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ORIGINAL ARTICLE

Anti-Inflammation Associated Protective Mechanism of Berberine and its Derivatives on Attenuating Pentylenetetrazole-Induced Seizures in Zebrafish

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Abstract

Epileptic seizures are characterized by synchronized discharges of neurons, leading to the activation of inflammatory responses that in turn contributes to seizure progression. Berberine (BBR), a bioactive constituent extracted from berberis, has been known to relieve seizures in rodent models. In this study, we synthesized two derivatives of berberine (BBR-D1 and BBR-D2) to compare their seizure reducing effect with BBR in pentylenetetrazole (PTZ)-induced seizures in zebrafish. We found a structure-activity relationship between hydrophilic/hydrophobic composition of the derivatives and their anticonvulsant activity. We also investigated the underlying mechanism related to their anti-inflammatory effect during seizures. BBR and its derivatives increased the seizure onset latency and suppressed the seizure-like behavior after PTZ treatment. Zebrafish larvae pretreated with BBR and its derivatives showed recovery on c-fos expression and neuronal discharges during seizures. The inflammatory responses occurred during the progression of seizures, including the recruitment of macrophages and neutrophils as well as an up-regulation of tumor necrosis factor alpha (TNFα), interleukin 1 beta (il1β), and interleukin 6 (il6). This effect was significantly suppressed by the pretreatment of BBR and its derivatives. Our results suggest that BBR and its derivatives attenuate PTZ-induced seizures and modulate anti-inflammatory effect to potentially protect zebrafish from the occurrence of further seizures. From the tested compounds, BBR-D1 (the hydrophilic berberrubine) showed the strongest seizure reducing effect.

Keywords Epilepsy · PTZ · Berberine · Anticonvulsant · Anti-inflammation · Zebrafish

Baoyue Zhang, Lizhen Wang and Xiuna Ji contributed equally to this work.

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Introduction

One of the most common neuronal disorders is epilepsy, which is characterized by the pathologically synchronized discharges of neurons, termed seizures. Seizures can manifest in convulsive behavior (Baraban et al. 2005; Stafstrom and Carmant 2015). Neuronal loss, inflammation, and oxidative stress induction are all observed in epileptic seizures (Choo et al. 2018; Fontana et al. 2018; Jin et al. 2018a; Okuneva et al. 2016; Vezzani et al. 2011). Epilepsy is found in approximately 1% of the general population and its treatment mainly relies on antiepileptic drugs (AEDs) (Devinsky et al. 2018). Unfortunately, one third of epileptic patients fail to response to existing AEDs, highlighting the need to find bioactive compounds to defeat this disease (Franco et al. 2016).

For centuries, berberis was widely used as traditional herb in oriental countries to treat miscellaneous diseases (Imenshahidi and Hosseinzadeh 2016; Siddiqui et al. 2017). Berberine (BBR), the active compound of berberis, has hypoglycemic, antioxidant, and anti-inflammatory effects, thus it

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could be used to treat neuronal disorders (Lin and Zhang 2018; Wang et al. 2017). BBR has shown the ability to protect neurons from Parkinson's disease-induced cell death in SH-SY5Y cell line by reducing reactive oxygen species generation and up-regulating heme oxygenase-1 expression (Bae et al. 2013). During the course of spinal cord injury rehabilitation in rats, injected berberine can relieve inflammation and enhance oligodendrocyte autophagy contributing to primary spinal neuronal recovery (Wang et al. 2017). In epilepsy studies BBR showed the ability to delay the onset of seizure-like convulsions in pilocarpine-treated rats (Gao et al. 2014). Additionally, BBR reduced the incidence of spontaneous spasmodic seizures in rats and restored nuclear factor κB (NF-κB), interleukin-1-beta (il1β), and tumor necrosis factor alpha (TNFα) expression in hippocampus, suggesting that BBR could ameliorate seizures mainly by suppressing inflammation (Sedaghat et al. 2017).

In clinical applications, the main problem of BBR is the low bioavailability, probably routing from its rigid structure and poor solubility in water (Lo et al. 2013; Maeng et al. 2002). To enhance its bioavailability and bioactivity, several berberine derivatives were designed and synthesized including 9-*O*-substituted and *C*-8,13-substituted berberine derivatives, 9-OH or 10-OH pseudoberberines, and the hydroxyberberines (Cheng et al. 2010; Ma et al. 2009; Pongkittiphan et al. 2015; Shan et al. 2013; Wang et al. 2018). However, the structure–activity relationship between the synthetic berberine derivatives and their anticonvulsant activity is lacking.

Zebrafish are widely used to investigate the pathophysiology and pharmacology of neurological diseases, including Parkinson's disease, motor neuron diseases, and epilepsy (Babin et al. 2014; Barbalho et al. 2016; Cronin and Grealy 2017; Kundap et al. 2017; Rosa-Falero et al. 2014). Compared with traditional rodent models, zebrafish has numerous advantages including high number of offspring, low maintenance cost, and fast brain development (Duy et al. 2017; Hortopan et al. 2010; Stewart et al. 2014; Torres-Hernandez et al. 2015). Although the structure of the nervous system of zebrafish is less complex than in mammalian species, it contains similar neurons to all higher vertebrate species enabling researchers to investigate the generation of abnormal neuronal discharges and the role of inflammation in seizures (Wullimann and Mueller 2004; Wyckhuys et al. 2009). In addition, the spontaneous swimming of zebrafish larvae is relatively developed on 5 days post fertilization (dpf), making it a suitable model for seizures related behavior research (Baraban et al. 2005).

Although the function of BBR on attenuating seizures has been already revealed, the anticonvulsant effect of two newly synthesized BBR derivatives remains unknown. This paper compared the anticonvulsant effect of berberine with its two derivatives, the hydrophilic berberrubine (hereafter referred to as BBR-D1) and the hydrophobic ethyl-2-(9-demethoxyberberine bromide-9-yl) hydroxyacetate (hereafter referred to as BBR-D2) (Fig. 1). Since BBR has been reported to suppress seizures in rodents mainly via restoring the anti-inflammatory mediators (Sedaghat et al. 2017), we investigated whether the two derivatives also exert anticonvulsant effect through regulating anti-inflammatory responses.

Materials and Methods

Animal Maintenance

All experimental manipulations concerning zebrafish were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (No.8023, amended in 1996). Protocols and procedures were reviewed and approved by the animal committee of the Biology Institute of Shandong Academy of Sciences and were in accord with the guideline for the Care and Use of Laboratory Animals of China. Adult zebrafish was raised separately according to their gender under a 14/10 h of light/darkness cycle photoperiod. Fish were fed twice a day with live artemia nauplii from commercial fish food supplier. Salinity of aquarium water was 450–500 μS, with a pH maintained at 7.0–7.5. Fertilized eggs of zebrafish were collected from natural mating of sexually mature zebrafish bred, then washed and transferred to 6-wells cell culture plates. 20 eggs per well were kept with 4 mL of aquarium water. Wild-type zebrafish larvae were used for HPLC analyses, seizure-like behavioral tests, macrophage migration tests, quantitative real-time PCR (qPCR), and electroencephalogram (EEG) recording. The larvae of neutrophil-specific zebrafish line *MPO: GFP* strain (green fluorescent protein expressed specifically in neutrophils driven by myeloperoxidase

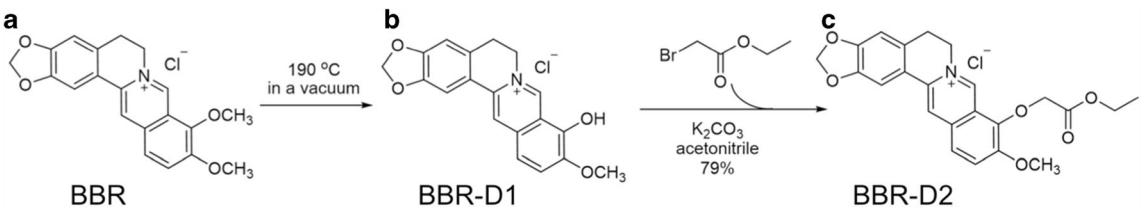


Fig. 1 Structure and synthesis of BBR-D1 and BBR-D2. **a** Structure of BBR. **b** High temperature and vacuum were used to generate BBR-D1. **c** Ethyl bromoacetate and K_2CO_3 at 80 °C were used to prepare BBR-D2.

promoter) was used for neutrophil migration assays (Renshaw et al. 2006).

