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RESEARCH ARTICLE

New Hydrazone Derivatives of Pyrazole-4-carboxaldehydes Exhibited Anti-inflammatory Properties

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ARTICLE HISTORY

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Abstract: ***Background*:** Several series of hydrazone derivatives of pyrazole-4-carboxaldehydes (**4-11**) were designed and synthesized to screen their inflammatory activity.

***Methods*:** The products were characterized by ^{1}H NMR, ^{13}C NMR and HRMS. *In vitro* LPS-induced TNF-α model and *in vivo* xylene-induced ear-edema model were used to evaluate their anti-inflammatory activity.

***Results and Conclusion*:** Bioassays indicated that most of the compounds markedly inhibited the expression of TNF-α at the concentration of 10 μg/mL. Compounds **7b** and **11c**, two of the most potent compounds, exhibited TNF-α inhibitory ability in a dose-dependent manner with IC_{50} values of 5.56 and 3.69 μM, respectively. *In vivo*, intraperitoneal administration with **7b** and **11c** markedly inhibited the ear edema at the doses of 20 and 50 mg/kg. Compound **11c**, inhibited edema by 49.59 % at the dose of 20 mg/kg, was comparable to the reference drug dexamethasone at the same dose (with an inhibition of 50.49 %). To understand the binding pattern, molecular docking of representative **7b** and **11c** was performed, which demonstrated that both compounds have a forceful binding with the TNF-α, and that the phenyl and hydrazide moieties of them play a significant role in binding with the target site.

Keywords: Anti-inflammatory, hydrazine, pyrazole, TNF-α, docking, antiinflammatory activity.

1. INTRODUCTION

Inflammation is part of the complex biological response of body tissues in response to the damage resulting from microbial pathogen infection, chemical stimuli, and physical trauma [1]. Although acute inflammation is required as a defense mechanism, persistent inflammation is harmful and should be suppressed. Dysregulation of inflammation may lead to various diseases such as arthritis, heart attacks, atherosclerosis, Alzheimer's disease, diabetes and even cancer [2-4]. Tumor Necrosis Factor alpha (TNF-α) has been well recognized as pro-inflammatory cytokine, which plays an important role in inflammatory-related diseases by amplifying inflammatory signals *via* multiple pathways [5, 6]. Hence, the inhibition of this cytokine provides a target for controlling and treating inflammatory diseases and attracts much attention in current anti-inflammatory drug discovery [7-9].

Pyrazoles are significant double nitrogen, five-membered, heterocyclic compounds. Since the discovery and approval of celecoxib as an anti-inflammatory agent in the 1990s, many medicinal chemists have focused on the design and synthesis of pyrazole derivatives as anti-inflammatory drugs [10-13]. Hydrazones are a class of organic compounds in the Schiff base family, which have also been extensively investigated for their uses in medicinal chemistry. Hydrazone and their derivatives are known to exhibit interesting diverse biological activities like antioxidant [14], anti-inflammatory [15-17], anticonvulsant [18, 19], antimicrobial [20-22], and anticancer [23-25]. Mohamed *et. al.* described some pyrazole-hydrazone derivatives, which showed potent anti-inflammatory agents *via* inhibiting COX-2 or 5-LOX enzymes [26-28]. These findings indicated that the combination of pyrazole and hydrazone moieties into one molecule is an alternation strategy to obtain potent anti-inflammatory agents.

In our previous work, a skeleton of pyrazole-hydrazone was established to evaluate their anti-inflammatory and antimicrobial activities [29]. Most of these compounds showed excellent TNF-α inhibitory activity, and some of them were shown to have comparable *in vivo* anti-inflammatory activity to the reference drug dexamethasone. As a continuation of the previous work, in this study, twenty-four muti-substituted

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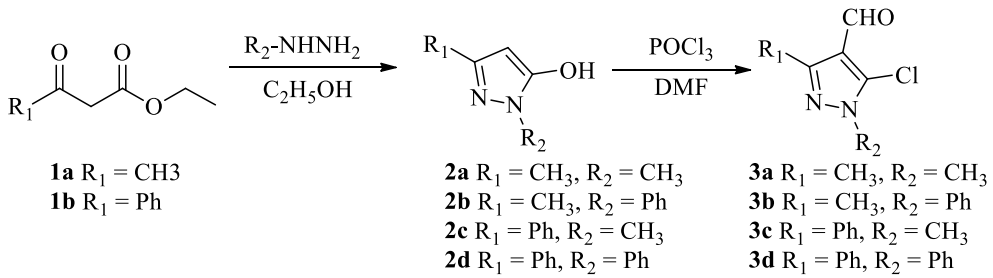
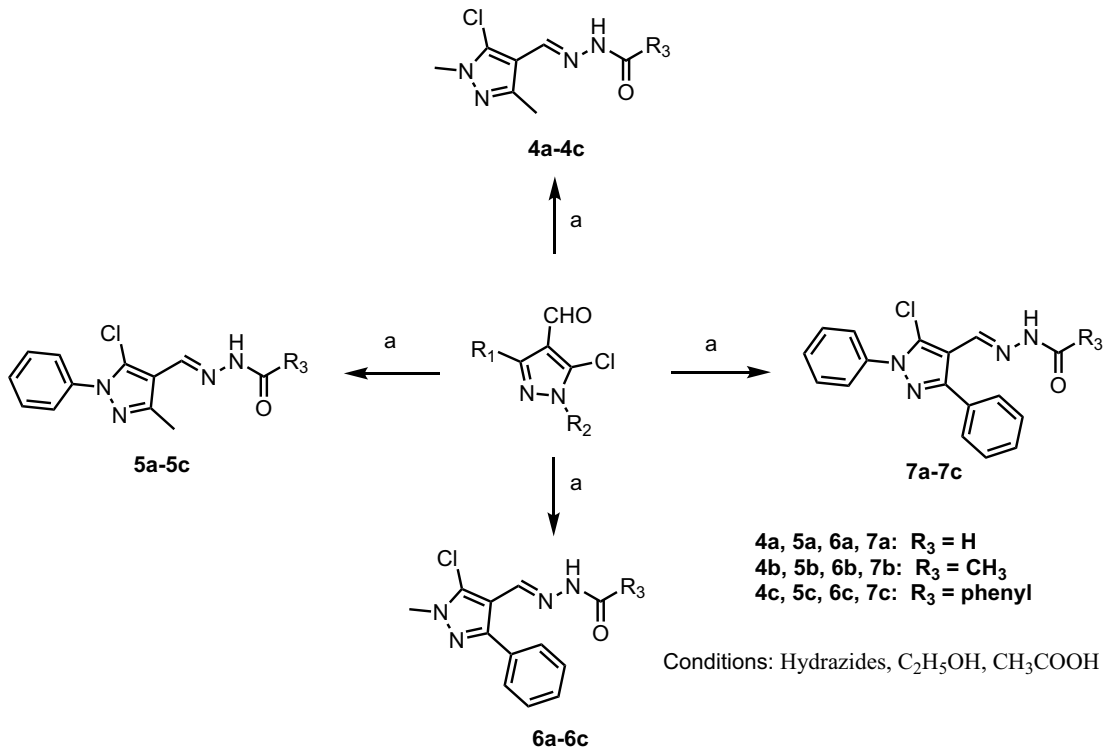


