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.

* These authors contributed equally to this work.

ABBREVIATIONS

ANOVA	analysis of variance
COX-2	cyclooxygenase-2
dpf	days post-fertilization
GFP	green fluorescent protein
H&E	hematoxylin and eosin
IκBα	inhibitor of kappa B
IL	interleukin
LPS	lipopolysaccharide
mpo	myeloperoxidase
MyD88	myeloid differentiation primary response 88
NF-κB	nuclear factor-kappa B
qRT-PCR	quantitative real-time polymerase chain reaction
STAT3	signal transducer and activator of transcription 3

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, pericoronitis, 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 initiatively 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-oxodesactelycinobutofalin (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.

► **Fig. 1** Chemical structures of bufadienolides isolated from toad venom.

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**,

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*.

► **Fig. 2** Bufadienolides reduce the accumulation of neutrophils in 3-dpf *Tg(mpo:GFP)* larvae subjected to LPS. **a** Representative images of zebrafish in the LPS-induced inflammatory model (scale bar = 200 µm). Dexamethasone (Dex) was utilized as the positive control. **b** The number of neutrophils in the region of interest (red circles) were counted. Data are represented as the mean ± SD of three independent experiments (n = 20). ###p < 0.001 vs. PBS group, ***p < 0.001 vs. LPS group by one-way ANOVA with Tukey's test.

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

Fig. 3 Bufadienolides, except for 1, 2, and 5, reduce the accumulation of neutrophils in 3-dpf *Tg(mpo:GFP)* larvae subjected to CuSO_4 . **a** Representative images of zebrafish in the CuSO_4 -induced inflammatory model (scale bar = 200 μm). 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.

study, transgenic line *Tg(mpo:GFP)*, 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 $\text{TNF-}\alpha$ 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 zebrafish 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 analgic 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

► **Fig. 4** Bufadienolides, except for 1, 2, and 5, reduce the accumulation of neutrophils in 3-dpf *Tg(mpo:GFP)* larvae subjected to tail transection. **a** Representative images of zebrafish in the tail transection-induced inflammatory model (scale bar = 200 µm). Dexamethasone (Dex) was utilized as the positive control. **b** The number of neutrophils in the region of interest (red squares) were counted. Data are represented as the mean ± 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.

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- κ B 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- κ B signaling can secrete cytokine IL-6 to directly stimulate the STAT3 pathway [39]. On the other hand, STAT3 can further enhance NF- κ B activity via prolonging its nuclear retention. These data suggest that NF- κ B

► **Fig. 5** Protective effect of gammabufotalin (**6**) in LPS-induced 3-dpf *Tg(mpo:GFP)* larvae. Each survival curve represents data pooled from three independent experiments ($n = 30$). Dexamethasone (Dex) was utilized as the positive control. $***p < 0.001$ vs. PBS group, $***p < 0.001$ vs. LPS group by one-way ANOVA with Tukey's test.

and STAT3 pathways are synergistically interacted in the inflammatory response [40]. We found that gammabufotalin significantly inhibited the mRNA levels of NF- κ B, I κ B α , 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- κ B 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- κ B 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

$\geq 95\%$. The chromatographic separation was performed on a COSMOSIL-C₁₈ column (250 mm $\times$ 4.6 mm, 5 μ m) at 30 $^{\circ}$ 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.

Zebrafish maintenance and embryos collection

Tg(mpo:GFP) [18] zebrafish were a kind gift from Professor Wenqing 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 $^{\circ}$ 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.).

► **Fig. 6** Gammabufotalin alleviates the inflammatory cell infiltration in LPS-induced 3-dpf *Tg(mpo:GFP)* larvae. The zebrafish larvae were microinjected with LPS in the presence or absence of gammabufotalin. Dexamethasone (Dex) was utilized as the positive control. After dehydration and embedding, paraffin sections (4 μ m) were stained by H&E. The black arrows represent the inflammatory cells (scale bar = 50 μ m, $n = 30$).

