substrate specificities, PI3Ks are divided into class I, II and III PI3Ks. The best known PI3Ks are Class I PI3Ks, which include subclass IA (consisting of PI3Kα, PI3Kβ and PI3Kδ isoforms) and subclass IB (consisting of PI3Kγ isoform only).[13,14] Among these isoforms, PI3Kδ and PI3Kγ are mainly expressed in the hematopoietic system and mediate immune responses. PI3Kγ (the only isoform of class IB) plays a pivotal role in inflammation, and it is involved in allergy, development of chronic inflammation, autoimmune diseases.[15,16] Therefore, there is important significance to discover a kind of drug that can influence PI3Kγ.

1,3,4-Oxadiazoles are an important class of heterocyclic compounds. The widespread use of them as a scaffold in medicinal chemistry establishes this moiety as a member of the privileged structures class.[17] 1,3,4-Oxadiazoles are associated with many

* Corresponding authors. Tel.: +86 25 8359 2572; fax: +86 25 8359 2672.
E-mail addresses: ghbxzh@yahoo.com.cn (H.-B. Gong), zhuhl@nju.edu.cn (H.-L. Zhu).
† These two authors contributed equally to this work.

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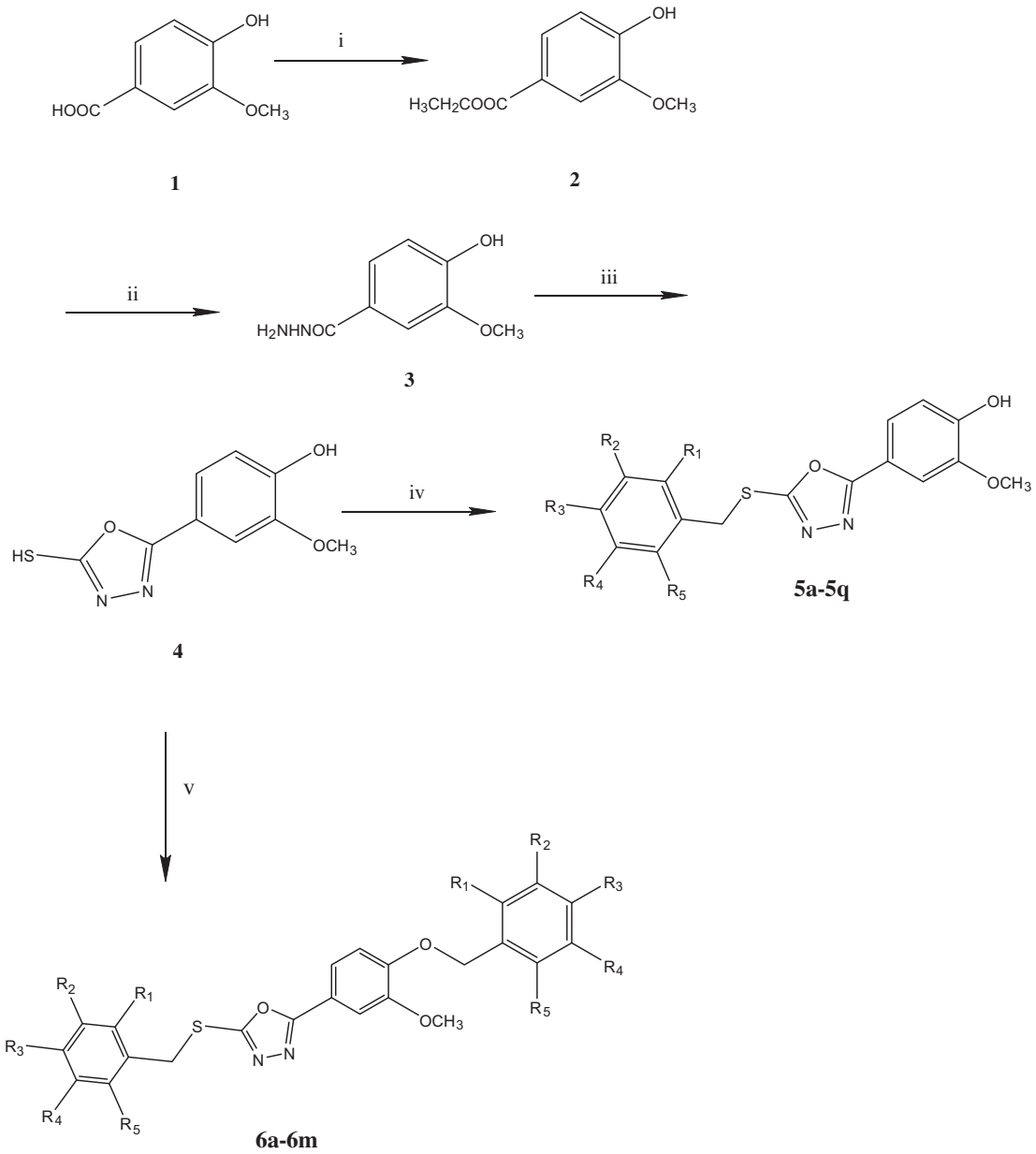
types of biological properties such as anti-inflammatory, hypoglycemic, antifungal and antibacterial activities.[18–21] Among these, a few differently substituted 1,3,4-oxadiazoles have exhibited potent immunosuppressive activities particually.[22–24] Therefore, oxadiazole derivatives have raised considerable attention to medicinal research, and a large number of investigations on their synthesis and biological activities have been reported during the last 10 years.[25–28]

Vanillic acid (4-hydroxy-3-methoxy benzoic acid), an oxidized form of vanillin which has been used as a chemical intermediate in the production of pharmaceuticals, is found in many traditional Chinese medicines, such as Rhizoma Picrorhizae, Ginseng, Propolis and BaiHao.[29] Vanillic acid has been reported to possess anti-inflammatory activity.[30] Thus, in this paper, we designed, synthesized and purified a series of novel 1,3,4-oxadiazole derivatives (**5a**–**5q**, **6a**–**6q**) derived from Vanillic acid. Docking simulations were performed to explore the probable molecular target of these compounds and studied their immunosuppressive activities and PI3Kγ inhibitory activity. Biological evaluation indicated that compounds **5o** and **6f** were potent inhibitors of PI3Kγ.

2. Results and discussion

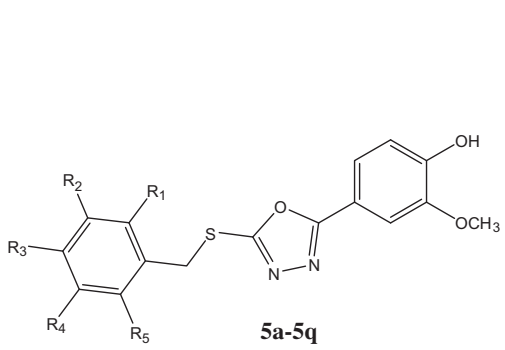
2.1. Chemistry

In this study, 34 1,3,4-oxadiazole derivatives (**5a**–**5q**, **6a**–**6q**) derived from 4-hydroxy-3-methoxy benzoic acid were firstly synthesized to screen for the anti-inflammatory activity. The synthetic route was based on the following sequence of reactions,[31] which was shown in Scheme 1. As an example, the reaction of 4-hydroxy-3-methoxybenzoic acid **1** with concentrated H_2SO_4 was refluxed in ethanol for 10 h to produce the corresponding ester **2**. Secondly, a mixture of the ester and hydrazine hydratein ethanol was refluxed overnight, by which the corresponding hydrazide **3**

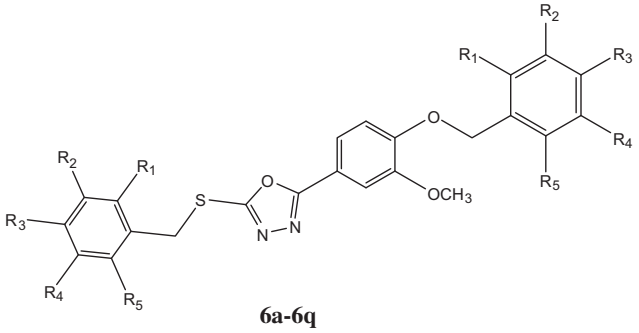


Scheme 1. General synthesis of compounds (**5a**–**6q**, **6a**–**6q**).

