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Synthesis and anti-inflammatory activity evaluation of a novel series of 6-phenoxy-[1,2,4]triazolo[3,4-*a*]phthalazine-3-carboxamide derivatives

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Abstract:

The transcription factor nuclear factor-κB (NF-κB) controls many physiological processes including inflammation, immunity, and apoptosis. In this study, a novel series of 6-phenoxy-[1,2,4]triazolo[3,4-*a*]phthalazine-3-carboxamide derivatives were synthesized as potent anti-inflammatory agents, which acted on tumor necrosis factor (TNF-α) as inhibitors of NF-κB activation. We showed that compounds **6h** (6-(2,4-dichlorophenoxy)-[1,2,4]triazolo[3,4-*a*]phthalazine-3-carboxamide) and **6i** (6-(3-tolyloxy)-[1,2,4]triazolo[3,4-*a*]phthalazine-3-carboxamide) showed more prominent anti-inflammatory activity than other compounds, with similar activities as the reference drug dihydrotanshinone; compound **6i** showed the lowest cellular toxicity among the tested compounds. *In vivo* evaluation of the anti-inflammatory activity showed that compound **6i** exhibited excellent anti-inflammatory activity with 58.19% inhibition at 50 mg/kg intraperitoneal (i.p.), with equal efficacy as the positive control indomethacin (100 mg/kg i.p; 59.21% inhibition).

Keywords: synthesis, 3-carboxamide triazole, phthalazine, anti-inflammatory, nuclear factor-κB, TNF-α, MTT

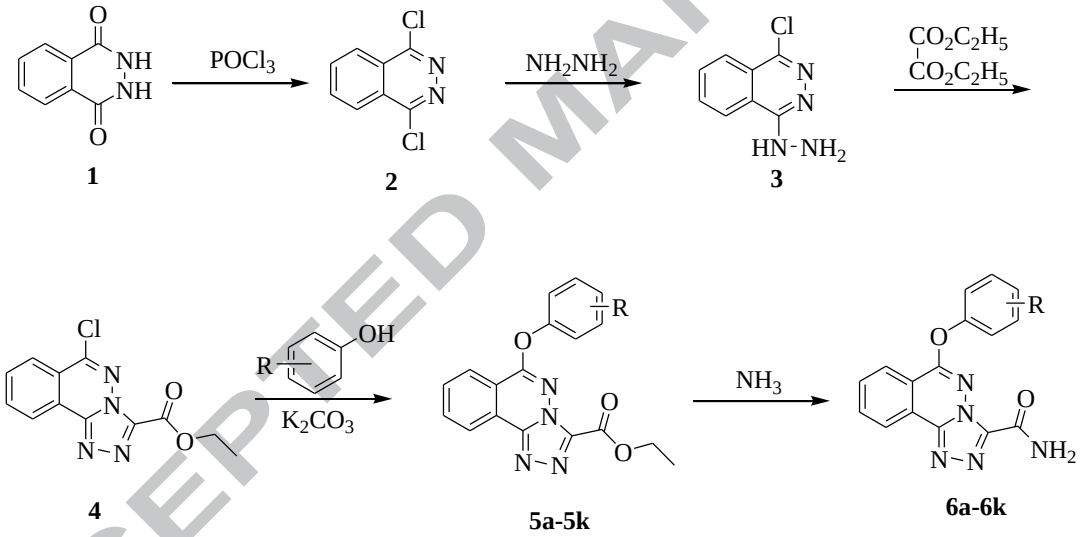
Inflammation is a common pathophysiological phenomenon that is involved in many diseases and is the most primitive protective response of the body to noxious stimuli.[1] There are many possible causes of inflammation, but the basic pathological changes are quite similar, such as tissue and cell degeneration, partial response for microvascular leakage of blood components, necrosis, hyperplasia, and repair; the clinical symptoms also show a similar trend and are characterized by redness, swelling, fever, pain, and dysfunction.[2-4] Non-steroidal anti-inflammatory drugs (NSAIDs) showed promising effect in the treatment of acute and chronic inflammation,[5] pain,[6] and fever,[7] through inhibition of cyclooxygenase (COX). However, their clinical usage is associated with undesirable and numerous side effects such as nephrotoxicity, gastrointestinal lesions, and bleeding;[8, 9] meanwhile, the resistance of body to anti-inflammatory drugs is widespread.[10] Therefore, it is particularly important to find and research new targets of prevention and treatment to inflammation with less adverse effects. Nuclear factor-kappaB (NF-κB) is a key factor