Synthesis of BBR-D1 and BBR-D2

Berberine chloride (5 g, 13.48 mmol) was heated at 190 °C under high vacuum (20–30 mmHg) for 30 min to give a crude solid, which was purified by column chromatography (CH_2Cl_2/CH_3OH = 5/1) to provide BBR-D1 as a purple solid. BBR-D1 (500 mg, 1.40 mmol), ethyl bromoacetate (701 mg, 4.20 mmol) and K_2CO_3 (580 mg, 4.20 mmol) in 5 ml of N,N-Dimethylformamide (DMF) were stirred at 80 °C for 6 h. The color of the mixture was turned from purple into yellow. The reaction product was diluted with 10 mL of methanol and filtered, then the filtrate was evaporated and the resulting solid was purified by column chromatography to give BBR-D2 (490 mg, 79%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.95 (s, 1 H, C*H*=N), 8.95 (s, 1 H, Ar*H*), 8.19 (d, *J* = 9.2 Hz, 1 H, Ar*H*), 8.01 (d, *J* = 9.2 Hz, 1 H, Ar*H*), 7.81 (s, 1 H, Ar*H*), 7.10 (s, 1 H, Ar*H*), 6.18 (s, 2 H, -OCH_2O-), 5.06 (s, 2 H, -CH_2COOCH_2CH*3*), 4.94 (t, *J* = 6.0 Hz, 2 H, CH_2), 4.19 (td, *J* = 7.2, 14.0 Hz, 2 H, -CH$_2$COOCH_2CH$_3$), 4.04 (s, 3 H, OCH_3), 3.21 (t, *J* = 6.0 Hz, 2 H, CH_2), 1.21 (t, *J* = 7.3 Hz, 3 H, -CH$_2$COOCH$_2$CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.30, 150.36, 149.68, 148.18, 146.19, 142.02, 138.07, 133.43, 131.16, 127.27, 123.95, 121.63, 120.88, 120.58, 108.91, 105.94, 102.57, 69.79, 61.26, 57.68, 55.91, 26.86, 14.52; ESI-TOF HRMS (m/z): calcd for $C_{23}H_{22}NO_6^+$, [M – Cl]$^+$, 408.1442; found, 408.1450.

Liquid Chromatography (HPLC) of BBR and its Derivatives

Samples of BBR, BBR-D1, and BBR-D2 (100 μM) containing aquarium water, which were used for pretreatment of 6 dpf zebrafish were collected and termed "environmental sample". The experimental samples refer to the pretreated zebrafish processed for HPLC investigation. By comparing the environmental samples with the corresponding experimental samples, we were able to test if BBR, BBR-D1, and BBR-D2 had been absorbed by zebrafish. For preparing the experimental samples, pretreated larvae (at least 200 fish per group) were collected into 1.5 mL tubes and immersed in methanol (1 mg per 20 μL) for ultrasonication. Both environmental samples and experimental samples were centrifuged at 12,000 rpm for 20 min under 4 °C. Supernatant was collected before blow-dried with nitrogen. The remaining residue was dissolved in 100 μL of 80% methanol, then centrifuged at 12,000 rpm for 15 min at 4 °C. 10 μL of the supernatant was used for HPLC analysis. HPLC was performed with Agilent 1260 infinity system (Agilent Technologies, USA) and carried out on YMC HPLC ODS-A column (250 * 4.6 mmL, 5 μm, 12 nm). During HPLC process, the temperature of column was maintained at 35 °C, and the flow rate was set to 0.8 mL/min. The mobile phase of this experiment was composed by solvent A (2.4% acetic acid solution with 20 mM ammonium acetate) and solvent B (acetonitrile). Elution condition was: 72% A and 28% B from 0 to 30 min. The detection wavelength is 345 nm. The retention time (RT; min) of BBR and its derivatives (hereafter referred to as BBR&Ds) in the environmental samples and experimental samples were recorded.

Pretreatment of BBR&Ds and PTZ Treatment

BBR (Berberine Chloride Hydrate; B0450, TCI; Tokyo, Japan) and its derivatives (BBR-D1 and BBR-D2) were solubilized in ddH_2O to prepare stock solution. The final concentration of BBR&Ds was 100 μM, which was selected based on our preliminary studies. Zebrafish larvae were incubated in 6-well plates (20 eggs per well), then treated with BBR&Ds at six different concentrations (1, 10, 100, 1000, 2000, 5000 μM). Then the median lethal concentration (LC_{50}) of BBR, BBR-D1, and BBR-D2 was determined. Since LC_1 was usually considered as a no-observed-effect concentration (NOEC) value, we tested anticonvulsant activity of BBR at the concentration below LC_1 (LC_1 of BBR is 308.9 μM). As a result, 100 μM was found to be the minimum concentration required to significantly inhibit the seizure-like behavior induced by PTZ (data not shown). Since the LC_{50} value of BBR-D1 and BBR-D2 was higher than that of BBR, we selected 100 μM for the subsequent experiments to compare the anti-seizure activity among these three compounds.

For drug treatment, larvae at 6 dpf were exposed to BBR&Ds in 6-well plates (20 eggs per well) for 24 h. At 7 dpf, larvae were exposed to 15 mM PTZ (Sigma-Aldrich; USA) to induce seizures (Baraban et al. 2005). Based on previous research results, migration of immune cells into zebrafish brain can be observed within 1 h after PTZ exposure (Jin et al. 2018b). In the present study, we found that there was no significant difference in *c-fos* mRNA expression levels and the number of migrated neutrophils among 3 min, 30 min, and 60 min after PTZ exposure. In order to investigate if the anti-seizure effect of BBR&Ds were indeed rooted in their anti-inflammatory activity, we chose two different PTZ exposure duration 3 min and 30 min for assays of inflammatory cells recruitment as well as inflammatory cytokines and c-fos expression (Fig. 2a). In addition, 100 μM of indomethacin (Sigma-Aldrich; USA), an anti-inflammatory agent which has been reported to have anti-seizure effect (Barbalho et al. 2016) was included in our study to provide a solid support for the causality between BBR&Ds-mediated anti-inflammation and their anti-seizure effect.