Scheme 1. The synthesis route of intermediates **3a-3d.**



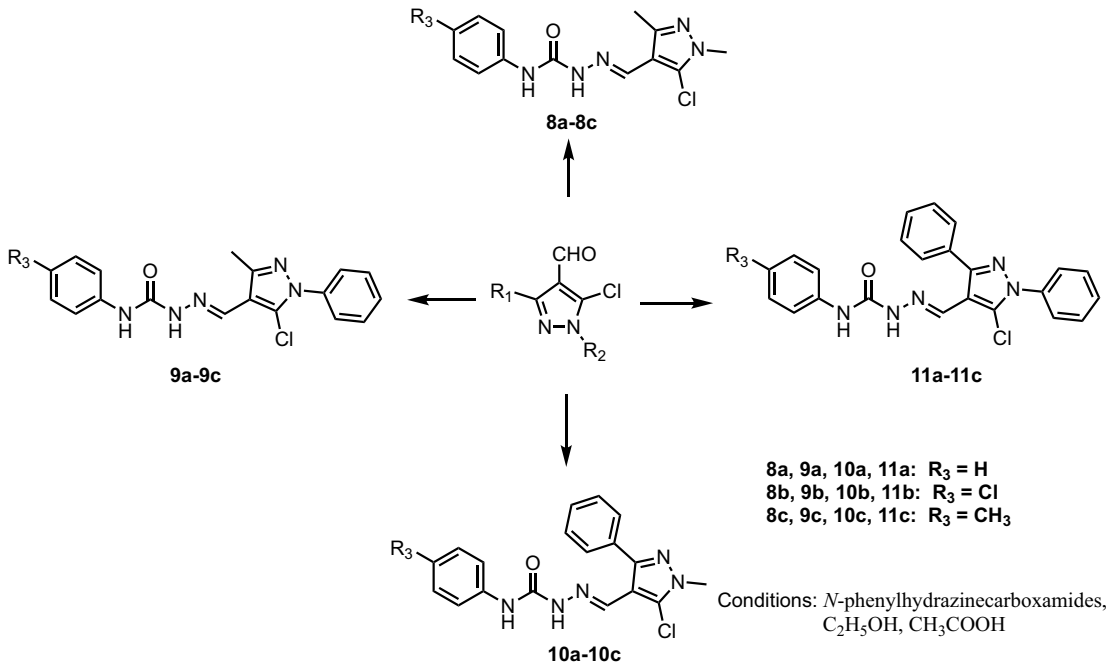
Scheme 2. The synthesis of target compounds **4a-c**, **5a-c, 6a-c**, and **7a-c.**

pyrazole-hydrazone derivatives were synthesized and screened for their anti-inflammatory properties.

2. CHEMISTRY

According to the designed structures, twenty-four pyrazole-hydrazone derivatives were divided into eight series, which were prepared from the intermediates **3**. The intermediates (**3a-3d**) were prepared according to Scheme **1**. The preparation of 1,3-substituted pyrazole-2-one (**2a-2d**) was carried out at 90 °C by reacting the ethyl acetoacetate (or ethyl benzoacetate) with methylhydrazine (or phenylhydrazine) in alcohol. Under Vilsmeier-Haack (DMF-$POCl_3$) conditions, compounds **2a-2d** were transformed into the corresponding 5-chloro-4-carboxaldehyde functionalized pyrazoles (**3a-3d**). The intermediates (**3a-3d)** reacted with formyl hydrazide, acetylhydrazide or benzoyl hydrazide in alcohol at room temperature in the presence of acetic acid to produce compounds **4a-4c**, **5a-5c**, **6a-6c**, and **7a-7c** (Scheme **2**). Compounds **8a-8c**, **9a-9c**, **10a-10c**, and **11a-11c** were obtained using the same conditions *via* reacting compounds **3a-3d** with *N*-phenylhydrazinecarboxamides (Scheme **3**). The structures of the desired products were confirmed by ^{1}H NMR, ^{13}C NMR, and mass spectrometry.

Compound **4c** was used as an example of the structure confirmation. In the ^{1}H-NMR spectrum, two singlets, due to the CH_3 on the pyrazole ring, was observed at 2.40 and 3.77 ppm. The aromatic protons of the benzene ring revealed peaks in the 7.50-7.92 ppm range. The absorption peak of C-H in imine was found at 8.38 ppm. The absorption peak of NH in the amide was observed at 11.65 ppm as a singlet. The absorption peak in the hydrogen spectrum was in complete conformity with the hydrogen signal in the structure. The ^{13}C NMR spectra also gave accurate information about the structure of compound **4c**, which has 11 types of carbons in different chemical environments. Moreover, the high-resolution mass spectrometry of **4c** displayed an $[M + H]^+$ signal at m/z



Scheme 3. The synthesis of target compounds **8a-c**, **9a-c, 10a-c**, and **11a-c.**

277.0848, corresponding to its molecular weight of 277.0851.

3. RESULTS AND DISCUSSION

3.1. Anti-inflammatory Activity

3.1.1. In Vitro Inhibition LPS-induced TNF-a Release

Lipopolysaccharide (LPS), an endotoxin of gram-negative bacterial cell walls, is thought to be a major risk factor in initiating the inflammatory processes by stimulating the release of inflammatory cytokines, such as tumor necrosis factor (TNF-α) [30]. The LPS-induced TNF-α release model was considered to be an effective screening method for new anti-inflammatory agents *in vitro* [31]. In the current study, compounds **4-11** were evaluated for their anti-inflammatory activity based on their ability to inhibit TNF-α release in LPS-stimulated RAW264.7 mouse macrophages. Dexamethasone was used as the standard, which was a corticosteroid that prevents the release of substances in the body that cause inflammation such as TNF-α [32, 33]. The cells were seeded and pretreated with the test compounds or dexamethasone (DXMS) at a concentration 10 μg/mL for 4 h before treatment with LPS (1 μg/mL) for 24 h at 37 °C. Cell-free supernatants were collected and analyzed for levels of TNF-α using an ELISA kit. The screen results of target compounds and positive control DXMS are shown in Fig. (**1**).