in the immune response triggered by a wide variety of molecules including inflammatory cytokines with higher specificity. This transcription factor represents a new target for the development of anti-inflammatory molecules.[11] Multiple studies also have demonstrated the central role of NF-κB in the regulation of many genes which involved in immunity and inflammation.[12-14] Therefore, the inhibitors of NF-κB function could modulate the inflammatory processes and thus to achieve the purposes of anti-inflammatory with less adverse effects.

Phthalazine derivatives were reported to possess anticonvulsant,[15] cardiotonic,[16] vasorelaxant,[17] antimicrobial,[18] antifungal,[19] anticancer,[20] and anti-inflammatory[21] applications, and our previous study demonstrated their anti-inflammatory activity.[22] As a leading compound, we continued to modify on it. Various derivatives of 1,2,4-triazole have been reported to possess anti-inflammatory activity.[23-27] Some studies have shown that many amide compounds have anti-inflammatory activity.[28, 29] Therefore, we designed a series of phthalazine derivatives with an amide group at the first position of the 1,2,4-triazole ring to examine if this could yield compounds with better activity. Followed the design concept above, we synthesized a series of 6-phenoxy-[1,2,4]triazolo[3,4-*a*]phthalazine-3-carboxamide derivatives and evaluated their anti-inflammatory activity. However, little attention has been paid to cytokines tumor necrosis factor (TNF-α) which play an important role in the inflammatory process and immune responses, even though the anti-inflammatory and immunomodulatory activities of the factors have been mentioned.[30,31] Therefore, we performed luciferase reporter assay (examination of the inhibitory effect of the synthesized compounds on the pro-inflammatory mediator TNF-α) to evaluate their experimental anti-inflammatory activities. Then, the most active compound was selected to evaluate its anti-inflammatory activity *in vivo*. We believed that these screening methods could help us discover potential anti-inflammatory agents.

Compounds were synthesized according to **Scheme 1**. On the basis of the previous studies carried out in our laboratory, we designed and synthesized 6-phenoxy-[1,2,4] triazolo[3,4-*a*]phthalazine-3-carboxamide derivatives (**6a-6k**). The starting material 2,3-dihydrophthalazine-1,4-dione (compound **1**) was reacted with

refluxing phosphorus oxychloride ($POCl_3$) to yield 1,4-dichlorophthalazine (compound **2**).[32] Compound **2** was further reacted with hydrazine hydrate in THF to yield 1-hydrazine-4-chrorophthalazin (compound **3**).[33] Then, compound **3** was added to diethyl oxalate and refluxed to yield ethyl 6-chloro-[1,2,4]triazolo[3,4-*a*] phthalazine-3-carboxylate (compound **4**). Then, compound **5** was reacted with the appropriate substituted phenols to yield 6-phenoxy-[1,2,4]triazolo[3,4-*a*]phthalazine-3 -carboxylate derivatives (**5a-5k)**. Finally, the target compounds, 6-phenoxy-[1,2,4]triazolo[3,4-*a*]phthalazine-3-carboxamide derivatives (**6a-6k**), were obtained by reacting compounds **5a-5k** with concentrated ammonia stirred at room temperature.[34]



R:

6a: -C_6H_4(*o*-F) 6b: -C_6H_4(*m*-F) 6c: -C_6H_4(*p*-F) 6d: -C_6H_4(*m*-Cl)

6e: -C_6H_4(*p*-Cl) 6f: -C_6H_4(*p*-Br) 6g: -C_6H_3(2, 4-2Cl) 6h: -C_6H_4(*o*-CH_3)

6i: -C_6H_4(*m*-CH_3) 6j: -C_6H_4(*p*-CH_3) 6k: -C_6H_4(*p*-OCH_3)

Reagents and conditions: (a) $POCl_3$, reflux, 4 h, yield 83.5%; (b) $NH_2NH_2 \cdot H_2O$, ethyl acetate, 25-60 ℃, yield 75.6%; (c) diethyl oxalate, reflux, 1h, yield 52.6%; (d) substituted phenol, K_2CO_3, ethyl acetate, 70℃, 4h, H_2O, 25℃, 0.5h, yield 51.2-61.3%; (e) NH_3 (28% wt. in H_2O), 45℃, 1h, yield 45.9-59.8%.