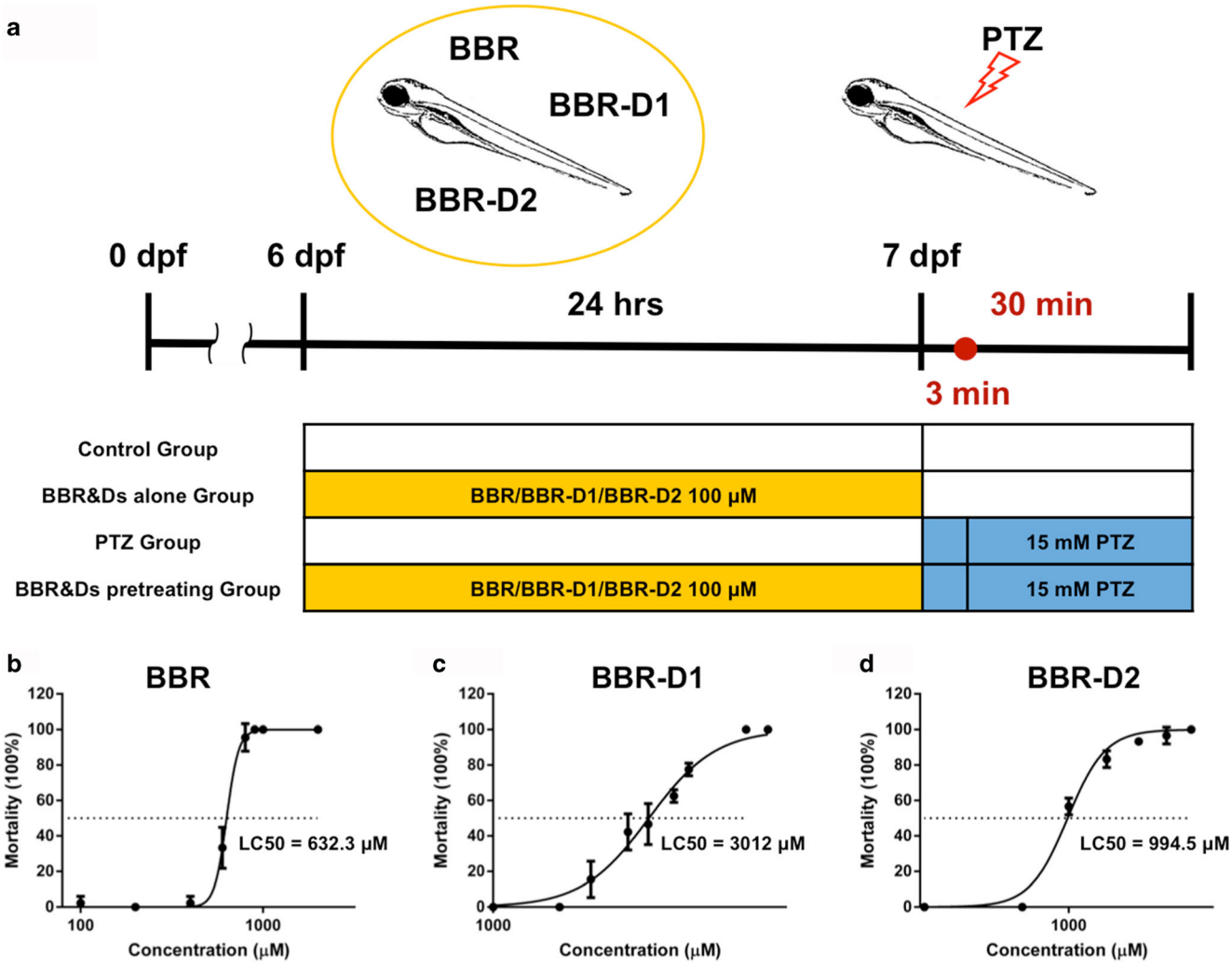


Fig. 2 The experimental work flow and LC_{50} evaluation of 6 dpf zebrafish treated with BBR&Ds. **a** Larvae at 6 dpf were pretreated with 100 μM BBR&Ds for 24 h. At 7 dpf, after 15 mM PTZ treatment for 30 min, pretreated zebrafish were subjected to behavioral tests, immune cell recruitment assays, and real-time qPCR. To investigate the association between the anti-seizure and anti-inflammatory activities of BBR&Ds, we also performed immune cell recruitment assays and tested the expression of c-fos and inflammatory cytokines 3 min after PTZ exposure. Untreated zebrafish and zebrafish pretreated with BBR&Ds while without PTZ were considered as the control (Ctl) and BBR&Ds alone groups, respectively. **b** The LC_{50} of BBR is 632.3 μM; **c** The LC_{50} of BBR-D1 is 3012 μM; **d** The LC_{50} of BBR-D2 is 994.5 μM. $n \geq 30$ per group.

Seizure Latency and Locomotor Activity Tests

Pretreated zebrafish larvae were transferred into 48-well plates at 7 dpf (one larva per well), then left in aquarium water (500 μL per well) for acclimation. After 30 min of acclimation, aquarium water was replaced by 500 μL of 15 mM PTZ. The locomotion activities of each larva were recorded immediately after PTZ treatment by Zeblab video-tracking system (Viewpoint, Lyon, France) for 30 min. Digital tracks and average speed were analyzed in every minute. At least 27 larvae in each experimental group were included in this experiment. Based on a previous report, latency period of PTZ-induced seizures was characterized by three stages (Baraban et al. 2005; Siebel et al. 2015). In stage I, larvae showed an increasing activity on swimming; in stage II, larvae presented a rapid whirlpool swimming; in stage III, larvae showed seizure-like convulsions followed by a loss of posture. The time duration from initial exposure to PTZ until zebrafish reach each convulsion stage was recorded to assess the influence of BBR&Ds pretreatment on seizure onset latency. At least 20 larvae in each group were tested individually.

Zebrafish Neutrophil and Macrophage Recruitment Assays

Larvae used for recruitment assays were treated with 200 mM 1-phenyl-2-thiourea (Sigma-Aldrich; USA) until performing immune cell recruitment assays to avoid pigment formation. At 6 dpf, zebrafish larvae were pretreated with BBR&Ds for 24 h. For neutrophil recruitment assays, BBR&Ds pretreated

MPO: GFP zebrafish larvae were exposed to 15 mM PTZ for 3 min and 30 min before imaging with a fluorescent microscope (AXIO Zoom V16; ZEISS; Germany). We also monitored neutrophil migration into the head of *MPO: GFP* zebrafish exposed to 15 mM PTZ for 3 min. For macrophage recruitment assays, pretreated zebrafish were exposed to 15 mM PTZ for 3 min and 30 min before neutral red staining to visualize macrophages (Herbomel et al. 2001). Larvae were washed, and then stained by 2.5 mg/mL neutral red dye for 1.5 h at 28.5 °C.

RNA Extraction and Real-Time Quantitative PCR

Larvae at 6 dpf were pretreated with BBR&Ds for 24 h. At 7dpf, larvae were exposed to PTZ for 3 min and 30 min, then washed and collected for qPCR. Total RNA was extracted from 20 heads of larvae in each treatment using EASY spin Plus RNA Mini Kit (RN2802; Aidlab Biotechnologies; Beijing, China). Extracted RNA of each group was reverse transcribed into cDNA according to the manufacturer's instructions of PrimeScript™ RT Master Mix (Takara; Tokyo, Japan). qPCR was set up to investigate the expression levels of four target gene (*c-fos*, *il1β*, *il6*, and *TNFα*).

qPCR was performed using SYBR® Premix DimerEraser™ (Takara; Tokyo, Japan) and the Light Cycler® 96 System (Light Cycler® Instrument; Roche; Switzerland). At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR product. All relative quantifications were carried out in triplicates, and then normalized to housekeeping gene *rpl13a*. Data were analyzed by the LC 96 Application Software and calculated according to the $2^{-\Delta\Delta Ct}$ method to quantify the gene expression (Livak and Schmittgen 2001). The primers used in this study were listed in Supplementary Table 1.

Western Blot Analysis

Heads of 7 dpf zebrafish ($n = 50$) from different groups were collected in cold RIPA lysis buffer (Thermo Fisher Scientific; USA). After centrifuging at 20,000 g for 10 min at 4 °C, the supernatant was transferred to a new tube. Protein concentration was determined by Enhanced BCA Protein Assay Kit (Solarbio; China). Samples were boiled in 5 × SDS-PAGE loading buffer (Beyotime, Beijing, China), and western blotting was carried out according to the Bio-Rad electrophoresis protocol with mouse anti-c-fos (1:100, Santa Cruz biosciences; USA) (Pena et al. 2017) and mouse anti-β-Actin (1:5000, Sigma-Aldrich) antibodies.

Enzyme Linked Immunosorbent Assay (ELISA)

In addition to the mRNA level measurement, ELISA was performed to test the protein levels of the inflammatory cytokine in animals with PTZ-induced seizures. Pretreated larvae were exposed to 15 mM PTZ for 3 min, washed, then the heads of zebrafish (n = 50) were used for protein extraction. The concentration of total protein was determined by Enhanced BCA Protein Assay Kit (Solarbio; China) according to the manufacturer's protocol. Zebrafish ELISA kits 1608 and 1612 (ZGene Biotech Inc.; China) were used to detect the protein levels of IL1β and TNFα, respectively. ELISA was performed according to the manufacturer's protocol.

Electroencephalogram (EEG) Recording

Zebrafish larvae were treated with BBR&Ds for 24 h before EEG recording. Each larva was immobilized by 2% low melting point agarose (Solarbio; China). Then larvae were treated with 2 mM tubocurarine (Tubocurarine hydrochloride pentahydrate; Sigma-Aldrich; USA) in order to reduce the artificial electric signal caused by occasional eye movements. After acclimation for 30 min, 15 mM PTZ was added to the prepared larva during EEG recording. 3% potassium acetate filled glass micropipettes (diameter, 5 μm) were used as electrodes. The signal was amplified (ELC-03XS; NPI Electronic; Tamm, Germany) and recorded, then analyzed with Lab Chart 7 (Lab Chart; ADInstruments, Australia).

Statistical Analysis

Data were analyzed using Graph Pad Prism 5.0 (GraphPad Software; CA, USA) by one-way ANOVA followed by Dunnett's Multiple Comparison Test and were presented as mean ± SEM. $P < 0.05$ was considered as significant.

Results

Comparison of the Toxicity of BBR and its Derivatives in Zebrafish

In order to compare the toxicity of BBR and its derivatives in zebrafish, the LC_{50} of BBR&Ds was assayed. Zebrafish larvae at 6 dpf were treated with various concentrations of BBR&Ds. As shown in Fig. 2b–d, the LC_{50} value of BBR, BBR-D1, and BBR-D2 were 632.3 μM, 3012 μM, and 994.5 μM, respectively. Our findings revealed that BBR-D1 and BBR-D2 exhibited lower toxicity than BBR.