As shown in Fig. (**1**), expect compounds **4b** and **8a**, all of the tested compounds significantly inhibited LPS-induced TNF-α generation *in vitro*. Among these compounds, **6a**, **6c**, **7a-7c**, **9-9c**, **10a-10c**, and **11a-11c** exhibited strong inhibition with inhibitory rates above 50%. Compounds **7b**, and **11c**, showing 67.18% and 70.73% TNF-α inhibitory activity, respectively, were two of the most promising compounds in this study. The positive drug DXMS exhibited an inhibition rate of 63.89% at the same concentration (10 μg/mL).

Some Structure-Activity Relationships (SAR) were obtained in comparison to the activities of the compounds with different substituents. The series of compounds **4a-4c** and **8a-8c**, with the methyl group in the 1- and 3- positions of pyrazole ring, showed relatively weaker anti-inflammatory activity than the other series of compounds. In the series of **4-7**, the activity order is **4<5<6<7**, which suggested that the phenyl group is more beneficial than methyl group in the 1- and 3- positions. The substituents on the hydrazide in compounds **4-7** are hydrogen (**4a, 5a, 6a, 7a**), methyl (**4b, 5b, 6b, 7b**), and phenyl (**4c, 5c, 6c, 7c**), respectively. There seems to be no regularity for the effects of these substituents on the cytokine-inhibitory ability, though the formyl hydrazine seems to be favorable to the anti-inflammatory activity, which gives four compounds (**4a, 5a, 6a, 7a**) with relatively higher cytokine-inhibitory ability. In the series of **8-11**, the activity order is **8<9≈10≈11**. In these series, compounds **8c**, **9c**, **10c**, **11c** containing methyl on phenyl ring, exhibited better inhibitory ability compared to the others.

3.1.2. Compound 7b and 11c Inhibit TNF-α Release in a Dose-dependent Manner

Among the active compounds discussed above, compounds **7b** and **11c** exhibited the highest cytokine-inhibitory ability. Thus, they were chosen for further evaluation of their dose-dependent inhibitory effects against LPS-induced TNF-α release. RAW 264.7 macrophages were pre-treated with **7b** and **11c** in a series of concentrations (30, 10, 3.3, 1.1 and 0.37 μM) for 4 h and were subsequently incubated with LPS (1 μg/mL) for 24 h. As shown in Fig. (**2**), pretreatment with **7b** and **11c** both exhibited a dose-dependent inhibition on TNF-α release induced by LPS. Accordingly, the IC_{50} values were calculated and shown in Fig. (**2**). Compound **7b** and

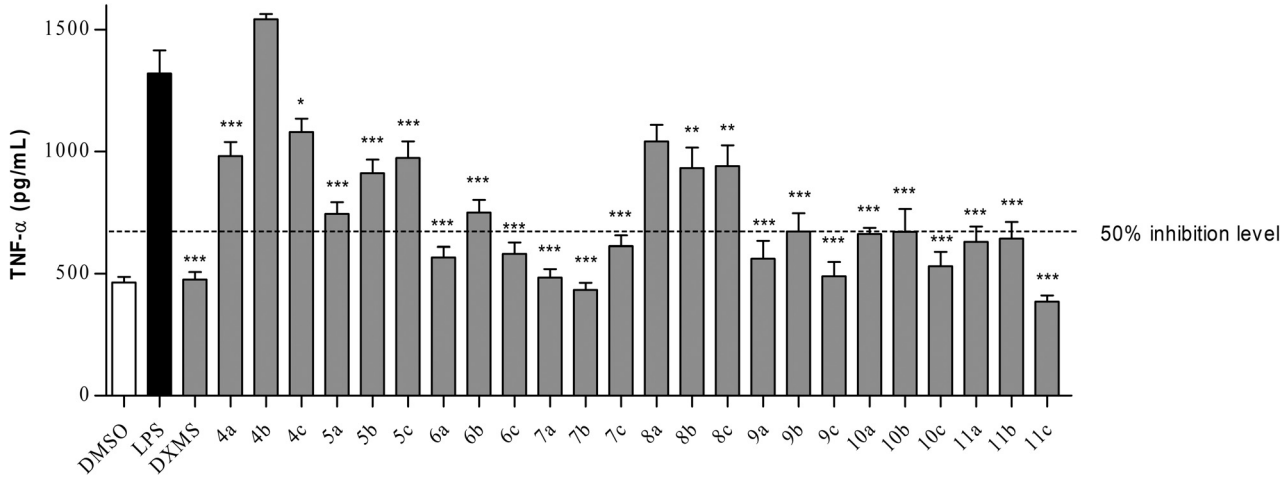


Fig. (1). Anti-inflammatory activity of the compounds 4-11 in the LPS-induced TNF-α release test in RAW264.7 macrophage. Cells were pre-treated with test compounds or dexamethasone (DXMS) at 10 μg/mL for 4 h, followed by LPS (1 μg/mL) treatment for 24 h. TNF-α levels in the culture medium were measured by ELISA. The results are expressed as the level of TNF-α. Each bar represents mean ± SEM of three parallel experiments. Values are significant at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared to the LPS group (all comparisons were made by ANOVA followed by Dunnett's test).

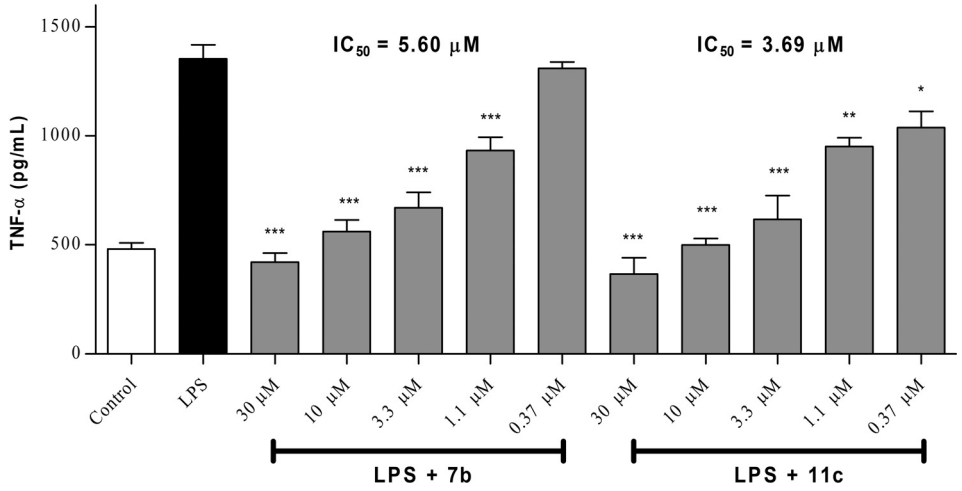


Fig. (2). Compounds 7b and 11c inhibited LPS-induced TNF-α release in a dose-dependent manner in RAW 264.7 macrophages. Cells were pre-treated with test compounds at designated concentrations for 4 h, followed by LPS (1 μg/mL) treatment for 24 h. TNF-α levels in the culture medium were measured by ELISA. The results are expressed as the level of TNF-α. Each bar represents mean ± SEM of three parallel experiments. Values are significant at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared to the LPS group (all comparisons were made by ANOVA followed by Dunnett's test).