Scheme 1: The synthesis of title compounds **6a-6k**.

All the target compounds were screened for their anti-inflammatory activity using the luciferase reporter assay. NF-κB-dependent luciferase activity was measured using the Dual Luciferase Reporter Assay system. Cytotoxicity was assessed by the MTT assay. MTT assay indicates cell viability in response to different compounds at various concentration, and the data reflected cytotoxicity of the compounds under evaluation. Then, compound (**6i**) was evaluated for its anti-inflammatory activity *in vivo* by the method of xylene-induced ear edema in Kunming mice (18-22 g body weight), purchased from the Laboratory of Animal Research, College of Pharmacy, Yanbian University, then we divided them into 3 groups: the drug treated group (compound **6i**), positive control group (Indomethacin) and normal control group, each group had 8 mice. The anti-inflammatory activity of compound **6i** was evaluated by intraperitoneal (i.p.) administration at the dose of 50 mg/kg. Then, compound **6i** (50 mg/kg) was administered orally (p.o.) to mice and its activities were evaluated at different intervals (1 h, 2 h, 3 h, 4 h, 5 h, and 6 h). The peak activity of **6i** was observed at 4 h after p.o. administration and compared with that of indomethacin (50 mg/kg, positive control).

It is known that NF-κB regulates several hundreds of genes, including those involved in immunity, inflammation, anti-apoptosis, cell proliferation, tumorigenesis, and the negative feedback of the NF-κB signal. NF-κB regulates the transcription of various inflammatory cytokines, including TNF-α. Therefore, pharmacological inhibition of NF-κB could be a valuable strategy to modulate the inflammatory processes. Therefore, in this study, we demonstrated the anti-inflammatory activity of the compounds (**6a-6k**) by examining their ability to inhibit TNF-α-induced NF-κB-dependent reporter gene expression.

To investigate the effect of the compounds on the expression of NF-κB induced by TNF-α, we performed NF-κB reporter assay. After cells were transiently transfected with the NF-κB-regulated luciferase reporter vector, the cells were further incubated with TNF-α in the presence of various concentrations of the compounds. Dihydrotanshinone is a component of the traditional Chinese medicinal plant *Salvia miltiorrhiza* Bunge. It has multiple therapeutic activities and is used to treat

vasculocardiac disease, hepatitis, inflammation, and cancer.[35, 36] Previous studies have demonstrated the anti-inflammatory activity of dihydrotanshinone and proved that the activity was reacted through inhibits TNF-α-induced NF-κB activation.[37, 38] So it was used as positive control in this study.

We found that all the eleven compounds **6a-6k** exhibited anti-inflammatory activity to a certain extent (**Fig. 1**, compared with the TNF-α-induced group). Among the halogen-substituted derivatives, 6-(2,4-dichlorophenoxy)-[1,2,4]triazolo[3,4-*a*] phthalazine-3-carboxamide (compound **6g**) showed better activity than the others and its activity was similar to the normal group (untreated with TNF-α). However, we found that the position of substituted group on the phenyl ring barely influenced the anti-inflammatory activity of these compounds and compound **6g** exhibited more cellular toxicity. Then, we focused our attention on the compounds containing electron donor group on the phenyl ring. Notable, 6-(3-tolyloxy)-[1,2,4]triazolo[3,4-*a*] phthalazine-3-carboxamide (compound **6i)** showed better activity than the normal group and had comparable anti-inflammatory activity with dihydrotanshinone. Taken together, **6i** was found to have the highest anti-inflammatory activity among the derivatives in this series.