The Absorption of BBR&Ds in Pretreated Zebrafish Larvae

Using an HPLC we investigated the absorption of BBR and its derivatives 1 and 2 in zebrafish. As shown in Fig. 3a, the adsorption peak of BBR, BBR-D1, and BBR-D2 was observed at 18.0 min, 13.3 min, and 6.5 min, respectively. Compared to the untreated zebrafish sample (Fig. 3b), BBR&Ds treated zebrafish (experimental samples) had the characteristic adsorption peaks (lower panels of Fig. 3c–e), which correspond to the peak in the environmental samples (upper panels of Fig. 3c–e).

The Effects of BBR&Ds on PTZ-Induced Seizure-like Behavior

To evaluate the effects of BBR&Ds on the development of PTZ-induced seizures, the latency for larvae to reach each seizure stage was recorded. In larvae pretreated with BBR-D1 and BBR-D2, a significantly prolonged latency to reach the stage III was found when compared to the larvae without pretreatment. However, there was no significant difference in latencies to reach stage I and II between larvae pretreated with BBR&Ds and PTZ group (Fig. 4a). Our results revealed that the pretreatment with two BBR derivatives delayed the occurrence of the first sign of seizure stage III, suggesting their ability to relieve seizures.

To investigate the effect of BBR&Ds on PTZ-induced seizure behavioral responses, locomotion plots were analyzed by measuring the total distance travelled of each zebrafish larva and the corresponding swimming speed. As a result, pretreatment with BBR and BBR-D1 showed a significant reduction on the increase of the total distance traveled triggered by PTZ (Fig. 4b). The average speed of BBR and BBR-D1 pretreated zebrafish was markedly decreased in comparison to that of PTZ group (Fig. 4c). Notably, there was an apparent difference in larval moving speed between the PTZ group and PTZ + BBR&Ds groups as early as 3 min after PTZ exposure, suggesting that BBR&Ds had anti-seizure effect at this time point. In contrast, no apparent change in the seizure-like behavior was found among the untreated zebrafish and those treated with BBR&Ds alone, proving that BBR&Ds pretreatments itself did not affect the locomotion activities of zebrafish larvae.

Decreased c-fos Expression after BBR&Ds Pretreatments in PTZ Treated Larvae

The mRNA levels of *c-fos* were analyzed after PTZ exposure to investigate whether BBR&Ds pretreatments could affect the expression of this neuronal activity marker. Since larval travelling speed have shown drastic difference between PTZ and BBR&Ds + PTZ groups as early as 3 min after PTZ exposure (Fig. 4c), PTZ exposure duration was set to 3 min and 30 min. In vivo, *c-fos* mRNA levels were increased during seizures (Buenafe et al. 2013; Morgan et al. 1987). Similarly, both 3 min and 30 min PTZ exposure periods generated a significantly increased expression of *c-fos*, while BBR&Ds markedly reduced this up-regulation (Fig. 4d, e). Moreover, BBR&Ds pretreatments reversed the increased protein expression of c-fos induced by 3 min PTZ treatment (Fig. 4f, g).

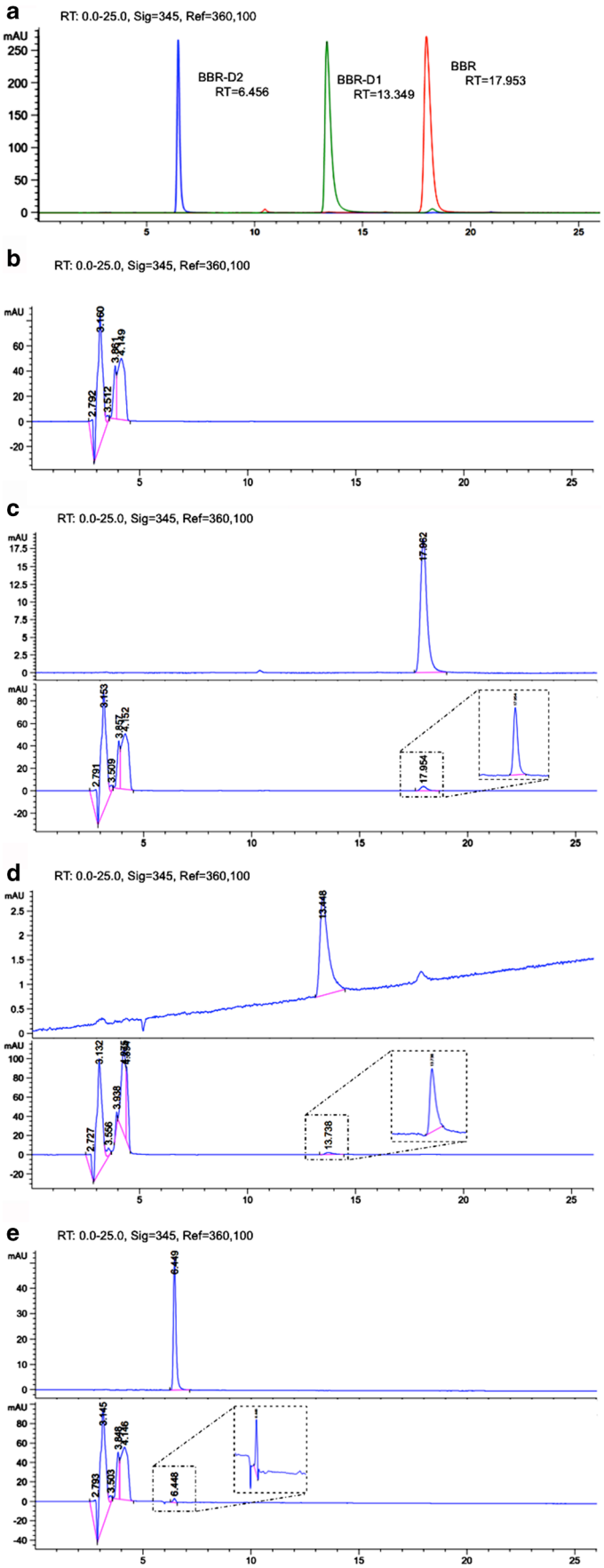
Effects of BBR&Ds on PTZ-Induced Abnormal Neuronal Discharge

Seizures are defined by the occurrence of abnormally synchronous electrical discharges (Trebuchon and Chauvel 2016). We performed EEG measurement to investigate the effect of BBR&Ds on PTZ-induced seizure-like activity directly recorded form the surface of the skull (Fig. 5a). Abnormal neuronal discharge manifested in the form of spikes was not observed during baseline recording periods prior to PTZ exposure. After the PTZ exposure, a long period of continuous spiking was observed while reduced epileptiform events were found in zebrafish pretreated with BBR&Ds (Fig. 5b). By comparing the signals recorded among BBR&Ds pretreatments, BBR-D1 showed the strongest ability to reduce the frequency of spikes (Fig. 5c).

The Effect of BBR&Ds on Neutrophil and Macrophage Recruitment Following PTZ-Induced Seizures

In zebrafish inflammatory responses result in a recruitment of immune cells, such as neutrophils and macrophages (Vezzani and Viviani 2015). After PTZ exposure for 3 min and 30 min an accumulation of neutrophils was observed in the brain region (Figs. 6a, b and 7a, b), indicating an inflammation action occurred as the result of seizures. BBR&Ds showed a strong ability to inhibit the neutrophil invasion into the brain induced by 3 min and 30 min PTZ treatment. We also monitored the migration of neutrophils into the head of zebrafish exposed to PTZ for 3 min by video recording and found the number and moving speed of neutrophils significantly decreased in BBR&Ds pretreatment groups (Supplementary movies). In addition, macrophage recruitment was performed to assess inflammatory responses during seizures (Figs. 6c, d and

Fig. 3 HPLC analysis of BBR and its derivatives. **a** The standard curves of BBR&Ds (loading amount 20 μL; BBR: RT = 17.953; BBR-D1: RT = 13.349; BBR-D2: RT = 6.456); **b** The adsorption peak of untreated zebrafish larvae sample; **c** BBR in the environmental sample (upper panel; loading amount 10 μL; RT = 17.962) and in larvae (lower panel; loading amount 10 μL; RT = 17.954). **d** BBR-D1 in environmental sample (upper panel; loading amount 10 μL; RT = 13.448) and in larvae (lower panel; loading amount 15 μL; RT = 13.738). **e** BBR-D2 in environmental sample (upper panel; loading amount 10 μL; RT = 6.449) and in larvae (lower panel; loading amount 10 μL; RT = 6.448).


a
RT: 0.0-25.0, Sig=345, Ref=360,100
mAU
BBR-D2
RT=6.456
BBR-D1
RT=13.349
BBR
RT=17.953
b
RT: 0.0-25.0, Sig=345, Ref=360,100
c
RT: 0.0-25.0, Sig=345, Ref=360,100
d
RT: 0.0-25.0, Sig=345, Ref=360,100
e
RT: 0.0-25.0, Sig=345, Ref=360,100

7c, d). 30 min of PTZ treatment increased the migration of macrophages to the brain, which was significantly reversed by BBR&Ds pretreatments. In contrast, there was no significant migration of macrophages after the short time treatment with PTZ.