Table 1
Structure of 1,3,4-oxadiazole derivatives



5a-5q



6a-6q

Compound	R_1	R_2	R_3	R_4	R_5
5a	H	H	NO_2	H	H
5b	H	Br	H	H	H
5c	H	H	Me	H	H
5d	NO_2	H	H	H	H
5e	H	NO_2	H	H	H
5f	F	H	H	H	H
5g	H	H	Cl	H	H
5h	F	H	H	H	H
5i	F	H	F	H	H
5j	H	Cl	H	H	H
5k	Br	H	H	H	H
5l	H	H	F	H	H
5m	Cl	H	H	H	H
5n	Me	H	H	H	H
5o	H	H	H	H	H
5p	H	OMe	H	H	H
5q	H	H	Br	H	H
6a	H	F	H	H	F
6b	Me	H	H	H	H
6c	H	H	Br	H	H
6d	Cl	H	H	H	H
6e	H	H	Cl	H	H
6f	F	H	H	H	H
6g	F	H	H	H	H
6h	H	H	F	H	H
6i	H	Br	H	H	H
6j	Br	H	H	H	H
6k	F	H	F	H	H
6l	H	H	H	H	H
6m	H	NO_2	H	H	H
6n	H	H	NO_2	H	H
6o	H	Cl	H	H	H
6p	H	H	Me	H	H
6q	H	OMe	H	H	H

was formed. Thirdly, treatment of the hydrazide **3** with carbon disulfide in the presence of KOH and 95% ethanol under reflux provided the key intermediate **4**. Finally the synthesis of compounds **5a**–**5q** was accomplished by refluxing **4** with equimolar amount different substituted benzyl bromides in the presence of NaOH in acetonitrile. The synthesis of compounds **6a**–**6q** was accomplished by refluxing **4** with excessive different substituted benzyl bromides in the presence of NaOH in acetonitrile. All target compounds were purified by recrystallisation. All of the synthetic compounds gave satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures. All structure of the synthetic compounds were shown in Table 1.

2.2. Biological activity

2.2.1. Cytotoxicity test

The inhibitory activity of the compounds is sometimes a result of their toxic effects and consequently might cause an erroneous conclusion. Prior to the following bioactivity analysis, all the synthesized 1,3,4-oxadiazole derivatives (**5a**–**5q**, **6a**–**6q**) were tested in vitro for their cytotoxicity on lymph node cells. The pharmacological results were summarized in Table 2. The data showed that most of the 1,3,4-oxadiazole derivatives had a quite low toxicity. The potential drug should has a low toxicity. Considering this factor, compound **5o** and **6f** may be a potential anti-inflammatory drug with low toxicity (CC_{50} = 211.04 μM and 170.84 μM).

2.2.2. Inhibitory activity assay

All the synthesized 1,3,4-oxadiazole derivatives (**5a**–**5q**, **6a**–**6q**) were evaluated for their inhibitory activity on murine lymphocyte proliferation induced by concanavalin A (ConA). The results were summarized in Table 2. Among them, compounds 5o and **6f** (**IC_{50} =** 1.25 μM and 4.75 μM for T cells) exhibited the most potent immunosuppressive activity.

Structure–activity relationships (SARs) were inferred from Table 2. We can arrive at the conclusion that the activity of the

tested compounds may be correlated to structure variation and modifications. A comparison of the *para* substituents on the benzene ring demonstrated that compounds (**5a**–**5q**) with electronic-donating substituent (methyl) (**5c**) showed more potent inhibitory activities than compounds only contain electronic-withdrawing substituent (halogen) (**5g**). Among the compounds with electronic-withdrawing substituents, the activities of fluorin substituent (**5c**) is a little higher activity than that of chlorin substituent (**5g**), which indicated that compounds (**5a**–**5q**) with small substituent (F) showed more potent inhibitory activities than compounds with big substituent (Cl). Meanwhile, compound **5o** displayed the best immune suppression activity, as it has the lowest IC_{50} (IC_{50} = 1.25 μM) and low toxicity (CC_{50} = 211.04 μM). For 5o is the compound with no substituent, we can arrive at the conclusion that the size of substituent has a great impact on the activity of the tested compounds (**5a**–**5q**), and the compounds with smaller substituent showed greater activity. As the compound **5o** showed the best activity, and **5o** is the compound with no substituent. So, we can come to a conclusion, comparing to the electronic character of substituent, the size of substituent has a greater impact on the activity of the tested compounds (**5a**–**5q**).

Compound (**6a**–**6q**) with different benzaldehyde substituents displayed different immunosuppressive activity. A comparison of the *ortho* substituents on the benzene ring demonstrated that compounds with halogen (**6f**) may have more potent inhibitory activities than compounds with methyl (**6b**). Furthermore, the inhibitory activity was governed by the position of substituents. The position of substituent son benzene ring also influenced the activities. Take the compounds with Br substituents for example, the order of the activities is that substituent at the *ortho* (**6j**) position > substituent at the *meta* (**6i**) position > substituent at the *para* (**6c**) position. Among them, compound **6f** with fluorin group at the *ortho* position displayed the best immunosuppression activity, as it has the lowest IC_{50} (IC_{50} = 4.76 μM) and low toxicity (CC_{50} = 170.84 μM).

There are some differences in structure between series **5**(**5a**–**5q**) and series **6**(**6a**–**6q**). The synthesis of compounds (**5a**–**5q**) were accomplished by the combination of 1,3,4-oxadiazole with equimolar amount benzyl bromides. Meanwhile, the synthesis of compounds (**6a**–**6q**) were accomplished by the combination of 1,3,4-oxadiazole with benzyl bromides and phenolic hydroxyl group with benzyl bromides. From the inhibitory activity result, we can find that the compounds of series **5** and the compounds of series **6** all showed potent inhibitory activities, so we can proof that the phenolic hydroxyl group of series **5** is not the activity site.

In summary, compounds **5o** and **6f** displayed the most potential biological activity against T cells (IC_{50} = 1.25 μM and 4.75 μM for T cells), and they may be a potential immunosuppressive agents.

Table 2
All the synthesized 1,3,4-oxadiazole derivatives (**5a**–**5q**, **6a**–**6q**) were tested in vitro for their cytotoxicity and inhibitory activity on lymph node cells

Compound	CC_{50} ± SD (μM)	IC_{50} ± SD (μM)	SI
5a	688.22±60.23	14.16 ± 0.21	48.60
5b	382.15 ± 37.93	48.06 ± 0.39	7.95
5c	549.26 ± 53.87	6.21 ± 0.07	10.19
5d	330.75 ± 33.14	9.60 ± 0.09	34.45
5e	392.24 ± 38.42	15.95 ± 0.17	24.59
5f	473.72 ± 46.84	5.79 ± 0.22	81.82
5g	479.83 ± 46.98	11.07 ± 0.14	43.35
5h	574.98 ± 56.89	10.07 ± 0.12	57.10
5i	520.08 ± 51.97	9.69 ± 0.08	53.67
5j	470.40 ± 46.18	8.29 ± 0.09	56.74
5k	363.31 ± 35.32	17.79 ± 0.18	20.42
5l	684.82 ± 69.24	8.19 ± 0.13	83.62
5m	564.32 ± 56.79	8.43 ± 0.07	66.94
5n	592.43 ± 59.36	8.42 ± 0.06	70.36
5o	211.04 ± 22.56	1.25 ± 0.01	168.83
5p	389.43 ± 39.56	7.41 ± 0.03	52.55
5q	455.46 ± 46.78	8.83 ± 0.10	51.58
6a	265.84 ± 25.85	14.37 ± 0.15	18.49
6b	195.49 ± 18.96	18.87 ± 0.17	15.19
6c	224.53 ± 21.87	17.28 ± 0.18	12.99
6d	144.26 ± 14.34	10.64 ± 0.15	8.67
6e	136.31 ± 14.23	17.18 ± 0.28	5.00
6f	170.84 ± 18.53	4.76 ± 0.05	35.89
6g	178.57 ± 16.78	5.22 ± 0.06	34.21
6h	153.30 ± 14.67	12.89 ± 0.13	11.89
6i	161.06 ± 15.89	9.12 ± 0.07	17.66
6j	150.95 ± 148.65	8.71 ± 0.09	17.33
6k	181.78 ± 17.96	12.89 ± 0.10	14.10
6l	196.67 ± 19.89	13.30 ± 0.14	14.79
6m	168.22 ± 17.05	12.23 ± 0.13	13.75
6n	155.61 ± 16.58	7.60 ± 0.06	20.48
6o	177.59 ± 18.06	15.81 ± 0.14	11.23
6p	275.25 ± 26.84	9.06 ± 0.08	30.38
6q	242.06 ± 23.95	10.07 ± 0.11	24.04
Csa	174.24 ± 17.34	2.12 ± 0.03	82.19

Table 3

Compound	PI3Kγ IC_{50} (μM)
5c	7.41
5n	6.48
5p	2.87
5o	1.34
6f	5.12
6g	5.74
LY294002	7.26[a]

[a] Reported value, Ref. 32.

