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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] *Key Laboratory of Natural Resources and Functional Molecules of the Changbai Mountain, Affiliated Ministry of Education, College of Pharmacy, Yanbian University, Yanji, Jilin 133002, China*
[b] *Medicinal Chemistry and Pharmacology Institute, Inner Mongolia University for the Nationalities, Tongliao, Inner Mongolia 208002, China*
[c] *Affiliated Hospital of Inner Mongolia University for Nationalities, Tongliao 028000, Inner Mongolia, China*

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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).

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

* Corresponding authors.
E-mail addresses: xjjin@ybu.edu.cn (X.-J. Jin), zsquan@ybu.edu.cn (Z.-S. Quan).

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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.

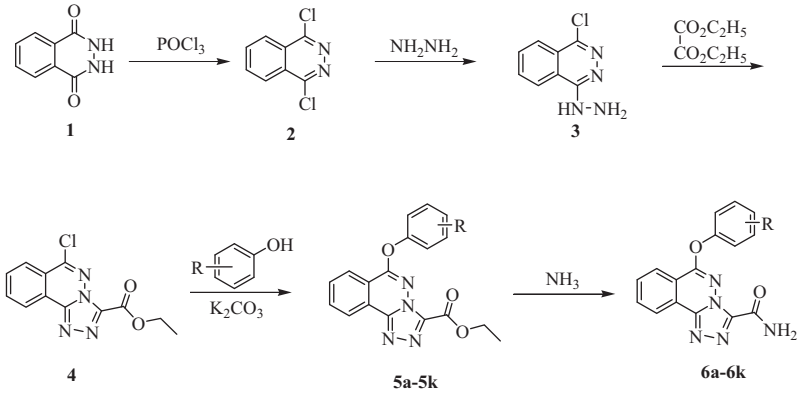
Compounds were synthesized according to Scheme 1. On the basis of the previous studies carried out in our laboratory, we designed and synthesized 6-phenoxy-[1,2,4]triazolo[3,4-*a*]phthalazine-3-carboxamide derivatives (**6a**–**6k**). The starting material 2,3-dihydrophthalazine-1,4-dione (compound **1**)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

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**)



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)	

Scheme 1. The synthesis of title compounds **6a**–**6k**. Reagents and conditions: (a) $POCl_3$, reflux, 4 h, yield 83.5%; (b) $NH_2NH_2{\cdot}H_2O$, ethyl acetate, 25–60 °C, yield 75.6%; (c) diethyl oxalate, reflux, 1 h, yield 52.6%; (d) substituted phenol, K_2CO_3, ethyl acetate, 70 °C, 4 h, H_2O, 25 °C, 0.5 h, yield 51.2–61.3%; (e) NH_3 (28% wt. in H_2O), 45 °C, 1 h, yield 45.9–59.8%.