Inhibitory Effect of BBR&Ds on PTZ-Induced TNFα, il1β, and il6 Up-Regulation

In zebrafish seizures increase the expression of TNFα, il1β, and il6 when seizure-induced inflammation occurs (Choo et al. 2018; Jin et al. 2018b; Vezzani and Viviani 2015). To evaluate the effect of BBR&Ds on inflammation caused by PTZ-induced seizures, the mRNA levels of *TNFα*, *il1β*, and *il6* were measured after seizure induction. The PTZ treated group (both 3 min and 30 min) showed an increased level of all three proinflammatory genes, whereas BBR&Ds pretreatments were capable of significantly reducing the mRNA expression of *TNFα*, *il1β*, and *il6* in comparison with PTZ group (Fig. 8a–f). Accordingly, BBR&Ds pretreatments inhibited the increased protein levels of TNFα and il1β, which were induced by 3 min PTZ treatment (Fig. 8g, h). The inhibitory effects of BBR&Ds on the up-regulation of TNFα, il1β, and il6 caused by PTZ-induced seizures suggested that BBR&Ds could reduce inflammation during seizures, and as a consequence suppress seizure progression.

Discussion

In the present study we synthesized two hydrophilic BBR derivatives, which were used to investigate the structure–activity relationship between their hydrophilic/hydrophobic structure and their action on seizure suppression. Our results showed that BBR and its derivatives could relieve PTZ-induced seizures in zebrafish via anti-inflammatory actions. Notably, the hydrophilic berberrubine BBR-D1 exhibited the strongest ability to attenuate PTZ-induced seizures.

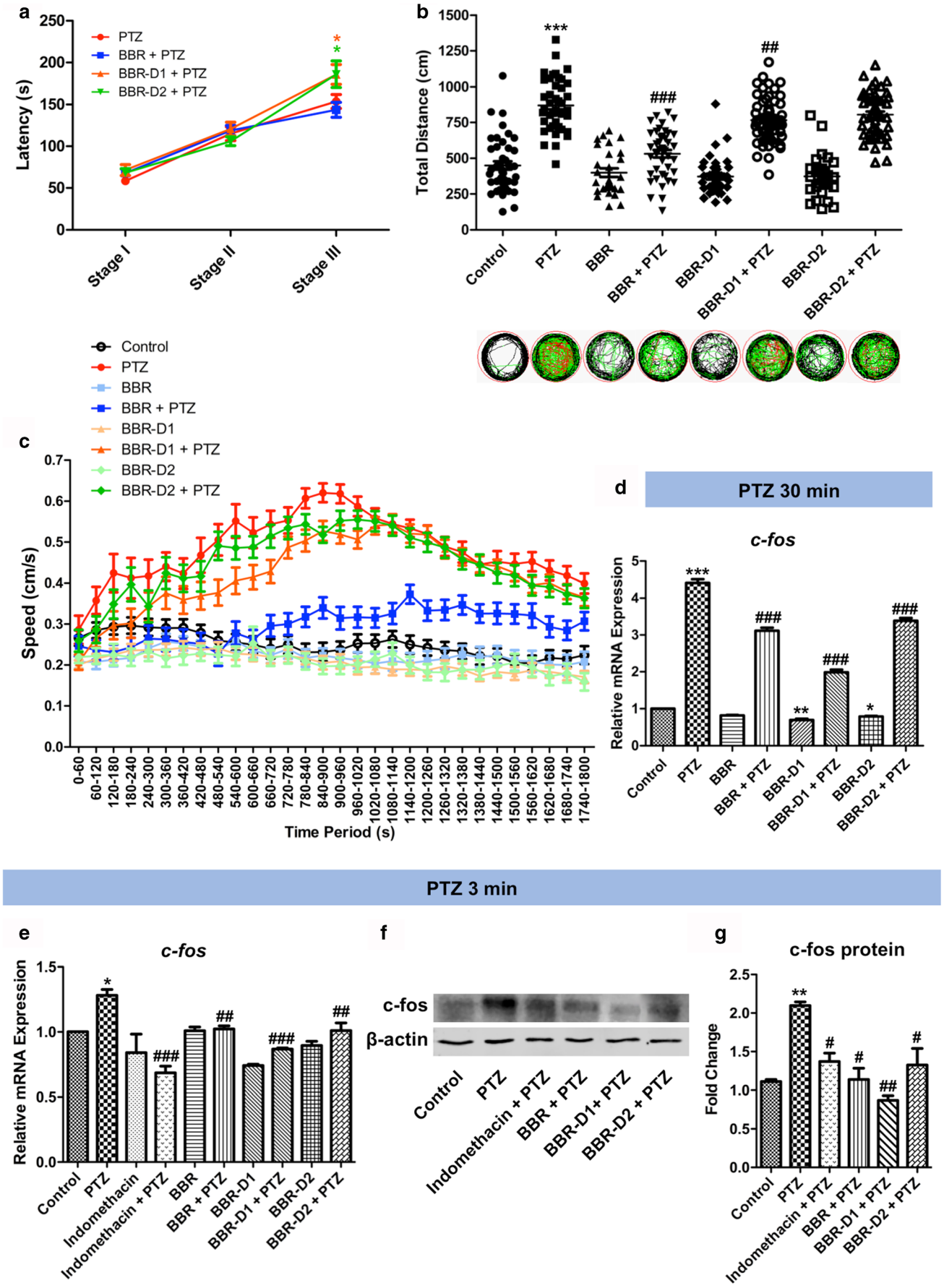
The Zebrafish Seizure Model Used in this Study

Unlike other neurodegenerative disorders for example Parkinson's and Alzheimer's disease, the early symptoms of epilepsy is the abnormal neuronal discharges without cell death, which could not be modeled in cell culture (Chakrabarti et al. 2016; Dauer and Przedborski 2003; Zhang et al. 2017). Thus, *in vivo* animal models with no or minimal ethical constrains are the ideal tools for investigating the underlying mechanisms of epilepsy. In addition, the minimal amount of drugs needed for testing, the short test periods used in assays, the manifestation of seizure-like behavior, the possibility of EEG recordings, and the transparent nature make zebrafish the *in vivo* animal model of choice in numerous epilepsy focused studies (Buenafe et al. 2013; Cho et al. 2017; Pisera-Fuster et al. 2017).

BBR&Ds Reverse PTZ-Induced Seizures-like Behavior, c-fos Up-Regulation, and Neuronal Excitability

After PTZ treatment larvae showed an increase in distance travelled along with an increasing swimming speed. In larvae pretreated with BBR and BBR-D1, PTZ-induced seizure-like behavior was suppressed, including a decrease of total distance and swimming speed. These results were consistent with that have been found in BBR treated rat seizure models (Ebrahimzadeh Bideskan et al. 2011). However, in zebrafish pretreated with BBR-D2, there was no obvious difference in total swimming distance whereas we observed significant prolonged latency to stage III of seizures. These findings suggested that although BBR-D1 and BBR-D2 are derived from BBR, they may perform different anticonvulsant actions during seizures. Based on our results of locomotor activity we found zebrafish pretreated with BBR&Ds showed an obvious reduced moving speed compared with PTZ group as early as 3 min after PTZ exposure, suggesting that BBR&Ds exerted anti-seizure action at this time point. Therefore, we tested c-fos expression and anti-inflammatory responses induced by BBR&Ds after PTZ exposure for 3 min to further verify if the anti-seizure effect of BBR&Ds were indeed rooted in their anti-inflammatory activity. The expression of *c-fos* was found to be associated with the hyperactivity levels of neurons during seizures (Baxendale et al. 2012; Beer et al. 1998; Herrera and Robertson 1996). We found that BBR&Ds pretreatments markedly reversed the increased expression of *c-fos* following PTZ treatment for 3 min and 30 min, respectively. EEG is known as the most direct measurement for investigating neuron discharge during seizures (Cho et al. 2017; Fujikawa et al. 2016; Lundt et al. 2016). In our study, BBR&Ds showed the ability to reduce PTZ-induced synchronized neuron discharges reflected in the decreased number of interictal spikes. All these findings indicated that BBR&Ds attenuated PTZ-induced seizures in zebrafish.