Fig. 1

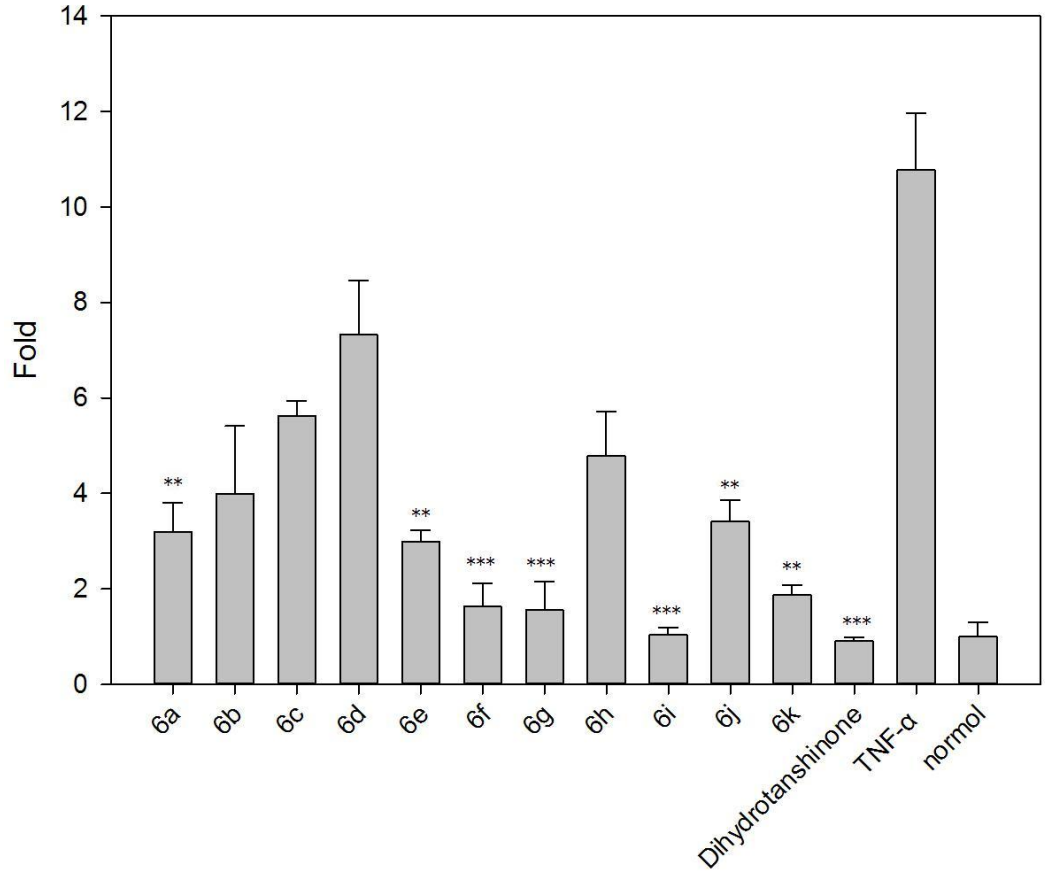


Fig. 1: HeLa cells were transiently transfected with a NF-κB-dependent reporter gene for 48 h and then pretreated for 8 h with the 100 μM of the compounds **6a-6k** followed by stimulation for 8 h with TNF-α (10 ng/ml), and the luciferase activity was determined as described in "6.2. Pharmacology". Data represented as mean ± standard deviation of three independent experiments. **P＜0.01, ***P＜0.001, significantly different when compared with TNF-α-stimulated normal cells.

Based on the results of primary screening, two most active derivatives **6g** and **6i** were chosen for evaluation in the further screening. We found that TNF-α-induced NF-κB reporter activity was substantially suppressed by compounds **6g** and **6i** in a dose-dependent manner (**Fig. 2**). Both of them showed strong anti-inflammatory activities at 100 μM, but when the dose was reduced, their activity markedly diminished. Nevertheless, they also had some anti-inflammatory activities at 30 μM, and compound **6i,** and not **6g,** exhibited anti-inflammatory activity even at 10 μM.