11c showed the IC_{50} values of 5.56 and 3.69 μM, respectively. The dose-dependent inhibition by **7b** and **11c** further suggested their potential as anti-inflammatory agents.

3.1.3. In vivo Inhibition of Xylene-induced Ear Edema

Xylene-induced edema leads to an acute inflammatory response and is known to cause severe vasodilation and edematous skin changes, partially associated with phospholipase A2 (PLA2) [34, 35]. Xylene treatment in mice increases the release of inflammatory mediators, such as histamine, serotonin, bradykinin, and TNF-α. Based on the *in vitro* inhibitory effects of our test compounds toward TNF-α expression, the *in vivo* anti-inflammatory activity of compounds **7b** and **11c** were evaluated using the xylene-induced ear edema mouse model. In this test, dimethyl sulfoxide (DMSO) was used as the vehicle, and DXMS was used as positive control. Anti-inflammatory activity was defined by the ability of each compound to prevent edema. As shown in Fig. (**3**), both tested compounds and DXMS inhibited the ear edema significantly in a dose-dependent manner. At the dose of 20 mg/kg, compound **11c** inhibited edema by 49.59%, which was comparable to DXMS at inhibiting xylene-induced ear edema (50.49%). The *in vivo* activities of the compound **11c** was superior to that of **7b**, which was in line with the previous *in vitro* data.

3.1.4. In silico Docking Studies

In the above data, the *in vivo* and *in vitro* anti-inflammatory ability of the target compounds have been described. To further confirm whether the anti-inflammatory

activity of the synthesized compounds mediates directly through TNF-α, and to assess their interactions with the target protein, the molecular docking of representative **7b** and **11c** with the TNF-α protein was performed by Discovery Studio 2019 version [36].

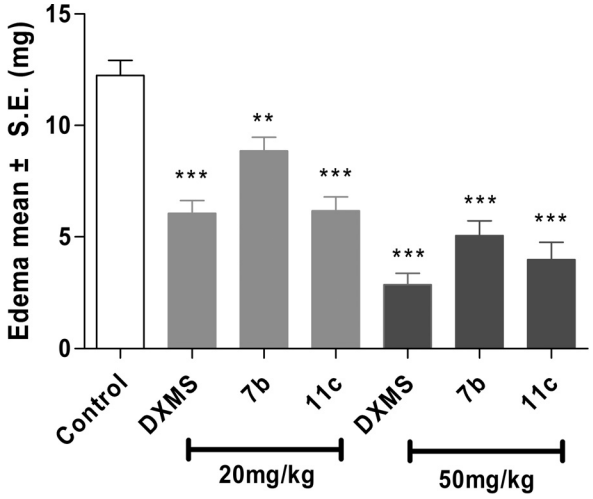


Fig. (3). Anti-inflammatory activity of the compounds 7b and 11c in the xylene-induced ear-edema test in mice. The test compounds and DXMS were administered *i.p.* at dose of 20 and 50 mg/kg, and edema was quantified after 30 min. **p<0.01 and ***p<0.001 compared with the control group (all comparisons were made by ANOVA followed by Dunnett's test).

Interactions of the compounds **7b** and **11c** with amino acids TNF-α protein were illustrated and showed in Fig. (**4**). As seen in Fig. (**4**) (left), the most important residues in the binding mode of compound **7b** are GLN125, VAL91, TYR87, ARG82, LEU93, PHE124 and LEU93. The hydrazide group of compound **7b**, as an H-bond donor, was involved in an interaction with the carbonyl group of GLN125, while the phenyl group in the 3- position was responsible for π-π, π-alkyl, and π-charge interaction with VAL91, TYR87, and ARG82. In addition to the above, the phenyl group in the 1- position and chlorine atom in 5- position also showed π-alkyl interactions with LEU93 and PHE124, respectively. As seen in Fig. (**4**) (right), LYS98, TYR119, LEU55, LEU197, PRO12, LYS11, and ALA156 were the critical residues in the binding mode of compound **11c**. The NH group of compound **11c**, as an H-bond donor, was involved in an interaction with carbonyl group of LEU55 and LEU197. The phenyl group in the 1- position was responsible for π-alkyl interaction with LYS98, while the phenyl group of N-phenylhydrazinecarboxamide moiety and the methyl on it exhibited π-charge and π-alkyl hydrophobic binding with LYS98, TYR87, and ARG82. These docking results indicated that phenyl group in 1- and 3- position of pyrazole ring and hydrazide moiety in the side chain may play a significant role in binding to the target site of TNF -α.

4. EXPERIMENTAL PROTOCOLS

4.1. Chemistry

Melting points were determined in open capillary-tubes and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded using an AV-300 spectrometer (Bruker, Switzerland), and all chemical shifts were given in parts per million relative to tetramethylsilane. High-resolution mass spectra were measured on an MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Germany). All the reagents and solvents were purchased from Aladdin (Shanghai, China) or Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), and were used as received.