Fig. 4 Effect of BBR&Ds on zebrafish locomotor activities and c-fos expression after PTZ treatment. **a** The latency for larvae to reach each seizure stage after PTZ treatment ($n \geq 20$). $^{*}P < 0.05$ vs PTZ. **b** The total distance travelled ($n \geq 27$ per group). $^{***}P < 0.001$ vs control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs PTZ. In digital tracking map (at the bottom of B), high-speed movement is represented in red lines; medium-speed movement is depicted in green lines; low-speed movement is represented in black lines. **c** The swimming speed of zebrafish larvae with different treatment. Average speed in every 60 s was calculated. **d** Relative *c-fos* mRNA expression levels after PTZ treatment for 30 min. **e** Relative *c-fos* mRNA expression levels after PTZ treatment for 3 min. **f** Protein levels of c-fos after PTZ treatment for 3 min revealed by western blotting. **g** Quantitative measurements of the relative densities of c-fos protein. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs PTZ.


a
Latency (s)
PTZ
BBR + PTZ
BBR-D1 + PTZ
BBR-D2 + PTZ
Stage I
Stage II
Stage III
b
Total Distance (cm)
Control
PTZ
BBR
BBR + PTZ
BBR-D1
BBR-D1 + PTZ
BBR-D2
BBR-D2 + PTZ
c
Speed (cm/s)
Time Period (s)
d
PTZ 30 min
c-fos
Relative mRNA Expression
PTZ 3 min
e
c-fos
Relative mRNA Expression
Indomethacin
Indomethacin + PTZ
f
c-fos
β-actin
g
c-fos protein
Fold Change

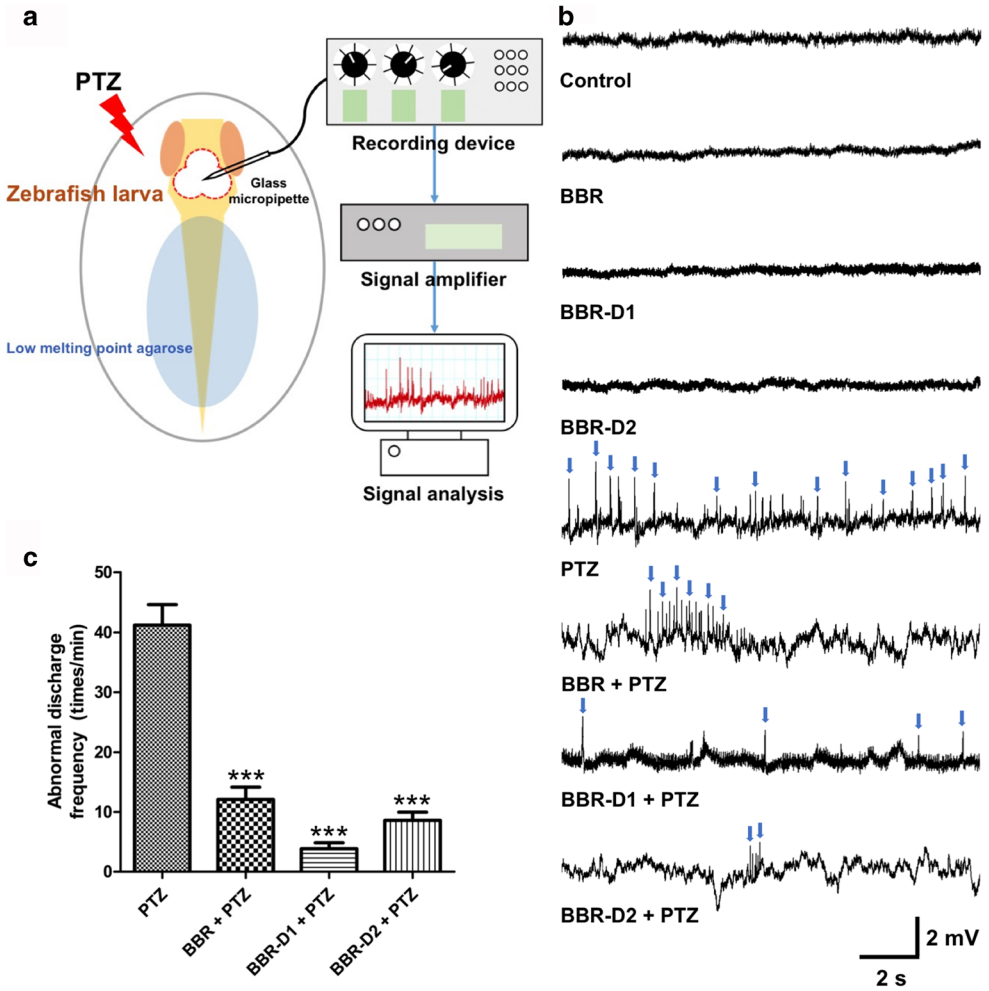


Fig. 5 EEG recordings of 7 dpf zebrafish larvae. **a** Schematic illustration of EEG recording methodology. **b** EEG recording of the brain activity in control conditions and BBR&Ds pretreatments. No abnormal activity was visible (in the control and BBR&Ds alone groups). PTZ-induced interictal spikes were represented as single unidirectional amplitude spikes. BBR&Ds reduced the number of spikes. Scale bar, 2 mV; 2 s. **c** The frequency of abnormal synchronous neuronal discharges. Spike with amplitude larger than 300% of the adjacent basal line was counted as one abnormal neuronal discharge (blue arrows indicated the distribution of these spikes), and the frequency was determined as the number of abnormal neuronal discharges in 1 min. ***$P < 0.001$ vs PTZ.

BBR-D1 Showed the Strongest Ability to Attenuate PTZ-Induced Seizures

In the present study BBR-D1 and BBR-D2 showed higher LC_{50} value as compared to BBR, suggesting their relatively low toxicity. Therefore, the bioactive effects of BBR-D1 and BBR-D2 were worth exploring. BBR-D1 can be separated from the plant *Berberis vulgaris* L. or obtained by pyrolysis of berberine, which has been reported to have the same pharmacological activities including antioxidant activity, antibacterial activity, and anticancer activity as berberine but with higher bioavailability (Kobayashi et al. 1995; Lo et al. 2013; Pongkittiphan et al. 2015). On the other hand, BBR-D2 has been studied as an intermediary product in a cellulose material research, while its biological activities are largely unknown. Here we showed the seizure suppressing effect of BBR-D1 and BBR-D2. By comparing the results of seizure indexes, the hydrophilic BBR derivative BBR-D1 showed stronger ability to attenuate PTZ-induced seizures compared to the hydrophobic BBR-D2. This is possibly due to the high metabolic capability, good solubility in water, and high stability in acid conditions of BBR-D1, which play critical roles during drug absorption (Huang et al. 2017; Yang et al. 2017). On the contrary, the hydrophobic BBR-D2 has poor solubility in water and most of the organic solvents, which may result in negative effects on drug absorption *in vivo*. Indeed, as shown in our HPLC assays, the content of BBR-D1 in zebrafish larvae was close to that in its corresponding environmental sample, suggesting its higher potential to be absorbed.