Fig. 2

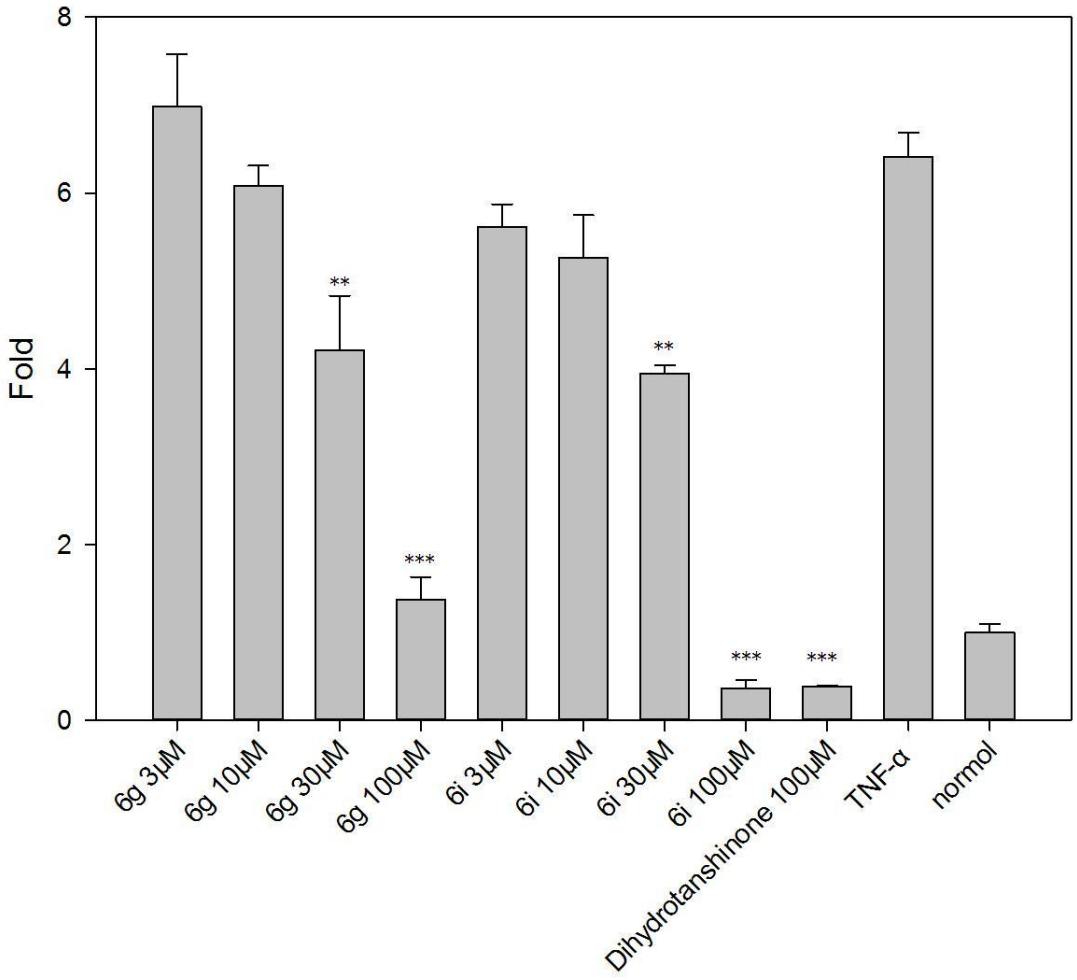


Fig. 2: HeLa cells were transiently transfected with a NF-κB-dependent reporter gene for 48 h and then pretreated for 8 h with the indicated concentrations of compounds **6i** and **6g** followed by stimulation for 8 h with TNF-α (10 ng/ml), and the luciferase activity was determined as described in "6.2. Pharmacology". Data represented as mean ± standard deviation of three independent experiments. **P＜0.01, ***P＜0.001, significantly different when compared with TNF-α-stimulated normal cells.

To evaluate the effect of compounds **6g** and **6i** on viability of HeLa cells, the MTT assay was performed. The cytotoxicity of both compounds decreased at decreasing dosage. Compound **6g** showed higher cytotoxicity, but compound **6i** exhibited slight cellular toxicity on HeLa cells at 100 μM (**Fig. 3**). Generally, compound **6i** showed reliable anti-inflammatory potential and lower toxicity than **6g**.