2.2.3. PI3Kγ inhibitory assay

The PI3Kγ inhibitory potency of oxadiazole derivatives was examined and the results were presented in Table 3. The tested compounds showed potent PI3Kγ inhibitory. The results of PI3Kγ inhibitory activity of the tested compounds were corresponding to the structure relationships (SARs) of their inhibitory effects on lymph node cells. The results suggested that inhibitory activity on lymph node cells of the compounds may by inhibiting PI3Kγ enzymatic activity.

2.2.4. Apoptosis assay

Apoptosis is an essential mechanism used to eliminate activated T cells during the shutdown process of excess immune responses and maintains proper immune homeostasis, while deficient apoptosis of activated T cells is associated with a wide variety of immune disorders. In order to study the preliminary anti-inflammatory mechanism of the compounds, we performed flow cytometry (FCM). As representative of these 1,3,4-oxadiazole derivatives, compound **5o** as studied in vitro. As shown in Figure 1, lymph node cells stimulated with ConA were treated with 0.25 μM, 2.5 μM and 25 μM of compound **5o** for 24 h. The compound increased the percentage of apoptosis by Annexin V-FITC/PI staining in a dose-dependent manner. The result indicated that compounds **5o** could induce apoptosis of ConA stimulated lymph node cells.

2.2.5. ELISA assay

To determine whether target compounds suppress activation of AKT (a key downstream effector of PI3K and important node in the PI3K/AKT/mTOR signaling pathway) and Caspase 3 (an import antenzyme in apoptosis). Selected compounds **5c**, **5n**, **5p**, **5o**, **6n**, **6f**, **6g** were tested with potent inhibitory activities against IL-1, IL-6 and IL-10 released in ConA-simulated mouse lymph node cells. As shown in Figure 6, among these seven compounds, **5o** and **6f** exhibited the highest inhibitory effects against ConA-induced IL-1, IL-6 and IL-10 expression.

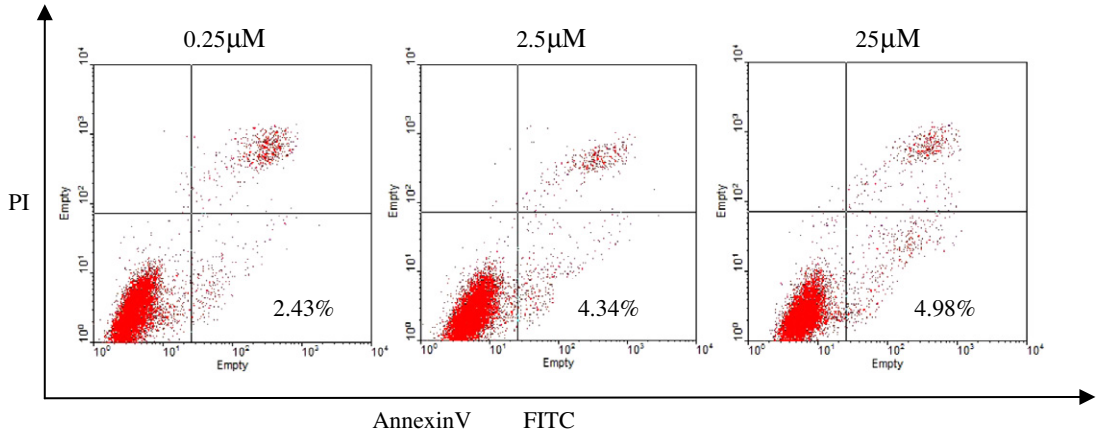


Figure 1. lymph node cells stimulated with ConA were treated with 0.25, 2.5 and 25 μM of compound **5o** for 24 h. The compound increased the percentage of apoptosis by Annexin V-FITC/PI staining in a dose-dependent manner. The result indicated that compounds **5o** could induce apoptosis of ConA stimulated lymph node cells.

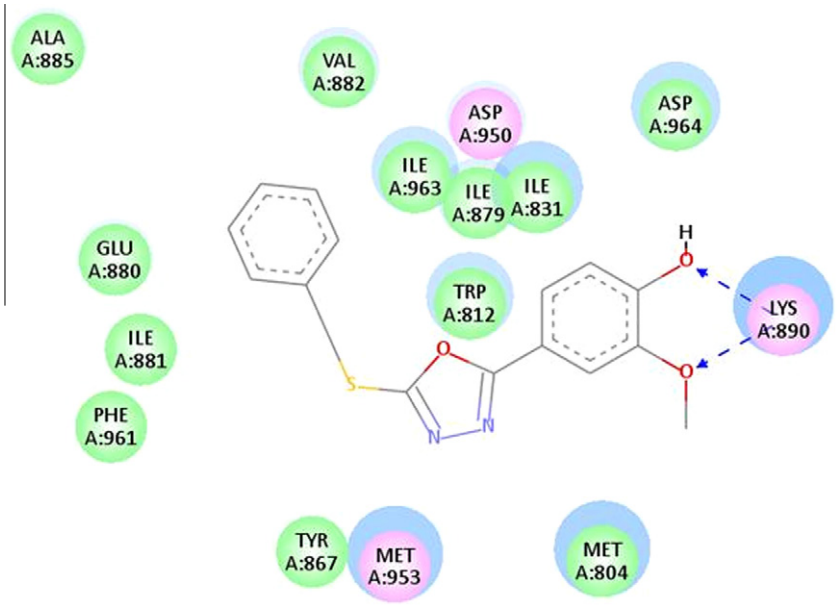


Figure 2. The molecular docking of compound **5o** into PI3Kγ. Compound **5o** is nicely bound to the PI3K via two hydrogen bonds (H–O···H: 2.04 Å, 112.2°, H–O···H: 2.02 Å, 139.2°).

2.3. Binding model of compounds 5o and 6f into PI3Kγ structure

To gain better understanding on the potency of the synthesized compounds and guide further structure–activity relationships (SARs) studies. The molecular docking was performed by potent inhibitor **5o** and **6f** into binding site of PI3Kγ. All docking runs were applied Discovery Studio3.1 (DS. 3.1). The binding modes of compound 5o and PI3Kγ were depicted in Figures 2 and 3. In the binding mode, compound **5o** is nicely bound to the PI3Kγ via three hydrogen bonds. The oxygen atom of 4-hydroxyl on the benzene group forms a hydrogen bond with primary hydrogen atom of LYS890 (H–O···H: 2.04 Å, 112.2°), the oxygen atom of 3-methoxyon the benzene group forms a hydrogen bond with primary hydrogen atom of LYS890 (H-O···H: 2.02 Å, 139.2°). The binding modes of compound 6f and PI3Kγ were depicted in Figures 4 and 5. In the binding mode, compound **6f** is nicely bound to the PI3Kγ via four hydrogen bonds. The oxygen atom of hydroxylon the benzene group form hydrogen bond with primary hydrogen atom of LYS890 (H–O···H: 1.64 Å, 129.8°), the oxygen atom on the benzene group forms a hydrogen bond with primary hydrogen atom of LYS890 (H–O···H: 1.85 Å, 134.7°), the fluorine atom on the benzene group form hydrogen bond with primary hydrogen atom of LYS833 (H–F···H: 2.05 Å, 111.9°), the fluorine atom on the benzene group form hydrogen bond with hydrogen atom of LYS802 (H–F···H: 2.07 Å, 149.5°). This molecular docking result, along with the biological assay data, suggested that compounds **5o** and 6f was a potential inhibitor of PI3Kγ.

3. Conclusions

In summary, a series of 1,3,4-oxadiazole derivatives have been synthesized and their immunosuppressive activities against ConA stimulated T cells proliferation were evaluated. Preliminary results showed that most of the compounds displayed enhanced inhibitory activities and low toxicity. Compounds **5o** and **6f** demonstrated the most potent inhibitory activity that inhibited the ConA stimulated T cells with IC_{50} = 1.25 μM and 4.75 μM for T cells

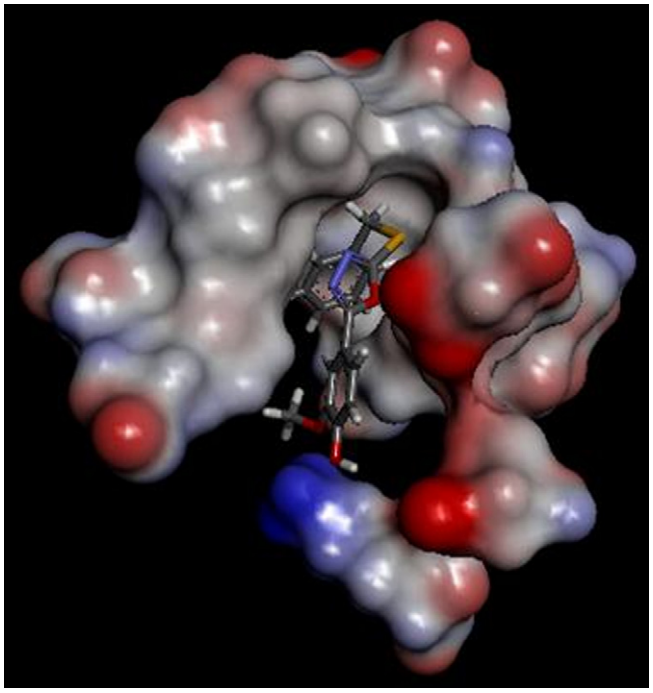


Figure 3. 3D model of the interaction between compound **5o** and PI3Kγ bonding site. The protein is represented by molecular surface. Compound **5o** is depicted by balls.

