**Screening of Bufadienolides from Toad Venom Identifies Gammabufotalin as a Potential Anti-inflammatory Agent**

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**ABSTRACT**
Toad venom (Chansu) is used in the treatment of infectious and inflammatory diseases in China and East/Southeast Asian countries. However, the anti-inflammatory components of toad venom have not yet been systematically evaluated and clearly defined. To investigate the anti-inflammatory effects of toad venom and identify new anti-inflammatory ingredients, we used zebrafish, an alternative drug screening model, to evaluate the anti-inflammatory effects of 14 bufadienolides previously isolated from toad venom. Most of the bufadienolides were found to exert significant anti-inflammatory effects on lipopolysaccharide-, CuSO4-, or tail transection-induced zebrafish inflammatory models. Moreover, gammabufotalin (6) inhibits lipopolysaccharide-induced inflammation by suppressing the myeloid differentiation primary response 88/nuclear factor-kappa B and STAT3 signal pathways. This study confirms the potential of zebrafish in drug screening, clarifies the anti-inflammatory effects of bufadienolides from toad venom, and indicates that gammabufotalin may be developed as a novel therapeutic agent for inflammatory diseases in the future.
Introduction

Toad venom (Chansu) is a traditional Chinese medicine that consists of the self-defensive secretions from the auricular and skin glands of the Asiatic toad _Bufo gargarizans_ or the Asian common toad _Duttaphrynus melanostictus_ (synonym _Bufo melanostictus_) [1,2]. It is widely used in China alone or combined with other drugs in the treatment of respiratory infections and inflammatory diseases, such as hepatitis, otitis media, tonsillitis, pulpitis, peri-coronitis, and arthritis [3,4]. Toad venom is usually processed by drying and pulverizing in order to be used in pills or topical formulations [5]. Bufadienolides are the main bioactive components of toad venom. Several studies have reported the anti-inflammatory activities of bufadienolides, especially on cancer-related inflammation [6,7]. Cinobufagin inhibited the NF-κB signaling pathway and decreased the production of TNF-α, IL-1β, and IL-12 in LPS-stimulated dendritic cells [8]. Bufalin attenuated the protein expression levels of the proinflammatory mediators TNF-α, IL-6, COX-2, and IL-1β through NF-κB signaling in carrageenan-induced paw edema in rats [6]. These findings highlight that toad venom has a promising future as an anti-inflammatory drug. However, the anti-inflammatory components of toad venom have not yet been systematically evaluated and clearly defined.

Zebrafish has emerged as a novel and alternative model for high-throughput screening for drug discovery [9,10]. Compared to cell lines, a zebrafish possesses fully developed vertebrate organ systems, and phenotype-based screening using a zebrafish model enables the study of a much broader range of biological processes [9]. Compared to murine models, experiments on zebrafish are easier to perform [11]. More importantly, accumulated evidence indicates that the immune system of the zebrafish is highly conserved with that of humans, including multiple immune cells (neutrophils, macrophages, and lymphocytes) and a large number of inflammatory genes (IL-6, TNF-α, NF-κB, etc.) [12–14].

MyD88, a critical adapter protein for toll-like receptor 4, leads to the activation of downstream NF-κB and the subsequent production of proinflammatory cytokines, such as TNF-α, IL-6, COX-2, and IL-1β [15]. STAT3 also plays a pivotal role in mediating the cascade response of inflammation [16]. In the current study, zebrafish inflammatory models were established to evaluate the anti-inflammatory effects of 14 bufadienolides previously isolated from toad venom [17] and the inhibitory effects of a selected bufadienolide, gammabufotalin, on proinflammatory mediators. The MyD88/NF-κB and STAT3 pathways were also examined.

Results

The structures of the bufadienolides isolated from toad venom are shown in Fig. 1. To identify their in vivo anti-inflammatory activities, a Tg(mpo-GFP) transgenic zebrafish line was used. In this zebrafish, mpo, tagged with GFP, is expressed in neutrophils, which makes it possible to track neutrophil behavior in vivo during the inflammatory process [18].

LPS is a typical proinflammatory agent that can trigger an inflammatory response and consequently activate a series of signaling cascades. Our research group initially established a zebrafish inflammatory model by microinjecting LPS into the yolk [19], which has been widely used in the rapid screening of anti-inflammatory drugs in vivo [20–22]. In this study, the anti-inflammatory effects of 14 bufadienolides at nontoxic doses (1, 4, and 12: 50 µM; 2, 13, and 14: 10 µM; 3 and 6: 5 µM; 5 and 8: 1 µM; 7, 9, and 11: 0.5 µM; 10: 4 µM) were evaluated for the first time using an LPS-stimulated zebrafish inflammatory model. As shown in Fig. 2, a large number of neutrophils can be found in the yolks after LPS microinjection, while bufadienolides or dexamethasone (positive control, 5 µg/ml) reduced the recruitment of neutrophils in the yolk sac. Gammabufotalin (6), bufalin (8), and 19-oxodes-actelycinobufotalin (12) showed the most remarkable inhibitory effects.