► **Fig. 7** Gammabufotalin reduces the mRNA expression levels of IL-6 and TNF- α in LPS-induced 3-dpf *Tg(mpo:GFP)* larvae. **a** The mRNA expression level of IL-6. **b** The mRNA expression level of TNF- α . Data are represented as the mean $\pm$ SD of three independent experiments ($n = 30$). Dexamethasone (Dex) was utilized as the positive control. ^{###} $P < 0.001$ vs. PBS groups, ^{**} $p < 0.01$ vs. LPS groups by one-way ANOVA with Tukey's test.

► **Fig. 8** The effects of gammabufotalin on the mRNA expression levels of NF- κ B, I κ B α , MyD88, and STAT3 in LPS-induced 3-dpf *Tg(mpo:GFP)* larvae. **a** The mRNA expression level of NF- κ B. **b** The mRNA expression level of I κ B α . **c** The mRNA expression level of MyD88. **d** The mRNA expression level of STAT3. Data are represented as the mean $\pm$ SD of three independent experiments ($n = 30$). Dexamethasone (Dex) was utilized as the positive control. ^{##} $P < 0.01$, ^{###} $p < 0.001$ vs. PBS group, ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs. LPS group by one-way ANOVA with Tukey's test.

Next, these larvae were divided randomly into a 24-well plate ($n = 20$ /well) and treated with various bufadienolides. Dexamethasone (5 μ g/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 μ M) in the presence or absence of various bufadienolides for 2 h. Untreated zebrafish larvae were used as the control group. Dexamethasone (5.0 μ g/mL) was used as the positive control and the images were captured to evaluate the recruitment of neutrophils.

► **Fig. 9** Possible anti-inflammatory mechanisms of gammabufotalin.

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- μ m sections. The specimens were subsequently stained with H&E (YuanYe Biotech) and observed under an 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 $2^{-\Delta\Delta C_t}$ method normalized to the expression level of β -actin. The primer sequences were as follows: β -actin, (forward) 5'-ATGGATGAG-GAAATCGCTG-3' and (reverse) 5'-A TGCCAACCATCAC TCCC

TG-3'; IL-6, (forward) 5'-AGACCGCTGCCTGTCTAAAA-3' and (reverse) 5'-TTTGATG TCGTTCACCAGGA-3'; TNF- α , (forward) 5'-GCTGGATCTTCAAAGTCGGGTGTA-3' and (reverse) 5'-TGTGAGT CTCAGCACACTTCCATC-3'; NF- κ B, (forward) 5'-GAGCCCTTTGTG CAAGAGAC-3' and (reverse) 5'-TGGGATACGTCCTCCTGTTC-3'; I κ B α , (forward) 5'-GGTG GAAAGACTCCTGAAAGC-3' and (reverse) 5'-TGTAAGGGAAGGTAAGAATG-3'; MyD88, (forward) 5'-GAGGATGGTGGTGCATCT-3' and (reverse) 5'-CGACAGG-GATTAG CCGTTTA-3'; STAT3, (forward) 5'-CCCTGGGACTAACTC TGGCA-3' and (reverse) 5'-AGAG GTCCTGGATTGGCCTC-3'.

Statistical analysis

Data are presented as the mean $\pm$ 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

Data collection: Y.R. Zheng, L.J. Deng, H.H. Cao, N.S. Xu, H.Y. Tian, B.J. Li, Z.B. Lu; design of the study: D.M. Zhang, W.C. Ye, L.Z. Yu, C.L. Fan, J.S. Liu; statistical analysis: Y.R. Zheng, L.J. Deng, H.H. Cao, N.S. Xu, D.M. Zhang, H.Y. Tian, B.J. Li, Z.B. Lu, W.C. Ye, L.Z. Yu, C.L. Fan, J.S. Liu; analysis and interpretation of the data: Y.R. Zheng, L.J. Deng, H.H. Cao, N.S. Xu, D.M. Zhang, H.Y. Tian, B.J. Li, Z.B. Lu, W.C. Ye, L.Z. Yu, C.L. Fan, J.S. Liu; drafting the manuscript: Y.R. Zheng, L.J. Deng, H.H. Cao, N.S. Xu, D.M. Zhang, H.Y. Tian, B.J. Li, Z.B. Lu, W.C. Ye, L.Z. Yu, C.L. Fan; critical revision of the manuscript: J.S. Liu.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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