4.1.1. General Procedure for the Preparation of Compounds 2a-2d

To a cooled (ice-bath) and stirred solution of methyl hydrazine (1.84 g, 0.04mol), the solution of ethyl acetoacetate (1.30 g, 0.01 mol) or ethyl benzoacetate (1.92 g, 0.01 mol) with 30 mL of absolute alcohol was added dropwise. Then, the mixture was refluxed and monitored by TLC (developing

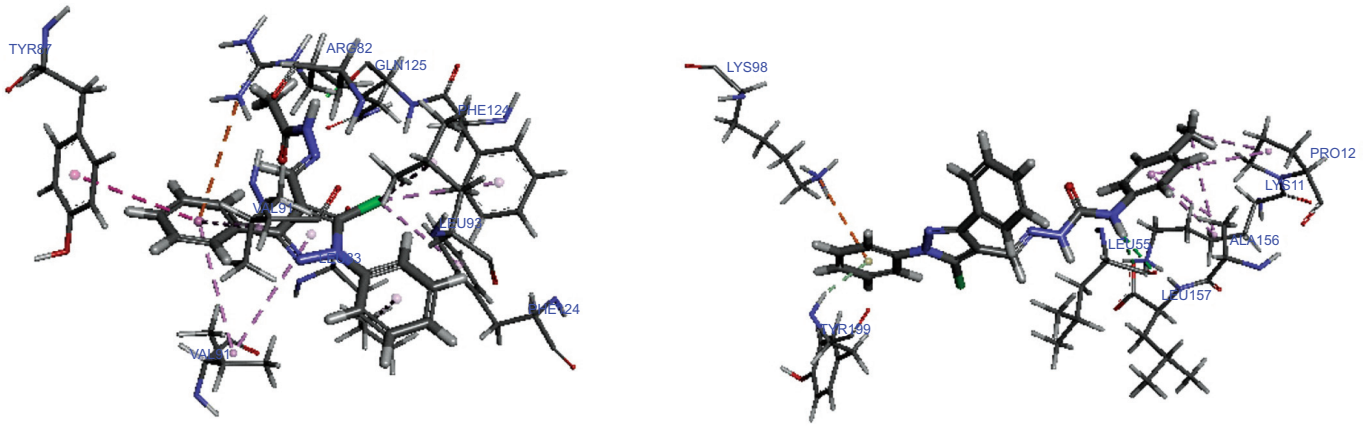


Fig. (4). Images of compounds **7b** and **11c** in the TNF-α active site (PDB: 2AZ5).

solvent: CH_2Cl_2/CH_3OH = 30/1). After completion of the reaction, the mixture was evaporated under reduced pressure to obtain products **2a** and **2c**.

To a cooled (ice-bath) and stirred solution of phenylhydrazine (4.32 g, 0.04 mol), the solution of ethyl acetoacetate (1.30 g, 0.01 mol) or ethyl benzoacetate (1.92 g, 0.01 mol) with 30 mL of absolute alcohol was added dropwise. Then, the mixture was refluxed and monitored by TLC (developing solvent: CH_2Cl_2/CH_3OH = 30/1). After completion of the reaction, the solid formed was filtrated and washed with small amount of cooled alcohol to give products **2b** and **2d**.

4.1.2. General Procedure for the Preparation of Compounds 3a-3d

To a cold, stirred solution of dimethylformamide (3.65 g, 0.05 mol), phosphorus oxychloride (23 g, 0.15 mol) was added dropwise. Then, compound **2** (0.05 mol) was added in batches. The mixture was stirred at 90 ^oC and monitored by TLC (developing solvent: CH_2Cl_2/CH_3OH = 30/1). After completion of the reaction, the mixture was cooled and poured into ice-cold water followed by extracting with CH_2Cl_2, which was dried to afford compounds **3a**-**3d**.

4.1.3. General Procedure for the Preparation of Compounds 4a-4c, 5a-5c, 6a-6c, and 7a-7c

To a solution of compound **3a** (1.59 g, 0.01 mol) in 20 mL of ethanol, an equimolar quantity of formyl hydrazide or acetylhydrazide or benzoyl hydrazide was added. Then, 0.5 mL of acetic acid was added. The mixture was stirred at room temperature and accompanied with TLC monitoring (developing solvent: CH_2Cl_2/CH_3OH = 30/1). After completion of the reaction, the solid formed was collected by filtration to give crude product, which was recrystallized from dichloromethane/ethanol (1:2) to afford the purified products (**4a-4c**). The compounds **5a-5c**, **6a-6c**, and **7a-7c** were prepared using the same procedures with the compounds **3b**, **3c**, and **3d** as the reactants, respectively.

4.1.4. General Procedure for the Preparation of Compounds 8a-8c, 9a-9c, 10a-10c, and 11a-11c

To a solution of compound **3a** (1.59 g, 0.01 mol) in 20 mL of ethanol, an equimolar quantity of *N*-phenylhydrazinecarboxamides was added. Then, 0.5 mL of acetic acid was added. The mixture was stirred at room temperature and accompanied by TLC monitoring (developing solvent: CH_2Cl_2/CH_3OH = 30/1). After completion of the reaction, the solid formed was collected by filtration to give crude product, which was recrystallized from dichloromethane/ethanol (1:2) to afford the purified products (**8a-8c**). The compounds **9a-9c**, **10a-10c**, and **11a-11c** were prepared using the same procedures with the compounds **3b**, **3c**, and **3d** as the reactants, respectively.

4.1.5. Characterization for the Target Compounds

4.1.5.1. N'-((5-Chloro-1,3-dimethyl-1H-pyrazol-4-yl)methylene)formohydrazide (4a)

Mp 177-178 ºC, yield 60%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 2.30 (s, 3H, CH_3), 3.73 (s, 3H, NCH_3), 7.91 (s, 1H, CH=N), 8.59 (d, 1H, J = 9.0 Hz, CHO), 11.48 (d, 1H, J = 9.0 Hz, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 164.88, 146.58, 137.91, 127.56, 111.14, 36.31, 14.46. ESI-HRMS calcd for C7H10ClN4O$^+$ ([M + H]$^+$): 201.0538; found: 201.0544.

4.1.5.2. N'-((5-Chloro-1,3-dimethyl-1H-pyrazol-4-yl)methylene)acetohydrazide (4b)

Mp 206-207ºC, yield 62%. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 2.32 (s, 3H, CH_3), 2.42 (s, 3H, $COCH_3$), 3.80 (s, 3H, NCH_3), 7.65 (s, 1H, CH=N), 9.25 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 173.23, 147.68, 135.41, 128.21, 111.16, 36.01, 20.46, 14.56. ESI-HRMS calcd for C8H12ClN4O$^+$ ([M + H]$^+$): 215.0694; found: 215.0685.

4.1.5.3. N'-((5-Chloro-1,3-dimethyl-1H-pyrazol-4-yl)methylene)benzohydrazide (4c)

Mp 179-181ºC, yield 56%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 2.40 (s, 3H, CH_3), 3.77 (s, 3H, NCH_3), 7.50-7.92 (m, 5H, Ph-H), 8.38 (s, 1H, CH=N), 11.65 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 163.05, 146.97, 140.75, 134.05, 132.05, 128.90, 127.95, 127.89, 111.62, 36.37, 14.57. ESI-HRMS calcd for C13H14ClN4O$^+$ ([M + H]$^+$): 277.0851; found: 277.0848.