CuSO₄ and tail transection can injure the lateral line neuromast areas or tails of zebrafish, and these two inflammatory models have been used for large-scale chemical screenings to seek new effective compounds [23–25]. Thus, the effects of 14 bufadienolides were further confirmed in CuSO₄- or tail transection-induced inflammatory models. Our results showed that the accumulation of neutrophils was significantly reduced in the injured areas after treatment with bufadienolides, except for 1, 2 and 5 (Figs. 3 and 4), which confirms that most bufadienolides have strong anti-inflammatory activities, especially 6 and 12.

To further identify the anti-inflammatory activities of bufadienolides and explore their underlying mechanisms, gammabufotalin (6) was chosen for the next experiments.

Survival rate analysis was carried out to visually measure the defensive efficacy of zebrafish on LPS. As shown in Fig. 5, all zebrafish died within 68 h post-injection in the LPS group, whereas more than 40% of zebrafish survived in gammabufotalin group with the same treatment duration, which indicates that gammabufotalin exhibits protective effects on zebrafish challenged by LPS.

Infiltration of inflammatory cells is a critical process during inflammation [26]. We observed the histopathological features of LPS-infected zebrafish by H&E staining. As shown in Fig. 6, infiltration of inflammatory cells was clearly observed in LPS-induced zebrafish. As expected, the histopathological features were ameliorated by gammabufotalin treatment, which further confirms that gammabufotalin exerts anti-inflammatory effects in LPS-microinjected zebrafish.
The proinflammatory cytokine mediators IL-6 and TNF-α play important roles in inflammation [27, 28] and can be overexpressed after LPS stimulation [29]. To investigate whether gammabufotalin regulates the expression levels of IL-6 and TNF-α, qRT-PCR analysis was employed to evaluate the mRNA levels of IL-6 and TNF-α in LPS-infected zebrafish. As shown in Fig. 7, γ-gammabufotalin significantly inhibited LPS-induced IL-6 and TNF-α upregulation, suggesting that gammabufotalin exerts its anti-inflammatory effects by inhibiting the expression levels of proinflammatory cytokines in vivo.

![Chemical structures of bufadienolides isolated from toad venom.](image)

**Fig. 7**

IL-6 and TNF-α mRNA levels were increased remarkably in LPS-microinjected zebrafish. Gammabufotalin significantly inhibited LPS-induced IL-6 and TNF-α upregulation, suggesting that gammabufotalin exerts its anti-inflammatory effects by inhibiting the expression levels of proinflammatory cytokines in vivo.
The NF-κB signaling pathway plays a vital role during the process of inflammation and can be activated by MyD88 in response to the LPS stimulus [30]. The phosphorylation and ubiquitylation of IκBα can promote the release and translocation of NF-κB into the nucleus, triggering the secretion of multiple inflammatory cytokines [31]. STAT3 is another important transcription factor in inflammation that can be activated by LPS and then translocate into the nucleus to regulate specific genes [16]. We thus examined the mRNA expression levels of NF-κB, IκBα, MyD88, and STAT3 to further elucidate the action mechanisms of gammabufotalin. Our results showed that gammabufotalin remarkably inhibited the up-regulation of NF-κB, IκBα, MyD88, and STAT3 in LPS-stimulated zebrafish (Fig. 8), suggesting that the anti-inflammatory effects of gammabufotalin are closely associated with inhibition of MyD88/NF-κB and STAT3 signal pathways (Fig. 9).

Discussion

In recent years, the zebrafish has become a prominent vertebrate model in biomedical research. Besides the advantages of small size, rapid growth, high productive rate, relatively transparent embryos, and amenability to genetic manipulation, the zebrafish has high similarities with humans in disease type and innate immune system [32]. Zebrafish embryos or larvae facilitate high-throughput in vivo experiments, and a large-scale screening on zebrafish has been performed for drug discovery [9]. In the present...
transgenic line \textit{Tg(mpo} : GFP\textit{)}, a neutrophil-labeled zebrafish line, was used. Neutrophils are major effectors of acute inflammation and innate immune responses. They can be recruited to infected or injured tissues by proinflammatory cytokines such as TNF-$\alpha$ and IL-6. Deregulation of neutrophils and their hyperactivity can lead to tissue damage in severe inflammation or trauma [33]. Neutrophil-labeled zebrafish allow us to observe the biological behavior of larvae neutrophils in real time, which facilitates anti-inflammatory drugs screening. LPS-, CuSO$_4$-, and tail transection-induced inflammatory models were established in our study. They respectively represent inflammations that are caused by bacterial infection and chemical and physical injuries. Our research group has successfully used these models in studying traditional Chinese medicines on inflammatory diseases [20, 34].