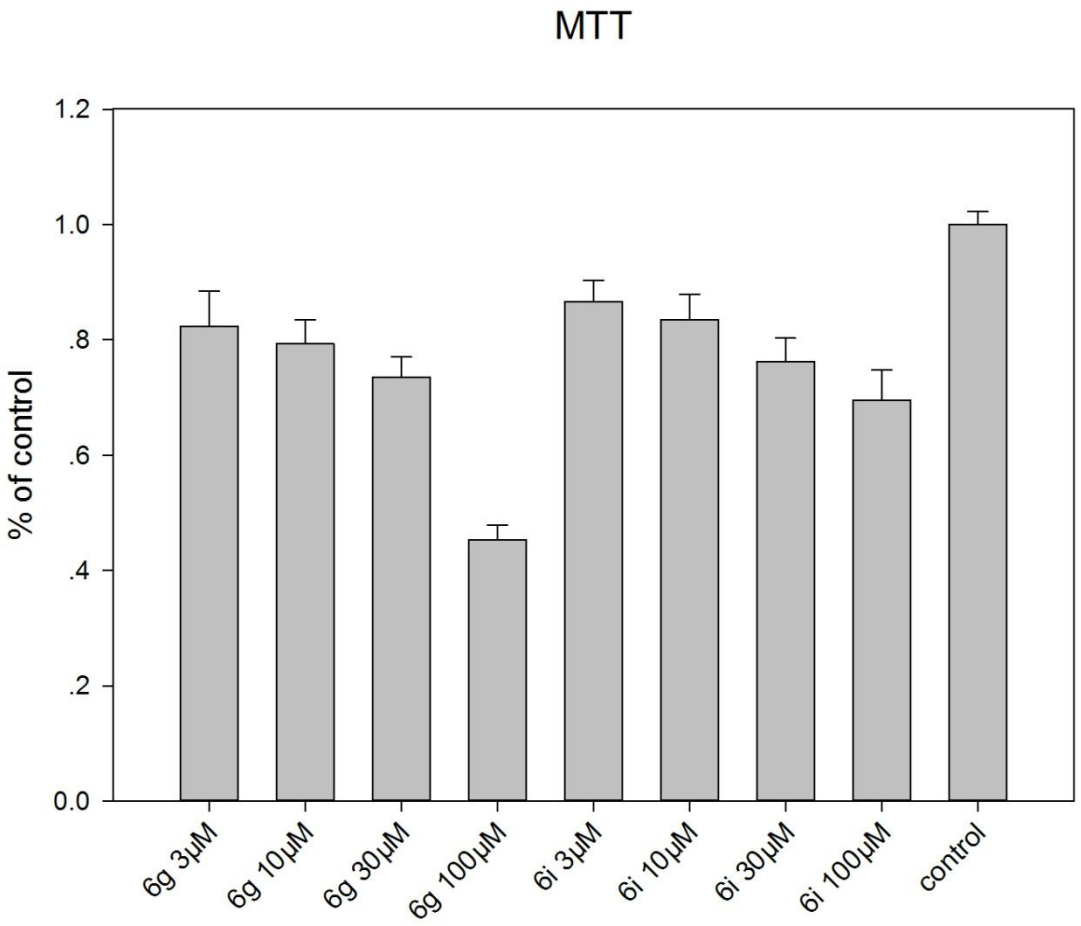


Fig. 3: HeLa cells were treated with the indicated concentrations of the compounds **6i** and **6g**. After 24 h incubation, cell viability was determined by MTT assays. Data represented as mean ± standard deviation of three independent experiments.

Based on the above results, the anti-inflammatory activity of compound **6i** further evaluated *in vivo*. In this experiment, we used indomethacin as the control positive drug. Indomethacin is a widely used non-steroidal anti-inflammatory drugs (NSAIDs) with anti-inflammatory, analgesic and antipyretic activity. Compound **6i** showed significant anti-inflammatory activity with 58.19% inhibition at 50 mg/kg (0.5 h after i.p.) in the Kunming mice. The results are shown in **Table 1**. Apparent statistical significance was observed between the test group and the control group. It is worth mentioning that compound **6i** and the control positive drug indomethacin (at a dosage of 100 mg/kg) showed equivalent anti-inflammatory activities. To determine the oral time to peak effect (TPE) of compound **6i**, we conducted a time-course test by

administering oral medications (p.o.; **Table 2**). As the interval lengthened, the anti-inflammatory activity of compound **6i** first increased and then declined at the dosage of 50 mg/kg; the peak activity was observed at the 4 h interval with 38.8% inhibition, which was lower than that observed with the positive control drug.

Table 1. Anti-flammatory activites of compound **6i** and the positive control administrated i.p.