4.1.5.4. N'-((5-Chloro-3-methyl-1-phenyl-1H-pyrazol-4-yl) methylene)formohydrazide (5a)

Mp 165-167 ºC, yield 51%. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 2.53 (s, 3H, CH_3), 7.43-7.56 (m, 5H, Ph-H), 7.84 (s, 1H, CH=N), 8.78 (d, 1H, J = 7.8 Hz, CHO), 9.64 (d, 1H, J = 7.8 Hz, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 165.06, 149.28, 138.15, 137.62, 129.17, 128.61, 128.17, 124.92, 112.57, 14.73. ESI-HRMS calcd for C12H12ClN4O$^+$ ([M + H]$^+$): 263.0694; found: 263.0701.

4.1.5.5. N'-((5-Chloro-3-methyl-1-phenyl-1H-pyrazol-4-yl) methylene)acetohydrazide (5b)

Mp 223-225 ºC, yield 57%. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 2.36 (s, 3H, $COCH_3$), 2.53 (s, 3H, CH_3), 7.43-7.56 (m, 5H, Ph-H), 7.79 (s, 1H, CH=N), 9.70 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 173.57, 149.14, 137.69, 135.50, 129.15, 128.53, 127.79, 124.91, 113.06, 20.50, 14.83. ESI-HRMS calcd for C13H14ClN4O$^+$ ([M + H]$^+$): 277.0851; found: 277.0854.

4.1.5.6. N'-((5-Chloro-3-methyl-1-phenyl-1H-pyrazol-4-yl) methylene)benzohydrazide (5c)

Mp 169-171ºC, yield 54%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 2.51 (s, 3H, CH_3), 7.49-7.94 (m, 10H, Ph-H), 8.49 (s, 1H, CH=N), 11.78 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 163.16, 148.87, 140.39, 137.75, 133.98, 132.14, 129.77, 129.14, 128.94, 128.00, 127.74, 125.41, 113.69, 14.86. ESI-HRMS calcd for C18H16ClN4O$^+$ ([M + H]$^+$): 339.1007; found: 339.1012.

4.1.5.7. N'-((5-Chloro-1-methyl-3-phenyl-1H-pyrazol-4-yl) methylene)formohydrazide (6a)

Mp 160-161 ºC, yield 40%. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 3.94 (s, 3H, NCH_3), 7.42-7.59 (m, 5H, Ph-H), 7.77 (s, 1H, CH=N), 8.68 (d, 1H, J = 7.8 Hz, CHO), 9.21 (d, 1H, J = 7.8 Hz, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 164.77, 151.08, 137.71, 131.94, 128.84, 128.59, 128.57, 128.05, 110.03, 36.59. ESI-HRMS calcd for C12H12ClN4O$^+$ ([M + H]$^+$): 263.0694; found: 263.0699.

4.1.5.8. N'-((5-Chloro-1-methyl-3-phenyl-1H-pyrazol-4-yl) methylene)acetohydrazide (6b)

Mp 144-147ºC, yield 56%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 2.05 (s, 3H, $COCH_3$), 3.89 (s, 3H, NCH_3), 7.44-7.63 (m, 5H, Ph-H), 8.16 (s, 1H, CH=N), 11.05 (d, 1H, J = 7.8 Hz, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 171.62, 149.18, 138.17, 134.46, 132.19, 128.40, 128.33, 126.87, 110.59, 36.50, 20.18. ESI-HRMS calcd for C13H14ClN4O$^+$ ([M + H]$^+$): 277.0851; found: 277.0844.

4.1.5.9. N'-((5-Chloro-1-methyl-3-phenyl-1H-pyrazol-4-yl) methylene)benzohydrazide (6c)

Mp 214-216ºC, yield 43%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 3.92 (s, 3H, NCH_3), 7.42-7.91 (m, 10H, Ph-H), 8.49 (s, 1H, CH=N), 11.69 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 163.15, 150.20, 140.65, 134.01, 132.43, 132.09, 128.98, 128.95, 128.89, 128.81, 128.00, 127.51, 111.05, 37.04. ESI-HRMS calcd for C18H16ClN4O$^+$ ([M + H]$^+$): 339.1007; found: 339.1010.

4.1.5.10. N'-((5-Chloro-1,3-diphenyl-1H-pyrazol-4-yl)methylene)formohydrazide (7a)

Mp 171-172ºC, yield 84%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 7.47-7.72 (m, 10H, Ph-H), 8.13 (s, 1H, CH=N), 8.58 (d, 1H, J = 7.5 Hz, CHO), 11.62 (d, 1H, J = 7.5 Hz, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 164.90, 151.49, 137.75, 137.41, 132.01, 129.83, 129.60, 129.36, 129.00, 128.97, 127.16, 125.87, 112.31. ESI-HRMS calcd for C17H14ClN4O$^+$ ([M + H]$^+$): 325.0851; found: 325.0849.

4.1.5.11. N'-((5-Chloro-1,3-diphenyl-1H-pyrazol-4-yl)methylene)acetohydrazide (7b)

Mp 184-186ºC, yield 81%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 2.05 (s, 3H, $COCH_3$), 7.47-7.72 (m, 10H, Ph-H), 8.07 (s, 1H, CH=N), 11.15 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 172.18, 151.21, 137.78, 134.54, 132.25, 129.82, 129.55, 129.24, 129.06, 128.85, 127.28, 125.83, 112.75, 20.65. ESI-HRMS calcd for C18H16ClN4O$^+$ ([M + H]$^+$): 339.1007; found: 339.1011.

4.1.5.12. N'-((5-Chloro-1,3-diphenyl-1H-pyrazol-4-yl)methylene)benzohydrazide (7c)

Mp 230-231ºC, yield 80 %. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 7.48-7.93 (m, 15H, Ph-H), 8.59 (s, 1H, CH=N), 11.80 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 163.22, 151.71, 140.30, 137.81, 133.91, 132.18, 132.04, 129.84, 129.60, 129.33, 129.08, 129.04, 128.92, 128.04, 127.47, 125.92, 112.77. ESI-HRMS calcd for C23H18ClN4O$^+$ ([M + H]$^+$): 401.1164; found: 401.1152.

4.1.5.13. 2-((5-Chloro-1,3-dimethyl-1H-pyrazol-4-yl)methylene)-N-phenylhydrazinecarboxamide (8a)

Mp 204-205ºC, yield 71%. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 2.47 (s, 3H, CH_3), 3.82 (s, 3H, NCH_3), 7.06-7.53 (m, 5H, Ph-H), 7.69 (s, 1H, CH=N), 8.07 (s, 1H, NH), 8.79 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 153.27, 147.21, 138.01, 133.62, 129.07, 127.84, 123.39, 119.21, 111.11, 36.08, 14.49. ESI-HRMS calcd for C13H15ClN5O$^+$ ([M + H]$^+$): 292.0960; found: 292.0963.