Toad venom has been traditionally used for treating infectious and inflammatory diseases in China for thousands of years. In the Chinese Pharmacopoeia, toad venom is considered a therapeutic agent for a carbuncle abscess, swollen sore throat, diarrhea with abdominal pain, and so on, with detoxicant, anti-inflammatory, and antalgic effects [35]. Bufadienolides are considered the major bioactive components of toad venom. However, except for bufalin and cinobufagin [36], there is little known about the biological effects of bufadienolides. In the present study, we evaluated the anti-inflammatory activities of 14 bufadienolides from toad venom in 3 zebrafish inflammatory models. Our data showed that

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Bufadienolides, except for 1, 2, and 5, reduce the accumulation of neutrophils in 3-dpf \textit{Tg(mpo} : GFP\textit{)} larvae subjected to CuSO$_4$. a Representative images of zebrafish in the CuSO$_4$-induced inflammatory model (scale bar = 200 $\mu$m). Dexamethasone (Dex) was utilized as the positive control. b The number of neutrophils in the region of interest (red irregular rectangles) were counted. Data are represented as the mean $\pm$ SD of three independent experiments (n = 15). ###$P < 0.001$ vs. control group, ***$p < 0.001$ vs. model group by one-way ANOVA with Tukey’s test.}
\end{figure}
these bufadienolides, except for 1, 2, and 5, have significant anti-inflammatory effects, which were indicated by reduced migration and recruitment of neutrophils to the injured sites. However, the inhibitory activities of these compounds were slightly different in the three zebrafish models, which may be attributed to the different inflammatory agents, inflammatory sites, or action time of bufadienolides. Gammabufotalin possessed the most significant anti-inflammatory activity among these bufadienolides. The anti-inflammatory properties of gammabufotalin were further confirmed by its effects on protecting zebrafish against LPS-induced death, alleviating inflammatory cell infiltration, and downregulating proinflammatory cytokines IL-6 and TNF-α. According to the results from all three zebrafish inflammatory models, 12 showed more potent effects than 4, suggesting that the introduction of an aldehyde group might be favorable to the anti-inflammatory activity. The difference of anti-inflammatory activities between 8 and 9 revealed that the presence of a hydroxyl group was unfavorable to the activity.

MyD88/NF-κB and STAT3 pathways that have been identified in zebrafish are the crucial hallmarks of inflammation and cancer [37, 38]. On one hand, activated MyD88/NF-κB signaling can secrete cytokine IL-6 to directly stimulate the STAT3 pathway [39]. On the other hand, STAT3 can further enhance NF-κB activity via prolonging its nuclear retention. These data suggest that NF-κB...
and STAT3 pathways are synergistically interacted in the inflammatory response [40]. We found that gammabufotalin significantly inhibited the mRNA levels of NF-κB, IκBα, MyD88, and STAT3 in LPS-infected zebrafish larvae. These results indicate that the anti-inflammatory activity of gammabufotalin may be due to its inhibitory effects on MyD88/NF-κB and STAT3 signaling pathways.

In summary, our study shows for the first time that bufadienolides from toad venom possess anti-inflammatory activities in zebrafish inflammatory models. Gammabufotalin (6) exerts the most potent inhibitory effects, which are related to the MyD88/NF-κB and STAT3 signaling pathways inhibition.

Materials and Methods

Material and reagents

The venom of *B. gargarizans* was bought from Baoyin Toad Breeding Base (Jiangsu, China). Bufadienolides were isolated and identified as previously reported [17]. Purity determined by HPLC was ≥95%. The chromatographic separation was performed on a COSMOSIL-C18 column (250 mm × 4.6 mm, 5 µm) at 30°C using MeOH and H2O as the mobile phase at a flow rate of 1.0 mL/min. The chromatogram was monitored at 296 nm. RNAiso Plus and an RT-PCR kit were provided by Takara. Dexamethasone was purchased from Guangzhou Baiyunshan Tianxin Pharmaceutical Co. Methylene blue was from Dalian Meilun Biotechnology Co and tricaine was from Shanghai Macklin Biochemical Co. DMSO, LPS, *Escherichia coli* O55: B5, and other reagents were obtained from Sigma-Aldrich.