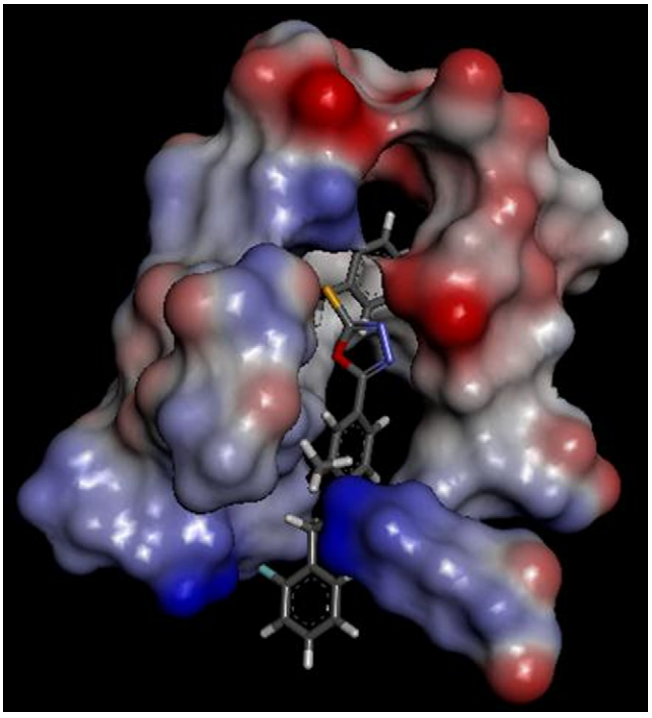


Figure 5. 3D model of the interaction between compound **6f** and PI3Kγ bonding site. The protein is represented by molecular surface. Compound **6f** is depicted by balls.

and inhibited the activity of PI3Kγ with IC_{50} of 1.34 μM. Moreover, the preliminary mechanism of compound **5o** inhibition effects was also detected by flow cytometry (FCM), and the compound exerted immunosuppressive activity via inducing the apoptosis of activated lymph node cells in a dose dependent manner. Molecular docking study indicated that compound **5o** and **6f** was nicely bound to the PI3Kγ with hydrogen bonds. All the results showed the great potential of compounds **5o** and **6f** as an immunosuppressant targeting PI3Kγ.

4. Experimental section

4.1. General

All chemicals used were purchased from Aldrich (USA). The reaction was monitored by thin layer chromatography (TLC). Melting points (uncorrected) were determined on an XT4 MP apparatus (Taike Corp., Beijing, China). ESI mass spectra were obtained on a Mariner System 5304 mass spectrometer, and ^{1}H NMR spectra

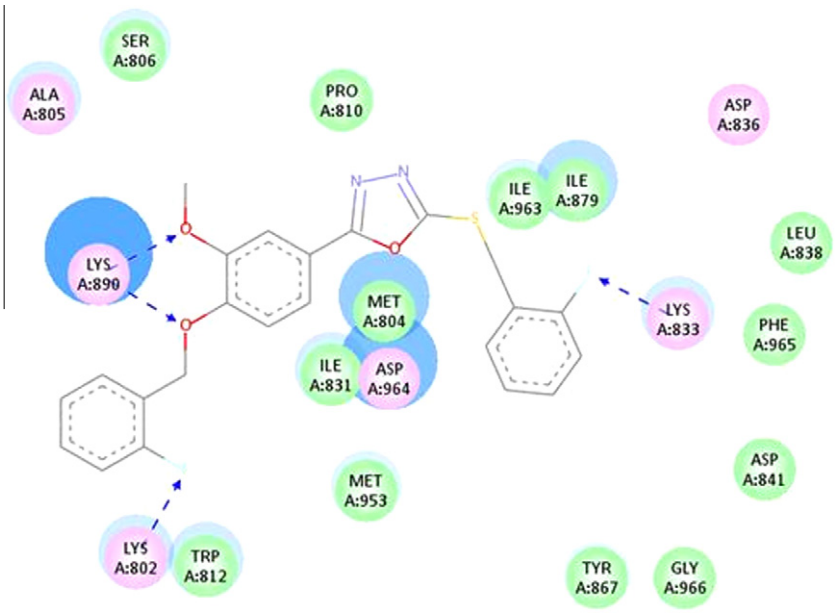


Figure 4. The molecular docking of compound **6f** into PI3Kγ. Compound **6f** is nicely bound to the PI3K via four hydrogen bonds (H–O···H: 1.64 Å, 129.8°, H–O···H: 1.85 Å, 134.7°, H–F···H: 2.05 Å, 111.9°, H–F···H: 2.07 Å, 149.5°).

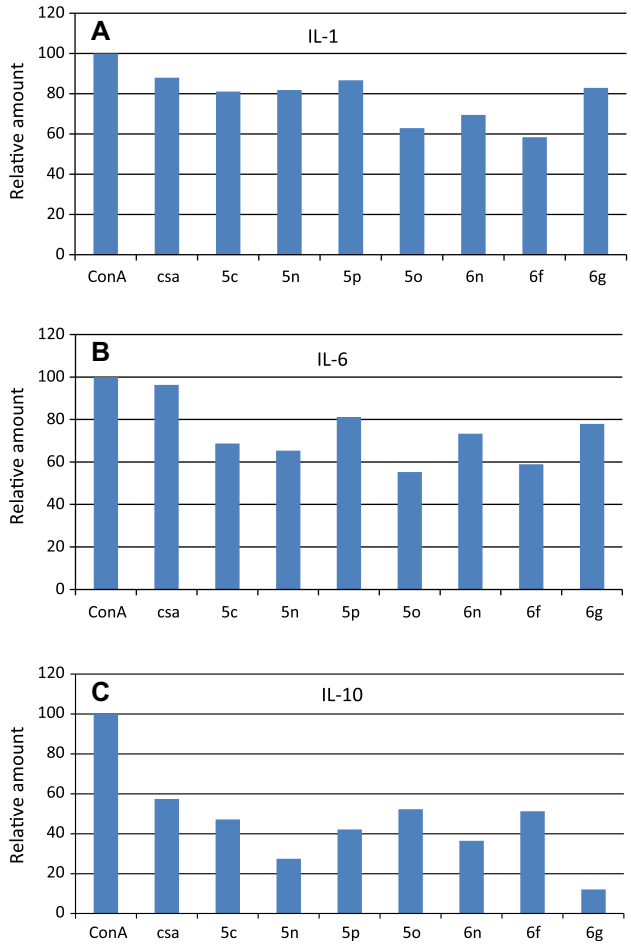


Figure 6. 1,3,4-Oxadiazole derivative inhibited ConA-induced IL-1, IL-6 and IL-10 secretion in Mouse lymph node cells, mouse lymph node cells were incubated in DMEM media (Gibco) supplemented with 10% FBS, 100 U/mL penicillin, and100 g/mL streptomycin at 37 °C with 5% CO2. Cells were pre-treated with 5 M of 1,3,4-oxadiazole derivatives, analogues or vehicle control for 2 h, then treated with ConA (5 g/mL) for 22 h. The culture media collected were centrifuged at 1000 rpm for 10 min. The levels of IL-1, IL-6 and IL-10 in the media were determined by ELISA using mouse IL-1, mouse IL-6 and mouse IL-10 ELISA Kits (BOSTER, USA).

were recorded on a Bruker PX 500 or DPX 300 spectrometer at 25 °C with TMS and solvent signals allotted as internal standards. Chemical shifts were reported in ppm (δ). Element analyses were performed on a CHN–O–Rapid instrument and were within 0.4% of the theoretical values.

4.2. Chemistry

4.2.1. General procedure for synthesis of ethyl 4-hydroxy-3-methoxybenzoate (2)

4-Hydroxy-3-methoxybenzoic acid (20 g, 119 mmol) in ethanol (50 mL) was treated with concentrated sulfuric acid (3 mL) under reflux for 10 h. The solvent was evaporated until no longer liquid outflow. Water (20 mL) was added, extracting by adding respectively 30 mL ethyl acetate. After washing the organic phase with saturated NaCl solution (40 mL), drying it with anhydrous Na_2SO_4 and evaporating the solvent under reduced pressure. Crude product were purified by column chromatography, product **2** appeared.