4.1.5.14. 2-((5-Chloro-1,3-dimethyl-1H-pyrazol-4-yl)methylene)-N-(4-chlorophenyl)hydrazine- carboxamide (8b)

Mp 223-224ºC, yield 78%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 2.40 (s, 3H, CH_3), 3.75 (s, 3H, NCH_3), 7.34 (d, 2H, J = 9.0 Hz, Ph-H), 7.60 (d, 2H, J = 9.0 Hz, Ph-H), 7.86 (s, 1H, CH=N), 8.50 (s, 1H, NH), 10.67 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 153.13, 146.74, 138.39, 134.13, 128.94, 127.20, 126.44, 121.05, 111.43, 36.31, 14.62. ESI-HRMS calcd for C13H14Cl2N5O$^+$ ([M + H]$^+$): 326.0570; found: 326.0568.

4.1.5.15. 2-((5-Chloro-1,3-dimethyl-1H-pyrazol-4-yl)methylene)-N-(p-tolyl)hydrazinecarbox- amide (8c)

Mp 213-215ºC, yield 69%. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 2.32 (s, 3H, Ph-CH_3), 2.46 (s, 3H, CH_3), 3.81 (s, 3H, NCH_3), 7.13 (d, 2H, J = 6.3 Hz, Ph-H), 7.40 (d, 2H, J = 6.3 Hz, Ph-H), 7.69 (s, 1H, CH=N), 7.99 (s, 1H, NH), 8.95 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 153.48, 147.20, 135.43, 133.53, 132.94, 129.54, 127.78, 119.36, 111.18, 36.06, 20.82, 14.48. ESI-HRMS calcd for C14H17ClN5O$^+$ ([M + H]$^+$): 306.1116; found: 306.1112.

4.1.5.16. 2-((5-Chloro-3-methyl-1-phenyl-1H-pyrazol-4-yl)methylene)-N-phenylhydrazinecarbox -amide (9a)

Mp 193-194ºC, yield 77%. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 2.58 (s, 3H, CH_3), 7.09-7.57 (m, 10H, Ph-H), 7.80 (s, 1H, CH=N), 8.09 (s, 1H, NH), 8.94 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 153.26, 148.63, 137.96, 137.65, 133.53, 129.18, 129.10, 128.63, 127.48, 124.96, 123.47, 119.24, 112.91, 14.78. ESI-HRMS calcd for C18H17ClN5O$^+$ ([M + H]$^+$): 354.1116; found: 354.1122.

4.1.5.17. 2-((5-Chloro-3-methyl-1-phenyl-1H-pyrazol-4-yl)methylene)-N-(4-chlorophenyl) hydrazinecarboxamide (9b)

Mp 220-221 ºC, yield 65%. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 2.57 (s, 3H, CH_3), 7.28-7.55 (m, 9H, Ph-H), 7.79 (s, 1H, CH=N), 8.08 (s, 1H, NH), 8.84 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 153.00, 148.59, 137.59, 136.58, 133.82, 129.20, 129.07, 128.68, 128.40, 127.56, 124.94, 120.37, 112.75, 14.77. ESI-HRMS calcd for C18H16Cl2N5O$^+$ ([M + H]$^+$): 388.0726; found: 388.0725.

4.1.5.18. 2-((5-Chloro-3-methyl-1-phenyl-1H-pyrazol-4-yl)methylene)-N-(p-tolyl)hydrazine- carboxamide (9c)

Mp 221-222ºC, yield 73%. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 2.32 (s, 3H, Ph-CH_3), 2.58 (s, 3H, CH_3), 7.14 (d, 2H, J = 6.0 Hz, Ph-H), 7.41 (d, 2H, J = 6.0 Hz, Ph-H), 7.49-7.58 (m, 5H, Ph-H), 7.78 (s, 1H, CH=N), 8.00 (s, 1H, NH), 8.83 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 153.28, 148.63, 137.66, 135.35, 133.31, 133.05, 129.58, 129.18, 128.61, 127.42, 124.96, 119.39, 112.94, 20.82, 14.78. ESI-HRMS calcd for C19H19ClN5O$^+$ ([M + H]$^+$): 368.1273; found: 368.1279.

4.1.5.19. 2-((5-Chloro-1-methyl-3-phenyl-1H-pyrazol-4-yl)methylene)-N-phenylhydrazine- carboxamide (10a)

Mp 221-222ºC, yield 69%. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 3.95 (s, 3H, NCH_3), 7.06-7.63 (m, 10H, Ph-H), 7.76 (s, 1H, CH=N), 7.85 (s, 1H, NH), 9.11 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 153.42, 150.46, 138.04, 132.75, 132.64,

128.91, 128.76, 128.71, 128.56, 127.86, 123.21, 119.20, 110.63, 36.56. ESI-HRMS calcd for $C18H17ClN5O^+$ ([M + H]$^+$): 354.1116; found: 354.1120.

4.1.5.20. 2-((5-Chloro-1-methyl-3-phenyl-1H-pyrazol-4-yl)methylene)-N-(4-chlorophenyl)- hydrazinecarboxamide (10b)

Mp 237-238ºC, yield72%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 3.91 (s, 3H, NCH_3), 7.33-7.61 (m, 9H, Ph-H), 7.96 (s, 1H, CH=N), 8.16 (s, 1H, NH), 10.74 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 152.87, 149.58, 138.07, 133.48, 132.83, 129.05, 129.03, 128.97, 128.81, 127.47, 126.54, 120.78, 110.90, 36.97. ESI-HRMS calcd for $C18H16Cl2N5O^+$ ([M + H]$^+$): 388.0726; found: 388.0721.

4.1.5.21. 2-((5-Chloro-1-methyl-3-phenyl-1H-pyrazol-4-yl)methylene)-N-(p-tolyl)hydrazine- carboxamide(10c)

Mp 222-223 ºC, yield 60 %. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 2.32 (s, 3H, Ph-CH_3), 3.94 (s, 3H, NCH_3), 7.09 (d, 2H, J = 6.3 Hz, Ph-H), 7.25 (d, 2H, J = 6.3 Hz, Ph-H), 7.44-7.64 (m, 5H, Ph-H), 7.75 (s, 1H, CH=N), 7.76 (s, 1H, NH), 9.09 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 153.51, 150.41, 135.44, 132.71, 132.67, 132.56, 129.40, 128.71, 128.53, 127.84, 126.8, 119.34, 110.69, 36.55, 20.81. ESI-HRMS calcd for $C19H19ClN5O^+$ ([M + H]$^+$): 368.1273; found: 368.1280.