Zebrfish maintenance and embryos collection

*Tg(mpo : GFP)* [18] zebrafish were a kind gift from Professor Wenging Zhang of South China University of Technology. They were maintained under a 14-h light/10-h dark cycle in a recirculating aquatic habitat system (pH 7.2–7.6, salinity 0.03–0.04%) following standard guidelines for maintenance protocols [41]. Adult zebrafish were housed in a breeding tank in a male-to-female ratio of 1:2 to collect a sufficient number of embryos, which were subsequently transferred to a clean petri dish filled with egg water containing 0.002% methylene blue as a fungicide. Finally, these embryos were kept in a warm oven at 28.5 °C for the following experiments.

Determination of nontoxic concentrations of bufadienolides in zebrafish

Larvae at 3 dpf were divided into 24-well plates (n = 20) and then treated with different concentrations of bufadienolides. Untreated zebrafish larvae served as the control group. After 24 h, the dead embryos were recorded and the concentrations that caused a survival rate over 95% were used as the nontoxic concentrations.

Lipopolysaccharide-induced inflammation model

Larvae at 3 dpf were anesthetized with 0.02% tricaine and immobilized in a clear petri dish coated with 2% agarose (Sigma-Aldrich). Subsequently 2 nL of LPS (0.5 mg/mL) were microinjected into the yolks to construct the inflammatory model [19, 20] by a cell microinjector (PM1000; MicroData Instrument, Inc.).
Next, these larvae were divided randomly into a 24-well plate (n = 20/well) and treated with various bufadienolides. Dexamethasone (5 µg/mL) served as a positive control and PBS was used as the vehicle control. Behavioral assessment of neutrophils was performed at 12 h post-LPS microinjection using a fluorescence microscope (MVX10; Olympus).

CuSO₄-induced inflammation model

Larvae at 3 dpf were randomly placed in a 24-well plate with 15 zebrafish/well and exposed to CuSO₄ (20 µM) in the presence or absence of various bufadienolides for 2 h. Untreated zebrafish larvae were used as the control group. Dexamethasone (5.0 µg/mL) was used as the positive control and the images were captured to evaluate the recruitment of neutrophils.
Tail transection-induced inflammation model
Larvae were injured at 3 dpf by transection of the caudal fin with a scalpel blade under a stereomicroscope (Olympus; SZX7). Next, these injured larvae were incubated with various bufadienolides for 6 h, mounted in 2% agarose, and imaged to observe the behavior of the fluorescent neutrophils. Untreated zebrafish larvae were used as the control group (n = 15).

Survival analysis
After microinjection, deceased zebrafish were counted every day and this experiment lasted for 3 days (n = 30) [19–21].

Histopathological analysis
Twelve hours after LPS microinjection with or without drug administration, the zebrafish (n = 30) were fixed in 4% (w/v) paraformaldehyde, dehydrated in graded ethanol, embedded with paraffin (Leica), and cut into 4-µm sections. The specimens were subsequently stained with H&E (Yuanye Biotech) and observed under a IX 53 light microscope (Olympus) [20].

Quantitative real-time polymerase chain reaction analysis
Total RNA was extracted from 30 larvae at 12 h post-microinjection with RNAiso Plus. Then CDNA was synthesized following the manufacturer’s instructions and the reverse transcription program was set as: 37°C for 15 min to start reaction, 85°C for 5 s to inactive enzymatic activity, 4°C to preserve. QRT-PCR was performed on a LightCycler 96 real-time PCR instrument (Roche) using TaKaRa Taq PCR kits. The amplification was performed at 95°C for 30 s followed by a total of 50 cycles at 95°C for 5 s, 60°C for 30 s, and a final extension at 95°C for 5 s, 65°C for 60 s, 95°C for 1 s. The gene expression ratios were calculated by the 2^ΔΔCT method normalized to the expression level of β-actin. The primer sequences were as follows: β-actin, (forward) 5’-AGACCGCTGCTGTCTAAA-3’ and (reverse) 5’-TTTGATG CTGTTACCCAGA-3’; NF-κB, (forward) 5’-GCTGGATCTCTAAGTGTTGTA-3’ and (reverse) 5’-TGATTGACCTCTCCGTGT-3’; IκBα, (forward) 5’-GGTG GAAAGACTCCTGAAAGC-3’ and (reverse) 5’-GAGATTTAGGGAAGTAAAGT-3’; MyD88, (forward) 5’-GAGATGTGGTGCTGTACCTC-3’ and (reverse) 5’-CGACAGG GATTAG CCGTTTA-3’; STAT3, (forward) 5’-CTCTGGGACTAACGTGGCA-3’ and (reverse) 5’-AGAGGCTCTGATTGCCC-3’.

Statistical analysis
Data are presented as the mean ± standard deviation (SD) from at least three independent experiments. Comparison of means among multiple groups was performed with one-way ANOVA using the statistical software GraphPad Prism 5.0. P < 0.05 was considered statistically significant.

Contributors’ Statement

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Conflict of Interest
The authors declare that they have no conflict of interest.

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