4.2.2. General procedure for synthesis of 4-hydroxy-3-methoxybenzohydrazide (3)

A stirred solution of compound **2** (50 mmol) in ethanol (50 mL) was treated with 85% hydrazine hydrate (20 mL), under reflux for 20 h. After cooling, removal of the solvent under reduced pressure gave a crude product, which was filtered off and purified by washing with saturated NaCl (40 mL) together with small quantity of ethanol to give compound **3**.

4.2.3. General procedure for synthesis of 2-methoxy-4-(1,3,4-oxadiazol-2-yl)phenol (4)

To a solution of **3** (16 g, 87.9 mmol) in alcohol (100 mL), carbon disulfide (45 mL), and potassium hydroxide (11.9 g, 21.2 mmol) were added and the resulting solution was heated to reflux for 20 h. The reaction mixture was concentrated and the residue was dissolved in water and acidified with hydrochloric acid. The resulting solid was filtered, dried and recrystallized from ethanol to afford compound **4** (12 g, 62.5 mmol) as a white solid.

4.2.4. General procedure for synthesis of the target compounds (5a–5q)

To a solution of 4(0.135 g, 0.7 mmol) in dry acetonitrile (10 mL), dry potassium hydroxide (0.03 g, 0.59 mmol) and corresponding benzyl bromides (0.7 mmol) were added and the resulting solution was heated to 85 °C for 3 h. The mixture was concentrated and taken in ethyl acetate (50 mL), washed with water (20 mL), saturated sodium chloride solution (20 mL) and dried over sodium sulfate. The resulting solution was concentrated and the purification of the residue by recrystallization from ethanol yielded the desired compounds **5a**–**6q**.

4.2.4.1. 2-Methoxy-4-(5-((4-nitrobenzyl)thio)-1,3,4-oxadiazol-2-yl)phenol (5a). Yellow solid. Yield: 68%. Mp: 151–153 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.98 (s, 3H), 4.57 (s, 2H), 5.99 (s, 1H), 7.01 (d, J = 8.25 Hz, 1H), 7.49 (d, J = 10.8 Hz, 2H), 7.68 (d, J = 8.6 Hz, 2H), 8.21 (d, J = 8.44 Hz, 2H). MS (ESI): 358.26 ($C_{16}H_{14}N_3O_5S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}N_3O_5S$: C, 53.48; H, 3.65; N, 11.69; O, 22.26; S, 8.92. Found: C, 53.34; H, 3.54; F, 5.75; N, 11.74; O, 23.06; S, 8.89.

4.2.4.2. 4-(5-((3-Bromobenzyl)thio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5b). Yellow solid. Yield: 89%. Mp: 129–131 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.99 (s, 3H), 4.47 (s, 2H), 7.01 (d, J = 8.22 Hz, 1H), 7.19–7.27 (m, 1H), 7.40–7.46 (m, 2H), 7.51 (d, J = 10.06 Hz, 2H), 7.63 (s, 1H). MS(ESI): 392.93 ($C_{16}H_{14}BrN_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}BrN_2O_3S$: C, 48.87; H, 3.33; Br, 20.32; N, 7.12; O, 12.21; S, 8.15. Found: C, 49.12; H, 3.34; Br, 20.17; N, 7.31; O, 11.67; S, 8.64.

4.2.4.3. 2-Methoxy-4-(5-((4-methylbenzyl)thio)-1,3,4-oxadiazol-2-yl)phenol (5c). White solid. Yield: 71%. Mp: 164–166 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 2.35 (s, 3H), 3.99 (s, 3H), 4.49 (s, 2H), 7.01 (d, J = 8.22 Hz, 1H), 7.16 (d, J = 7.86 Hz, 2H), 7.35 (d, J = 8.04 Hz, 2H), 7.51 (dd, J = 8.22 Hz, J = 2.02 Hz, 1H), 7.54 (d, J = 1.84 Hz, 1H). MS(ESI): 329.13 ($C_{17}H_{17}N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{16}N_2O_3S$: C, 48.87; H, 3.33; Br, 20.32; N, 7.12; O, 12.21; S, 8.15. Found: C, 49.12; H, 3.34; Br, 20.17; N, 7.31; O, 11.67; S, 8.64.

4.2.4.4. 2-Methoxy-4-(5-((2-nitrobenzyl)thio)-1,3,4-oxadiazol-2-yl)phenol (5d). Yellow solid. Yield: 84%. Mp: 144–146 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.96 (s, 3H), 4.84 (s, 2H), 5.99 (s, 1H), 6.99 (d, J = 8.76 Hz, 1H), 7.46–7.50 (m, 3H), 7.57–7.62 (m, 1H), 7.86 (d, J = 6.78 Hz, 1H), 8.14 (d, J = 8.04 Hz, 1H). MS(ESI): 358.64 ($C_{16}H_{14}N_3O_5S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}N_3O_5S$: C, 53.48; H, 3.65; N, 11.69; O, 22.26; S, 8.92. Found: C, 53.82; H, 3.67; N, 10.94; O, 23.02; S, 8.98.

4.2.4.5. 2-Methoxy-4-(5-((3-nitrobenzyl)thio)-1,3,4-oxadiazol-2-yl)phenol (5e). Yellow solid. Yield: 90%. Mp: 95–97 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.97 (s, 3H), 4.57 (s, 2H), 6.99 (d,

J = 8.02 Hz, 1H), 7.47–7.61 (m, 3H), 7.85 (d, *J* = 7.68 Hz, 1H), 8.02 (s, 1H), 8.16 (d, *J* = 8.4 Hz, 1H), 8.35 (s, 1H). MS(ESI): 358.93 ($C_{16}H_{14}N_3O_5S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}N_3O_5S$: C, 53.48; H, 3.65; N, 11.69; O, 22.26; S, 8.92. Found: C, 54.13; H, 3.64; N, 11.95; O, 22.02; S, 8.91.

4.2.4.6. 4-(5-((2-Fluorobenzyl)thio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5f). White solid. Yield: 81%. Mp: 134–136 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.98 (s, 3H), 4.53 (s, 2H), 5.98 (s, H), 7.00 (d, *J* = 8.04 Hz, 1H), 7.04–7.13 (m, 2H), 7.26–7.32 (m, 1H), 7.49–7.61 (m, 3H). MS(ESI): 331.35 ($C_{16}H_{14}FN_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}FN_2O_3S$: C, 57.82; H, 3.94; F, 5.72; N, 8.43; O, 14.44; S, 9.65. Found: C, 57.99; H, 3.93; F, 5.76; N, 8.41; O, 14.48; S, 9.62.

4.2.4.7. 4-(5-((4-Chlorobenzyl)thio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5g). White solid. Yield: 65%. Mp: 179–181 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.95 (s, 3H), 4.46 (s, 2H), 5.16 (s, 2H), 6.92 (d, *J* = 8.4 Hz, 1H), 7.28–7.31 (m, 2H), 7.36–7.41 (m, 6H), 7.47 (dd, *J* = 8.26 Hz, *J* = 2.0 Hz, 1H), 7.51 (d, *J* = 2.04 Hz, 1H). MS(ESI): 474.12 ($C_{16}H_{14}ClN_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}ClN_2O_3S$: C, 58.36; H, 3.83; Cl, 14.98; N, 5.92; O, 10.14; S, 6.77. Found: C, 59.03; H, 3.81; Cl, 14.99; N, 5.89; O, 10.25; S, 6.79.

4.2.4.8. 4-(5-((2,6-Difluorobenzyl)thio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5h). White solid. Yield: 73%. Mp: 185–187 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.98 (s, 3H), 4.54 (s, 2H), 6.89–6.94 (t, *J* = 7.77 Hz, 2H), 7.01 (d, *J* = 8.22 Hz, 1H), 7.26–7.30 (m, 2H), 7.51–7.55 (m, 2H). MS(ESI): 350.34 ($C_{16}H_{13}F_2N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{12}F_2N_2O_3S$: C, 54.85; H, 3.45; F, 10.85; N, 8.00; O, 13.70; S, 9.15. Found: C, 54.78; H, 3.46; F, 10.81; N, 7.95; O, 13.61; S, 9.11.

4.2.4.9. 4-(5-((2,4-Difluorobenzyl)thio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5i). White solid. Yield: 69%. Mp: 142–144 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.97 (s, 3H), 4.49 (s, 2H), 5.95 (s, 1H), 6.82–6.85 (m, 2H), 7.03 (d, *J* = 12.63 Hz, 1H), 7.47–7.55 (m, 3H). MS(ESI): 350.34 ($C_{16}H_{13}F_2N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{12}F_2N_2O_3S$: C, 54.85; H, 3.45; F, 10.85; N, 8.00; O, 13.70; S, 9.15. Found: C, 54.75; H, 3.43; F, 10.91; N, 8.02; O, 13.67; S, 9.19.