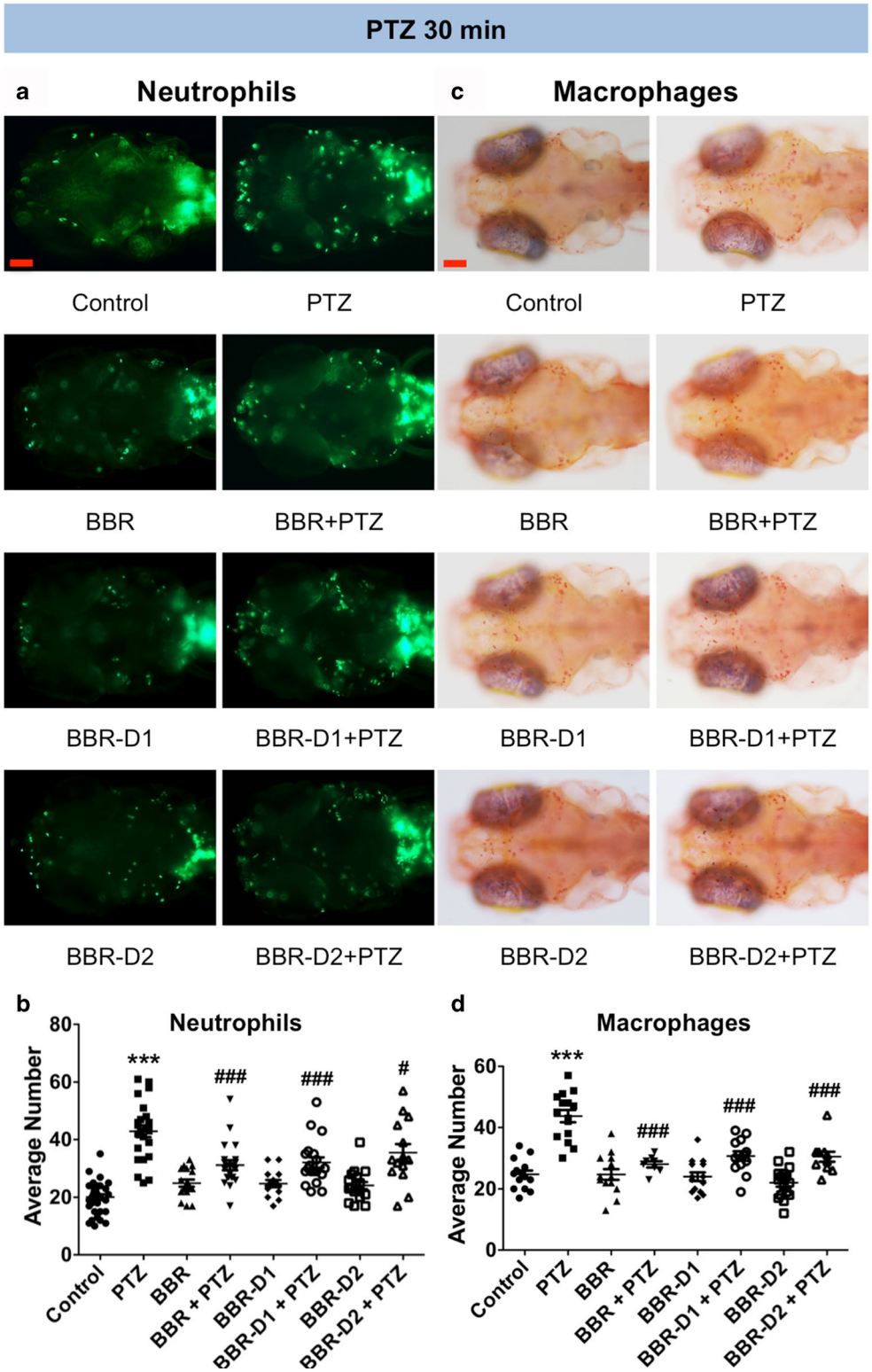
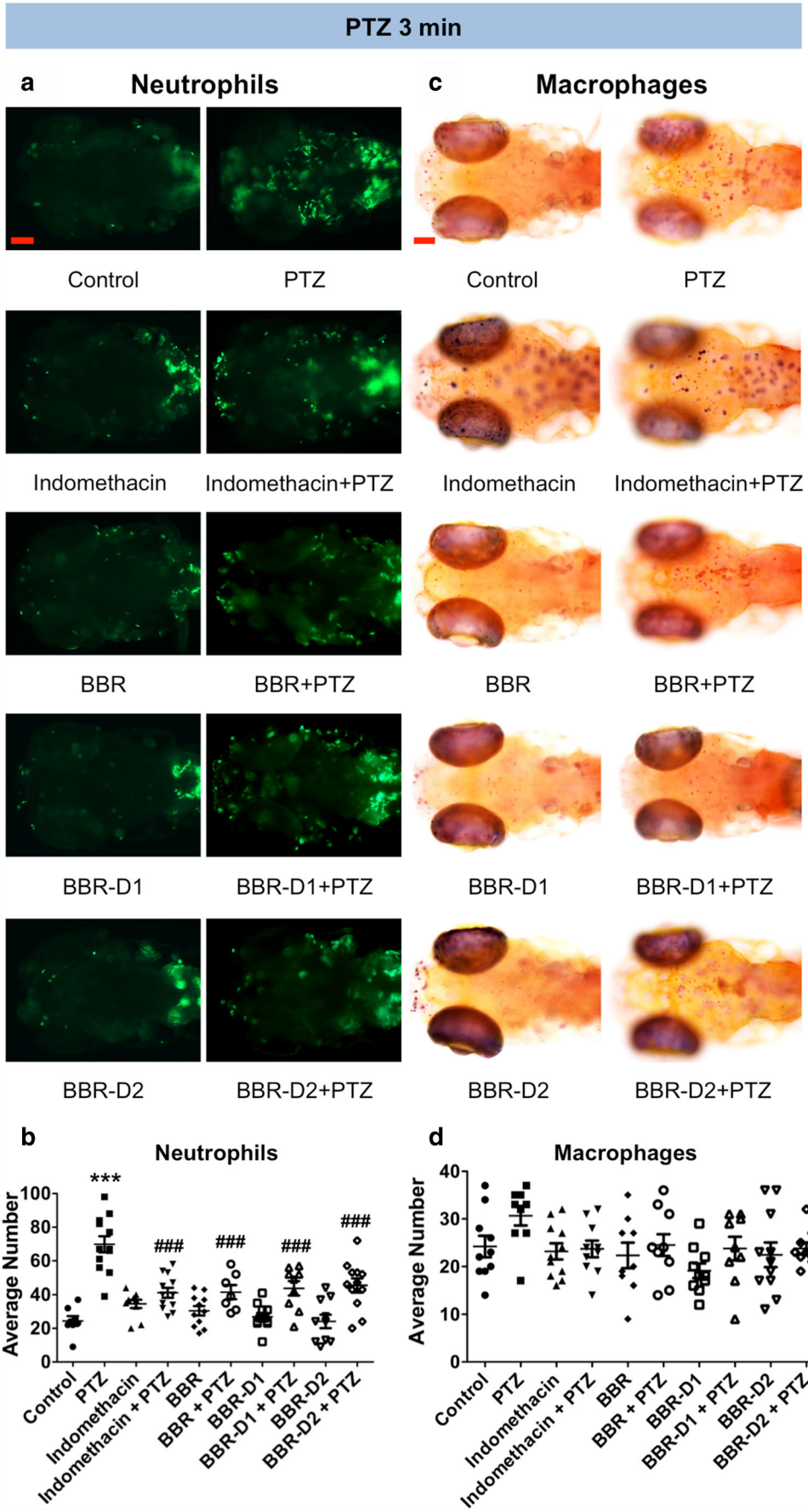


Fig. 6 BBR&Ds reduce the recruitment of neutrophils and macrophages in the zebrafish brain after PTZ treatment for 30 min **a** Migration of neutrophils (green signal) into the head of larvae after 30 min PTZ treatment. **b** The number of neutrophils in larvae heads after 30 min PTZ treatment. **c** Infiltration of macrophages (orange signal) into the head of larvae after 30 min PTZ treatment. **d** The number of macrophages in larvae heads after 30 min PTZ treatment. $n \geq 8$ per group. $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs PTZ. Scale bar, 100 μm.