Comp.	Dose (mg/kg)	Number of mice	Edema mean ±SEM.(mg)	Inhibition rate(%)
6i	50	8	3.70±0.73*	58.19
Indomethacin	100	8	3.61±0.49*	59.21
DMSO	-	8	8.85±1.00	-

*$p < 0.05$ compared with vehicle control group.

Table 2. anti-inflammatory activity of compound **6i** administrered orally at different times before xylene application.

Time (h)	Inhibition	
	6i (Dose 50 mg/kg)	Indomethacin (Dose 100 mg/kg)
1	5.63	19.05
2	8.92	22.49
3	22.73	27.67*
4	38.85*	63.39*
5	22.44	53.46*
6	8.19	29.65

*$p < 0.05$ compared with vehicle control group at the corresponding time.

In conclusion, a new series of anti-inflammatory 6-phenoxy-[1,2,4]triazolo[3,4-*a*] phthalazine-3-carboxamide derivatives were synthesized and their anti-inflammatory activities were evaluated by the luciferase reporter assay. Two compounds **6g** and **6i**

showed promising anti-inflammatory activity, which was much better than the TNF-α-induced group and slightly more potent than the normal control group. It is worth mentioning that compound **6i** had comparable activity with the reference drug dihydrotanshinone, and showed lower cellular toxicity than compound **6g**, indicating that **6i** is most likely safer than **6g**. Then, compound **6i** was selected for anti-inflammatory evaluation *in vivo*. The activity screening indicated that compound **6i** at 50 mg/kg showed 58.19% inhibition compared with the control positive drug at the dose of 100 mg/kg in mice at 0.5 h after i.p.. It means that compound **6i** could achieve a similar anti-inflammatory activity with a lower dosage than Indomethacin, the widely used NSAIDs. We then reported that intraperitoneal administration of the drug was superior to oral one (only with 38.8 % inhibition by p.o.). We inferred that the anti-inflammatory activity of compound **6i** might be affected by the first-pass effect.

Conflict of interest: We declare that we have no conflict of interest with respect to this study.

Acknowledgments

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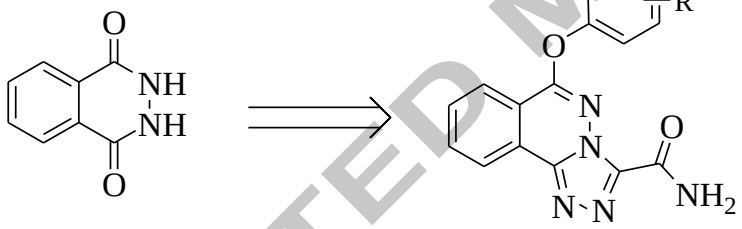
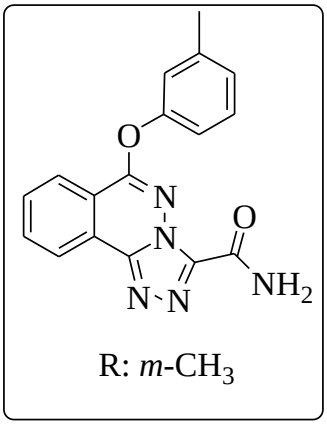
Synthesis and anti-inflammatory activity evaluation of a novel series of 6-phenoxy-[1,2,4]triazolo[3,4-*a*]phthalazine-3-carboxamide derivatives

Da-Chuan Liu [a], Guo-Hua Gong [b, c], Cheng-Xi Wei [b], Xue-Jun Jin [a, *], Zhe-Shan Quan [a, *]

A new series of anti-inflammatory 6-phenoxy-[1,2,4]triazolo[3,4-a]phthalazine-3 -carboxamide derivatives were synthesized and their anti-inflammatory activities were evaluated by the luciferase reporter assay. 6-(3-tolyloxy)-[1,2,4]triazolo[3,4-*a*]phthalazine -3-carboxamide (compound **6i**) had comparable activity with the reference drug dihydrotanshinone with low cellular toxicity. Then, compound **6i** was selected for anti-inflammatory evaluation in vivo. The activity screening indicated that compound 6i at 50 mg/kg showed 58.19% inhibition at 0.5 h after i.p. and 38.8 % inhibition by p.o..



Target compound



R: *m*-CH_3