4.2.4.10. 4-(5-((3-Chlorobenzyl)thio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5j). White solid. Yield: 79%. Mp: 133–135 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.96 (s, 3H), 4.46 (s, 2H), 6.99 (d, *J* = 4.95 Hz, 1H), 7.26–7.27 (m, 2H), 7.34 (s, 1H), 7.46–7.51 (m, 3H). MS(ESI): 348.80 ($C_{16}H_{14}ClN_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}ClN_2O_3S$: C, 55.09; H, 3.76; Cl, 10.16; N, 8.03; O, 13.76; S, 9.19. Found: C, 54.99; H, 3.73; Cl, 10.18; N, 8.05; O, 13.73; S, 9.17.

4.2.4.11. 4-(5-((2-Bromobenzyl)thio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5k). White solid. Yield: 87%. Mp: 169–171 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.98 (s, 3H), 4.62 (s, 2H), 5.95 (s, 1H), 6.99 (d, *J* = 4.95 Hz, 1H), 7.15–7.18 (m, 1H), 7.26–7.28 (m, 1H), 7.48–7.51 (m, 2H), 7.59 (d, *J* = 4.86 Hz, 1H), 7.63 (d, *J* = 4.77 Hz, 1H). MS(ESI): 393.26 ($C_{16}H_{14}BrN_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}BrN_2O_3S$: C, 48.87; H, 3.33; Br, 20.32; N, 7.12; O, 12.21; S, 8.15. Found: C, 48.91; H, 3.31; Br, 20.40; N, 7.13; O, 12.25; S, 8.22.

4.2.4.12. 4-(5-((4-Fluorobenzyl)thio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5l). White solid. Yield: 84%. Mp: 161–163 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.99 (s, 3H), 4.48 (s, 2H), 6.99 (d, *J* = 5.04 Hz, 1H), 7.14 (d, *J* = 4.68 Hz, 2H), 7.33 (d, *J* = 4.77 Hz, 2H), 7.49 (d, *J* = 4.92 Hz, 1H), 7.53 (s, 1H). MS(ESI): 332.35 ($C_{16}H_{14}FN_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}FN_2O_3S$:C, 57.82; H, 3.94; F, 5.72; N, 8.43; O, 14.44; S, 9.65. Found: C, 57.79; H, 3.93; F, 5.74; N, 8.39; O, 14.44; S, 9.68.

4.2.4.13. 4-(5-((2-Chlorobenzyl)thio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5m). White solid. Yield: 91%. Mp: 160–162 °C. ^{1}H NMR (CDCl3, 300 MHz): 3.97 (s, 3H), 4.52 (s, 2H), 6.97 (d, *J* = 8.22 Hz, 1H), 7.33–7.39 (m, 4H), 7.42–7.54 (m, 7H). MS(ESI): 348.80 ($C_{16}H_{14}ClN_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{13}ClN_2O_3S$: C, 55.09; H, 3.76; Cl, 10.16; N, 8.03; O, 13.76; S, 9.19. Found: C, 55.15; H, 3.73; Cl, 10.22; N, 8.07; O, 13.67; S, 9.15.

4.2.4.14. 2-Methoxy-4-(5-((2-methylbenzyl)thio)-1,3,4-oxadiazol-2-yl)phenol (5n). White solid. Yield: 82%. Mp: 131–133 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.47 (s, 3H), 3.96 (s, 3H), 4.56 (s, 2H), 7.01 (d, *J* = 8.04 Hz, 1H), 7.18–7.24 (m, 3H), 7.41 (d, *J* = 6.78 Hz, 1H), 7.51–7.55(m, 2H). MS(ESI): 328.39 ($C_{17}H_{17}N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{16}N_2O_3S$: C, 62.18; H, 4.91; N, 8.53; O, 14.62; S, 9.76. Found: C, 62.26H, 4.94 N, 8.53; O, 14.65; S, 9.68.

4.2.4.15. 4-(5-(Benzylthio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5o). White solid. Yield: 75%. Mp: 114–116 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.97(s, 3H), 4.47 (s, 2H), 6.05 (s, 1H), 7.02 (d, *J* = 8.28 Hz, 1H), 7.47 (d, *J* = 10.8 Hz, 2H); 7.55–7.61 (m, 3H); 8.02 (d, *J* = 8.44 Hz, 2H). MS(ESI): 314.30 ($C_{16}H_{15}N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{16}H_{14}N_2O_3S$: C, 61.13; H, 4.49; N, 8.91; O, 15.27; S, 10.20. Found: C,59.07; H, 4.50; N, 8.89.

4.2.4.16. 2-Methoxy-4-(5-((3-methoxybenzyl)thio)-1,3,4-oxadiazol-2-yl)phenol (5p). White solid. Yield: 75%. Mp: 93–95 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.79 (s, 3H), 3.97 (s, 3H), 4.48 (s, 2H), 6.84 (d, *J* = 4.83 Hz, 1H), 6.98–7.05 (m, 3H), 7.35 (s, 1H), 7.46–7.51 (m, 3H). MS(ESI): 344.38 ($C_{17}H_{17}N_2O_4S$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{16}N_2O_4S$: C, 59.29; H, 4.68; N, 8.13; O, 18.58; S, 9.31. Found: C, 59.19; H, 4.66; N, 8.15; O, 18.63; S, 9.29.

4.2.4.17. 4-(5-((4-Bromobenzyl)thio)-1,3,4-oxadiazol-2-yl)-2-methoxyphenol (5q). White solid. Yield: 78%. Mp: 186–188 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.97 (s, 3H), 4.44 (s, 2H), 6.01 (s, 1H), 6.99 (d, *J* = 4.92 Hz, 1H), 7.33–7.36 (m, 2H), 7.45–7.49 (m, 4H). MS(ESI): 344.38 ($C_{17}H_{17}N_2O_4S$, $[M+H]^+$). Anal. Calcd for $C_{17}H_{16}N_2O_4S$:C, 59.29; H, 4.68; N, 8.13; O, 18.58; S, 9.31. Found: C, 59.16; H, 4.67; N, 8.15; O, 18.53; S, 9.29.

4.2.5. General procedure for synthesis of the target compounds (6a–6q)

To a solution of **4** (0.135 g, 0.7 mmol) in dry acetonitrile (10 mL), dry potassium hydroxide (0.03 g, 0.59 mmol) and corresponding benzyl bromides (1.4 mmol) were added and the resulting solution was heated to 85 °C for 3 h. The mixture was concentrated and taken in ethyl acetate (50 mL), washed with water (20 mL), saturated sodium chloride solution (20 mL) and dried over sodium sulfate. The resulting solution was concentrated and the purification of the residue by recrystallization from ethanol yielded the desired compounds **6a**–**6q**.

4.2.5.1. 2-(4-((3,5-Difluorobenzyl)oxy)-3-methoxyphenyl)-5-((3,5-difluorobenzyl)thio)-1,3,4-oxadiazole (6a). White solid. Yield: 79%. Mp: 137–139 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.94 (s, 3H), 4.49 (s, 2H), 5.20 (s, 2H), 6.81–6.93 (m, 4H), 6.99 (d, *J* = 8.22 Hz, 1H), 7.46–7.60 (m, 4H). MS(ESI): 476.52 ($C_{23}H_{17}F_4N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{16}F_4N_2O_3S$: C, 57.98; H, 3.38; F, 15.95; N, 5.88; O, 10.07; S, 6.73. Found: C, 56.45; H, 3.36; F, 14.33; N, 5.84; O, 10.36; S, 6.79.

4.2.5.2. 2-(3-Methoxy-4-((2-methylbenzyl)oxy)phenyl)-5-((2-methylbenzyl)thio)-1,3,4-oxadiazole (6b). Yellow solid. Yield: 75%, Mp: 76–78 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 2.46(s, 3H), 3.94 (s, 3H), 4.55 (s, 2H), 5.17 (s, 2H), 6.98 (d, J = 8.22 Hz, 1H), 7.17–7.26 (m, 7H), 7.32 (s, 1H), 7.41 (d, J = 7.68 Hz, 2H), 7.49–7.61 (m, 3H). MS(ESI): 431.92 ($C_{25}H_{25}N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{25}H_{24}N_2O_3S$: C, 69.42; H, 5.59; N, 6.48; O, 11.10; S, 7.41. Found: C, 68.89; H, 5.61; N, 6.65; O, 12.16; S, 7.38.