4.1.5.22. 2-((5-Chloro-1,3-diphenyl-1H-pyrazol-4-yl)methylene)-N-phenylhydrazinecarboxamide (11a)

Mp 220-222ºC, yield 80%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 6.99-7.74 (m, 15H, Ph-H), 8.03 (s, 1H, CH=N), 8.05 (s, 1H, NH), 10.08 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 152.89, 151.13, 138.97, 137.76, 132.71, 132.56, 129.85, 129.56, 129.36, 129.23, 129.07, 129.02, 127.34, 125.78, 123.00, 119.18, 112.74. ESI-HRMS calcd for $C23H19ClN5O^+$ ([M + H]$^+$): 416.1273; found: 416.1275.

4.1.5.23. 2-((5-Chloro-1,3-diphenyl-1H-pyrazol-4-yl)methylene)-N-(4-chlorophenyl)hydrazine- carboxamide (11b)

Mp 240-241ºC, yield 75%. ^{1}H-NMR (DMSO-d_6, 300MHz): δ 7.32-7.73 (m, 14H, Ph-H), 8.04 (s, 1H, CH=N), 8.17 (s, 1H, NH), 10.85 (s, 1H, NH). ^{13}C-NMR (DMSO-d_6, 75MHz): δ 152.85, 151.17, 138.04, 137.75, 133.05, 132.49, 129.86, 129.59, 129.52, 129.40, 129.05, 128.95, 127.41, 126.60, 125.79, 120.81, 112.70. ESI-HRMS calcd for $C23H18Cl2N5O^+$ ([M + H]$^+$): 450.0883; found: 450.0889.

4.1.5.24. 2-((5-Chloro-1,3-diphenyl-1H-pyrazol-4-yl)methylene)-N-(p-tolyl)hydrazinecarbox- amide (11c)

Mp 201-202ºC, yield 78 %. ^{1}H-NMR ($CDCl_3$, 300MHz): δ 2.31 (s, 3H, Ph-CH_3), 7.09 (d, 2H, J = 6.3 Hz, Ph-H), 7.24 (d, 2H, J = 6.3 Hz, Ph-H), 7.47-7.71 (m, 10H, Ph-H), 7.72 (s, 1H, CH=N), 7.83 (s, 1H, NH), 9.06 (s, 1H, NH). ^{13}C-NMR ($CDCl_3$, 75MHz): δ 153.41, 151.60, 137.68, 135.37, 132.79, 132.48, 132.25, 129.41, 129.21, 129.03, 128.95, 128.91, 128.52, 127.63, 125.27, 119.36, 112.28, 20.81. ESI-HRMS calcd for $C24H21ClN5O^+$ ([M + H]$^+$): 430.1429; found: 430.1431.

5. PHARMACOLOGY

5.1. Cell Line and Reagents

Mouse RAW264.7 macrophage cell line was obtained from the China Cell Bank (Beijing, China). Fetal bovine serum was purchased from Biological Industries (Kibbutz Beit-Haemek, Israel). LPS was purchased from Sigma (St. Louis, MO, USA), and was dissolved in PBS. DXMS and compounds were dissolved in DMSO for *in vitro* experiments.

5.2. Cell Treatment and ELISA Assay (TNF-α)

Mouse RAW264.7 macrophages were cultured in RPMI 1640 medium (Zhejiang Tianhang Biotechnology Co., Ltd.) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ^{o}C with 5% CO_2. Cells were pretreated with test compounds, DXMS or vehicle control for 4 h, then treated with LPS (1μg/ml) for 24 h. After treatment, the supernatant was separated and used for the determination of levels of TNF-α by ELISA using mouse TNF-α ELISA Kits Biolegend (San diego, CA, USA).

5.3. Xylene-Induced Ear-Edema Model

Animal experiments were carried out on KunMing mice (half male and female) weighing 20-26 g. The mice were housed collectively in polycarbonate cages in groups of ten, where they were maintained on a 12 h light/dark cycle in a temperature-controlled (25 ± 2 ºC) laboratory with free access to food and water. Each animal was used only once. Procedures involving animals and their care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition, National Academies Press, Washington, DC. Local ethical committee approval was also obtained. In this screening, selected compounds were evaluated for their anti-inflammatory activity *via in vivo* inhibition assay of xylene-induced ear edema in mice [37]. Compounds and DXMS were dissolved in DMSO and administered to mice intraperitoneally at a dose of 50 mg/kg and 20 mg/kg (0.05 mL/20 g body weight). Control group received vehicle only. Thirty minutes after administration, mice were used in the xylene-induced ear-edema test (20 μL xylene was daubed by a micropipette on the surface of the right ear of each mouse). After 30 min, mice were sacrificed, and a cylindrical tissue plug (7 mm diameter) was excised from both ears of mice. Edema was quantified by measuring the difference in weight between the plugs from the right and left ears. Anti-inflammatory activity was expressed as the percent reduction in edema in comparison with the control group. DXMS was tested in parallel as a reference drug.

5.4. *In Silico* Docking Studies

The docking studies were carried out using Discovery Studio (release 2019) and the 3D crystal structure of 2AZ5 (Crystal Structure of TNF-alpha with a small molecule inhibitor) was downloaded from Protein Data Bank. The three-dimensional structures of **7b** and **11c** were constructed using Chem. 3D ultra 12.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2010)], then it was energetically minimized by using MMFF94 with 5000

iterations and minimum RMS gradient of 0.10. For protein preparation, the hydrogen atoms were added, and the water and ligands were removed. Types of interactions of the docked protein with **7b** and **11c** were analyzed after the end of molecular docking. Several docking patterns were obtained and ranked and by DOCKER_INTERACTION_ ENERGY. The lowest energy conformation of ligand-protein complex was analyzed.

CONCLUSION

In summary, several types of pyrazole-hydrazone derivatives were synthesized and their anti-inflammatory activities were evaluated. Majority of the synthetic compounds exhibited significant inhibitory activities against LPS-induced TNF-α expression in RAW 264.7 macrophages. Compounds **7b** and **11c**, two of most potent compounds, exhibited TNF-α inhibitory ability in a dose-dependent manner with IC_{50} values in μM level. *In vivo*, intraperitoneal administration with **7b** and **11c** markedly inhibited the ear edema at the doses of 20 and 50 mg/kg. To understand the binding pattern, molecular docking of representative **7b** and **11c** was performed, which demonstrated that both compounds have a forceful binding with the TNF -α, and that the phenyl and hydrazide moieties of them may play a significant role in binding with the target site.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by Medical ethics committee of Jinggangshan University (approval number: 20180558).

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. Procedures involving animals and their care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition, National Academies Press, Washington DC.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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