4.2.5.3. 2-(4-((4-Bromobenzyl)oxy)-3-methoxyphenyl)-5-((4-bromobenzyl)thio)-1,3,4-oxadiazole (6c). White solid. Yield: 83%, Mp: 167–169 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.97 (s, 3H), 4.46 (s, 2H), 5.17 (s, 2H), 6.93 (d, J = 5.68 Hz, 1H), 7.32–7.37 (m, 4H), 7.46–7.54 (m, 6H). MS(ESI): 563.12 ($C_{23}H_{19}Br_2N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{18}Br_2N_2O_3S$: C, 49.13; H, 3.23; Br, 28.42; N, 4.98; O, 8.54; S, 5.70. Found: C, 49.94; H, 3.25; Br, 27.82; N, 4.73; O, 8.84; S, 5.91.

4.2.5.4. 2-(4-((2-Chlorobenzyl)oxy)-3-methoxyphenyl)-5-((2-chlorobenzyl)thio)-1,3,4-oxadiazole (6d). White solid. Yield: 79%. Mp: 101–103 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.97 (s, 3H), 4.61 (s, 2H), 5.31 (s, 2H), 6.93 (d, J = 8.44 Hz, 1H), 7.22–7.26 (m, 2H), 7.27–7.28 (m, 2H), 7.40 (d, J = 9.34 Hz, 2H), 7.48 (dd, J = 8.4 Hz, J = 2.00 Hz, 1H), 7.54–7.55 (m, 2H), 7.62 (dd, J = 6.58 Hz, J = 2.56 Hz, 1H). MS(ESI): 472.74 ($C_{23}H_{19}Cl_2N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{18}Cl_2N_2O_3S$: C, 58.36; H, 3.83; Cl, 14.98; N, 5.92; O, 10.14; S, 6.77. Found: C, 58.67; H, 3.85; Cl, 13.69; N, 5.99; O, 10.44; S, 6.64.

4.2.5.5. 2-(4-((4-Chlorobenzyl)oxy)-3-methoxyphenyl)-5-((4-chlorobenzyl)thio)-1,3,4-oxadiazole (6e). White solid. Yield: 65%. Mp: 149–152 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.95 (s, 3H), 4.46 (s, 2H), 5.16 (s, 2H), 6.92 (d, J = 8.4 Hz, 1H), 7.28–7.31 (m, 2H), 7.36–7.41 (m, 6H), 7.47 (dd, J = 8.26 Hz, J = 2.0 Hz, 1H), 7.51 (d, J = 2.04 Hz, 1H). MS(ESI): 474.12 ($C_{23}H_{19}Cl_2N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{18}Cl_2N_2O_3S$: C, 58.36; H, 3.83; Cl, 14.98; N, 5.92; O, 10.14; S, 6.77. Found: C, 59.03; H, 3.81; Cl, 14.99; N, 5.89; O, 10.25; S, 6.79.

4.2.5.6. 2-(4-((2-Fluorobenzyl)oxy)-3-methoxyphenyl)-5-((2-fluorobenzyl)thio)-1,3,4-oxadiazole (6f). White solid. Yield: 81%. Mp: 98–100 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.95 (s, 3H), 4.53 (s, 2H), 5.27 (s, 2H), 6.98–7.18 (m, 5H), 7.26–7.32 (m, 2H), 7.49–7.54 (m, 4H). MS(ESI): 441.12 ($C_{23}H_{19}F_2N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{18}F_2N_2O_3S$: C, 62.72; H, 4.12; F, 8.63; N, 6.36; O, 10.90; S, 7.28. Found: C, 62.54; H, 4.17; F, 8.93; N, 6.19; O, 10.98; S, 7.51.

4.2.5.7. 2-(4-((2,6-Difluorobenzyl)oxy)-3-methoxyphenyl)-5-((2,6-difluorobenzyl)thio)-1,3,4-oxadiazole (6g). White solid. Yield: 73%. Mp: 123–136 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.91 (s, 3H), 4.54 (s, 2H), 5.24 (s, 2H), 6.89–6.96 (m, 4H), 7.11 (d, J = 8.44 Hz, 1H), 7.26–7.38 (m, 2H), 7.54–7.57 (m, 2H). MS(ESI): 475.82 ($C_{23}H_{17}F_4N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{16}F_4N_2O_3S$: C, 57.98; H, 3.38; F, 15.95; N, 5.88; O, 10.07; S, 6.73. Found: C, 56.71; H, 3.38; F, 14.29; N, 5.83; O, 10.29; S, 6.69.

4.2.5.8. 2-(4-((4-Fluorobenzyl)oxy)-3-methoxyphenyl)-5-((4-fluorobenzyl)thio)-1,3,4-oxadiazole (6h). White solid. Yield: 85%. Mp: 137–139 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.95 (s, 3H), 4.47 (s, 2H), 5.16 (s, 2H), 6.93–7.10 (m, 5H), 7.39–7.52 (m, 6H). MS (ESI): 442.07 ($C_{23}H_{19}F_2N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{18}F_2N_2O_3S$: C, 62.72; H, 4.12; F, 8.63; N, 6.36; O, 10.90; S, 7.28. Found: C, 63.11; H, 4.15; F, 8.89; N, 6.29; O, 10.88; S, 7.34.

4.2.5.9. 2-(4-((3-Bromobenzyl)oxy)-3-methoxyphenyl)-5-((3-bromobenzyl)thio)-1,3,4-oxadiazole (6i). White solid. Yield: 83%. Mp: 127–129 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.96 (s, 3H), 4.45 (s, 2H), 5.17 (s, 2H), 6.93 (d, J = 8.58 Hz, 1H), 7.18–7.26 (m, 2H), 7.36–7.53 (m, 6H), 7.61(s, 2H). MS(ESI): 565.03 ($C_{23}H_{19}Br_2N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{18}Br_2N_2O_3S$: C, 49.13; H, 3.23; Br, 28.42; N, 4.98; O, 8.54; S, 5.70. Found: C, 49.94; H, 3.25; Br, 27.82; N, 4.73; O, 8.84; S, 5.91.

4.2.5.10. 2-(4-((2-Bromobenzyl)oxy)-3-methoxyphenyl)-5-((2-bromobenzyl)thio)-1,3,4-oxadiazole (6j). White solid. Yield: 76%. Mp: 121–123 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.94 (s, 3H), 4.47 (s, 2H), 5.16 (s, 2H), 6.90 (d, J = 8.74 Hz, 1H), 7.16–7.24 (m, 2H), 7.35–7.52 (m, 6H), 7.63 (s, 2H). MS(ESI): 565.03 ($C_{23}H_{19}Br_2N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{18}Br_2N_2O_3S$: C, 49.13; H, 3.23; Br, 28.42; N, 4.98; O, 8.54; S, 5.70. Found: C, 49.85; H, 3.31; Br, 27.88; N, 4.69; O, 8.81; S, 5.94.

4.2.5.11. 2-((2,4-Difluorobenzyl)thio)-5-(4-(2,4-difluorophenoxy)-3-methoxyphenyl)-1,3,4-oxadiazole (6k). White solid. Yield: 89%. Mp: 135–137 °C.^{1}H NMR ($CDCl_3$, 300 MHz): 3.94 (s, 3H), 4.49 (s, 2H), 6.82–6.87 (m, 3H), 6.88–6.92 (m, 1H), 6.99 (d, J = 4.95 Hz, 1H), 7.47–7.55 (m, 4H). MS(ESI): 462.42 ($C_{22}H_{15}F_4N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{22}H_{14}F_4N_2O_3S$: C, 57.14; H, 3.05; F, 16.43; N, 6.06; O, 10.38; S, 6.93 Found:C, 57.19; H, 3.06; F, 16.38; N, 6.08; O, 10.31; S, 6.96.

4.2.5.12. 2-(4-(Benzyloxy)-3-methoxyphenyl)-5-(benzylthio)-1,3,4-oxadiazole (6l). White solid. Yield: 82%. Mp: 115–117 °C.1H NMR ($CDCl_3$, 300 MHz): 3.97 (s, 3H), 4.52 (s, 2H), 5.23 (s, 2H), 6.97 (d, J = 8.22 Hz, 1H), 7.33–7.37 (m, 4H), 7.39 (m, 7H). MS(ESI): 404.48 ($C_{23}H_{21}N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{20}N_2O_3S$: C, 68.30; H, 4.98; N, 6.93; O, 11.87; S, 7.93 Found:C, 68.25; H, 4.95; N, 6.92; O, 11.91; S, 7.89.

4.2.5.13. 2-(3-Methoxy-4-((3-nitrobenzyl)oxy)phenyl)-5-((3-nitrobenzyl)thio)-1,3,4-oxadiazole (6m). White solid. Yield: 79%. Mp:149–151 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.97 (s, 3H), 4.57 (s, 2H), 5.28 (s, 2H), 6.95 (d, J = 5.13 Hz, 1H), 7.48–7.59 (m, 4H), 7.82 (dd, J = 18.93 Hz, J = 18.93 Hz, 2H), 8.18 (dd, J = 12.27 Hz, J = 12.09 Hz, 2H), 8.34 (d, J = 3.75 Hz, 2H). MS(ESI): 494.48($C_{23}H_{19}N_4O_7S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{18}N_4O_7S$: C, 55.87; H, 3.67; N, 11.33; O, 22.65; S, 6.48 Found:C, 55.95; H, 3.64; N, 11.29; O, 22.71; S, 6.45.

4.2.5.14. 2-(3-Methoxy-4-((4-nitrobenzyl)oxy)phenyl)-5-((4-nitrobenzyl)thio)-1,3,4-oxadiazol (6n). White solid. Yield: 86%. Mp: 156–158 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.95 (s, 3H), 4.55 (s, 2H), 5.29 (s, 2H), 6.94 (d, J = 5.13 Hz, 1H), 7.45–7.56 (m, 4H), 7.81 (dd, J = 18.87 Hz, J = 18.96 Hz, 2H), 8.16 (dd, J = 12.31 Hz, J = 12.04 Hz, 2H), 8.36 (d, J = 3.74 Hz, 2H). MS(ESI): 494.48 ($C_{23}H_{19}N_4O_7S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{18}N_4O_7S$: C, 55.87; H, 3.67; N, 11.33; O, 22.65; S, 6.48 Found:C, 55.79; H, 3.69; N, 11.33; O, 22.68; S, 6.51.

4.2.5.15. 2-(4-((3-Chlorobenzyl)oxy)-3-methoxy)phenyl)-5-((3-chlorobenzyl)thio)-1,3,4-oxadiazole (6o). White solid. Yield: 76%. Mp: 124–126 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.96 (s, 3H), 4.66 (s, 2H), 5.35 (s, 2H), 6.91 (d, J = 8.44 Hz, 1H), 7.26–7.32 (m, 2H), 7.28–7.29 (m, 2H), 7.44 (d, J = 9.34 Hz, 2H), 7.51 (dd, J = 8.3 Hz, J = 2.00 Hz, 1H), 7.56–7.57 (m, 2H), 7.65 (dd, J = 6.59 Hz, J = 2.52 Hz, 1H). MS(ESI): 472.74 ($C_{23}H_{19}Cl_2N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{23}H_{18}Cl_2N_2O_3S$: C, 58.36; H, 3.83; Cl, 14.98; N, 5.92; O, 10.14; S, 6.77. Found: C, 58.62; H, 3.88; Cl, 13.73; N, 5.89; O, 10.43; S, 6.62.

4.2.5.16. 2-(3-Methoxy-4-((4-methylbenzyl)oxy)phenyl)-5-((4-methylbenzyl)thio)-1,3,4-oxadiazole (6p). Yellow solid. Yield: 75%, Mp: 76–78 °C. ^{1}H NMR ($CDCl_3$, 300 MHz):2.67 (s, 3H), 3.95 (s, 3H), 4.56 (s, 2H), 5.16 (s, 2H), 6.99 (d, J = 8.24 Hz, 1H), 7.16–7.25 (m, 7H), 7.35 (s, 1H), 7.42 (d, J = 7.68 Hz, 2H), 7.48–7.60 (m, 3H). MS(ESI): 431.92 ($C_{25}H_{25}N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{25}H_{24}N_2O_3S$: C, 69.42; H, 5.59; N, 6.48; O, 11.10; S, 7.41. Found: C, 68.69; H, 5.67; N, 6.63; O, 12.18; S, 7.33.

4.2.5.17. 2-(3-Methoxy-4-((3-methoxybenzyl)oxy)phenyl)-5-((3-methoxybenzyl)thio)-1,3,4-oxadiazole (6q). White solid. Yield: 79%. Mp: 112–114 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 3.14 (s, 3H), 3.93 (s, 3H), 4.57 (s, 2H), 5.14 (s, 2H), 6.92 (d, J = 8.79 Hz, 2H), 7.11–7.22 (m, 5H), 7.36 (s, 2H), 7.51 (d, J = 7.64 Hz, 3H), 7.49–7.61 (m, 2H). MS(ESI): 431.92 ($C_{25}H_{25}N_2O_3S$, $[M+H]^+$). Anal. Calcd for $C_{25}H_{24}N_2O_3S$: C, 69.42; H, 5.59; N, 6.48; O, 11.10; S, 7.41. Found: C, 68.89; H, 5.61; N, 6.65; O, 12.16; S, 7.38.

4.3. Cytotoxicity test

Fresh lymph node cells were obtained from BALB/C mice. Cells were cultured in a 96-well plate at a density of 5×10^5 cells per well. Different compounds were added to each well respectively at the concentration of 10 μM. Cells without treated by compounds were used as the control. The incubation was performed in a humidified, 37 °C, 5% CO_2-containing incubator for 24 h. 20 μL of MTT (Sigma, MO; 4 mg/mL in PBS) was added to each well 4 h before the end of the incubation. MTT formazan production was dissolved in 200 μL DMSO replacing the medium at 37 °C for 30 min. The absorbance was read on an ELISA reader (Tecan, Austria) at 570 nm (OD570).

4.4. Inhibitory activity assay

Fresh lymph node cells were obtained from BALB/C mice. 5×10^5 cells per well were cultured in a 96-well plate at the same conditions to that mentioned above. The cultures were stimulated with 5 μg/mL of concanavalin A (ConA) to induce T cell proliferative responses for 24 h. After that, cells were left untreated or treated with compounds at the concentration of 1 μM for 72 h. Twenty microliters of MTT (Sigma, MO; 4 mg/mL in PBS) was added to each well 4 h before the end of the incubation. After moving the supernatant, 200 μL DMSO was added to dissolve the formazan crystals. The absorbance at 570 nm (OD570) was read on an ELISA reader (Tecan, Austria). Inhibitory activity of each compound was calculated using the following formula:

$$\text{Inhibitory activity} = [\text{Control(OD570 nm)} - \text{Compounds(OD570 nm)}]/\text{Control(OD570 nm)} \times 100\%$$

Cells untreated with compounds were used as the control.

4.5. Apoptosis assay

Lymph node cells were incubated in a 24-well plate at a density of 3×10^6 cells per well and stimulated with 5 μg/mL of ConA for 24 h. Then different concentrations of compound **5o** were added to each well. Twenty-four hours later, cells were harvested and stained with Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI). And then samples were analyzed by FACS Calibur flow cytometer (Becton Dickinson, SanJose, CA).

4.6. PI3Kγ inhibitory assay

Thirty-four 1,3,4-oxadiazole derivatives were tested in a search for small molecule inhibitors of PI3Kγ,which was purchased from R&D Systems (Minneapolis, MN). PI3Kγ was incubated for 4 h at room temperature with or without the presence of the oxadiazoles derivatives, the final concentration of drug as 3 μM, 10 μM, 30 μM and 50 μM. The results were summarized in Table 3.

4.7. Cell treatment and ELISA assay

Mouse lymph node cells were incubated in DMEM media (Gibco) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. Cells were pretreated with 5 μM of 1,3,4-oxadiazole derivatives, analogues or vehicle control for 2 h, then treated with ConA (5 μg/mL) for 22 h. The culture media collected were centrifuged at 1000 rpm for 10 min. The levels of IL-1, IL-6 and IL-10 in the media were determined by ELISA using mouse IL-1, mouse IL-6 and mouse IL-10 ELISA Kits (BOSTER, USA). After centrifugation, collect use a sterile container, centrifugation 20-min at the speed of 2000–3000 rpm. Remove supernatant, detect the composition of cells, dilute cell suspension with PBS (pH 7.2–7.4). Cell concentration reach 1 million/mL, repeated freeze-thaw cycles, damage cells and release of intracellular components, centrifugation 20-min at the speed of 2000–3000 rpm. Remove supernatant, if precipitation appeared, centrifugal again. The results were shown in Figures 6.

4.8. Molecular docking

The PI3Kγ-LXX protein–ligand complex crystal structure (3L54.pdb) was chosen as the template for the modeling study of compounds **5o** and **6f** bound to PI3Kγ. The crystal structure was obtained from the RCSB Protein Data Bank. The molecular docking procedure was performed by using LigandFit protocol within Discovery Studio 3.1. For ligand preparation, the 3D structures of **5o** and **6f** were generated and minimized using Discovery Studio 3.1. For enzyme preparation, the hydrogen atoms were added, and the water and impurities were removed. The whole PI3Kγ enzyme was defined as a receptor and the site sphere was selected based on the ligand binding location of LXX, then the LXX molecule was removed and **5o** and **6f** were placed during the molecular docking procedure. Types of interactions of the docked enzyme with ligand were analyzed after the end of molecular docking.

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