BBR&Ds Inhibit Inflammatory Responses during Seizure Progression

PTZ has been reported to cause inflammatory responses in the brain of zebrafish, promoting a recruitment of immune cells, such as neutrophils and macrophages (Vezzani et al. 2011). Although the migration of macrophages was not observed after the short treatment with PTZ, the number of macrophages in the brain was significantly increased after 30 min treatment with PTZ. One possible reason is that it takes at least


PTZ 3 min
a
Neutrophils
c
Macrophages
Control
PTZ
Control
PTZ
Indomethacin
Indomethacin+PTZ
Indomethacin
Indomethacin+PTZ
BBR
BBR+PTZ
BBR
BBR+PTZ
BBR-D1
BBR-D1+PTZ
BBR-D1
BBR-D1+PTZ
BBR-D2
BBR-D2+PTZ
BBR-D2
BBR-D2+PTZ
b
Neutrophils
Average Number
***
###
###
###
###
Control
PTZ
Indomethacin
Indomethacin + PTZ
BBR
BBR + PTZ
BBR-D1
BBR-D1 + PTZ
BBR-D2
BBR-D2 + PTZ
d
Macrophages
Average Number
Control
PTZ
Indomethacin
Indomethacin + PTZ
BBR
BBR + PTZ
BBR-D1
BBR-D1 + PTZ
BBR-D2
BBR-D2 + PTZ

◀ **Fig. 7 Effect of BBR&Ds on the recruitment of neutrophils and macrophages in the zebrafish brain after PTZ treatment for 3 min.** **a** Migration of neutrophils (green signal) into the head of larvae after 3 min PTZ treatment. **b** The number of neutrophils in larvae heads after 3 min PTZ treatment. **c** Migration of macrophages (orange signal) into the head of larvae after 3 min PTZ treatment. **d** The number of macrophages in larvae heads after 3 min PTZ treatment. $n \geq 8$ per group. $^{***}P < 0.001$ vs control; $^{###}P < 0.001$ vs PTZ. Scale bar, 100 μm

90 min to visualize macrophages by using neutral red staining, by that time the number of macrophages in the brain had returned to the normal level. It has been reported that inflammation is activated by PTZ in rodents. Injection of PTZ increased inflammatory cytokine levels in the cerebral cortex and hippocampus of mice (Hoda et al. 2017; Temp et al. 2017). In PTZ-kindled rats, a markedly up-regulation in the expression of hippocampal inflammatory cytokines was

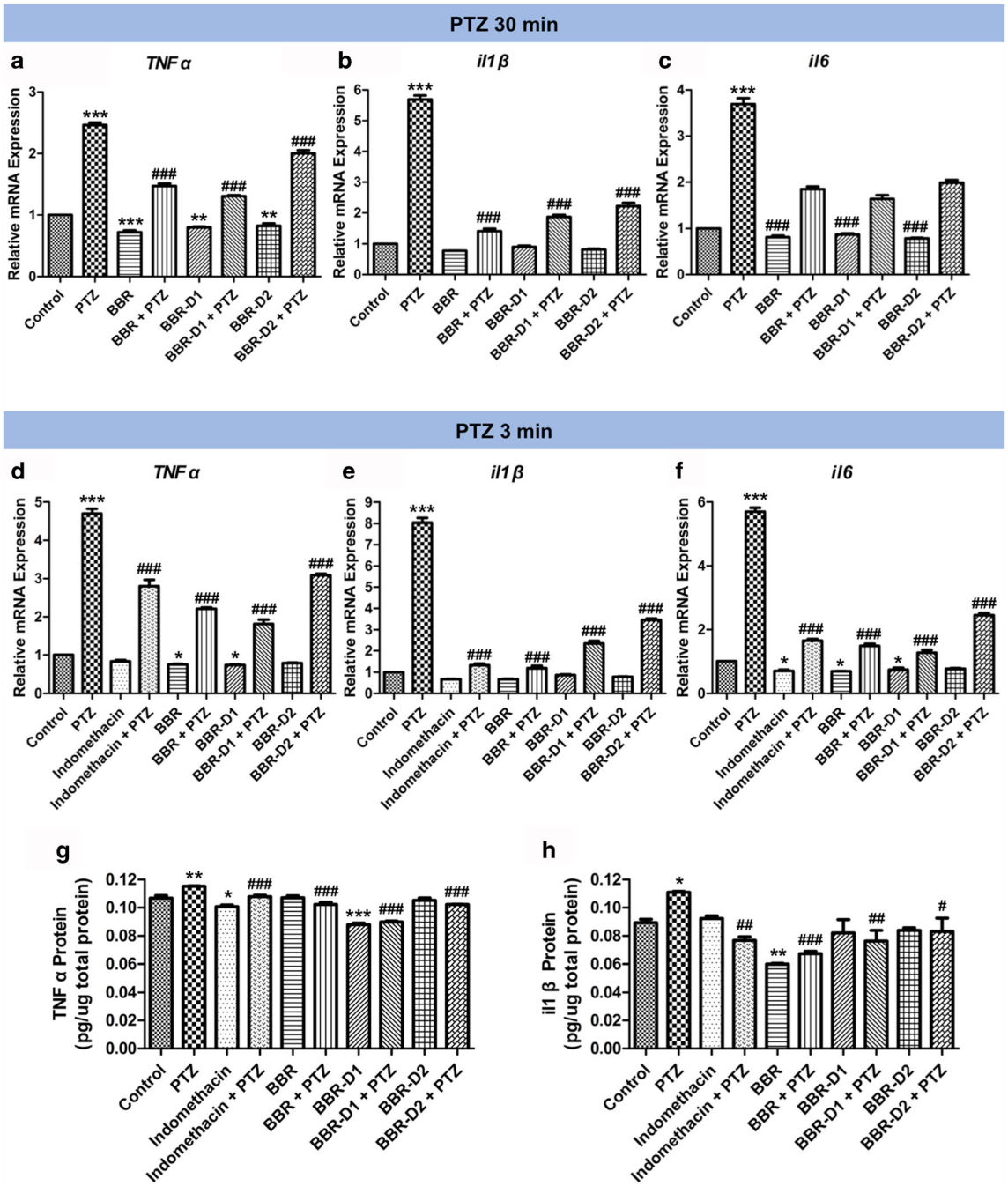


Fig. 8 Effect of BBR&Ds on the expression of inflammatory cytokines after PTZ-induced seizures. **a–c** Relative mRNA expression levels of *TNFα*, *il1β*, and *il6* after 30 min PTZ treatment. **d–f** Relative mRNA expression levels of *TNFα*, *il1β*, and *il6* after 3 min PTZ treatment. **g** Protein levels of TNFα and il1β after PTZ treatment for 3 min revealed by ELISA assays. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs PTZ.

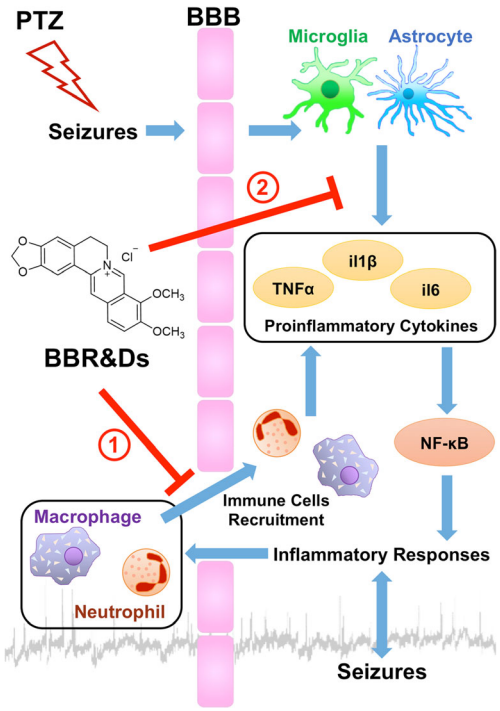


Fig. 9 Schematic of anti-inflammation associated protective mechanism of BBR&Ds on attenuating PTZ-induced seizures. PTZ-induced seizures enhance the activation of microglias and astrocytes, promoting the secretion of proinflammatory cytokines TNFα, il1β, and il6. Proinflammatory cytokines activate NF-κB, which cause inflammation responses and immune cell recruitment in the brain, thereby aggravating seizures. BBR&Ds could relieve PTZ-induced seizures via:① reversing the recruitment of immune cells and/or ② inhibiting the expression of proinflammatory cytokines.

revealed (Liu et al. 2018; Riazi et al. 2008; Xia et al. 2018). Accordingly, we found that inflammatory biomarkers TNFα, il1β, and il6 were increased after PTZ treatment in zebrafish. We also found that this increase was reversed by BBR pretreatment, which is consistent with previous findings on glial cells and in rat seizure models (Chen et al. 2014; Vieira et al. 2014). Similarly, BBR-D1 and BBR-D2 caused a significant decrease in the recruitment of inflammatory cells and expression of inflammatory cytokines. Although BBR&Ds showed similar anti-inflammatory effect during seizures, their inhibitory effect on total swimming distance were variable, suggesting that anti-inflammatory activity of BBR&Ds is one of the contributors to seizure suppression. Since we did not demonstrate that the anti-seizure effect of BBR&Ds was exclusively due to their anti-inflammatory activity, further studies are needed to clarify the mechanisms.

Anti-Inflammation Associated Protective Mechanism of BBR&Ds on Attenuating PTZ-Induced Seizures

It has been reported that inflammatory responses play pivotal roles in seizures progression (Jin et al. 2018b; Vezzani et al. 2012, 2016). When seizures occur, key proinflammatory cytokines, such as il1β, il6, and TNF-α are expressed in activated astrocytes and microglia (Bonaventura et al. 2018; Dupuis and Auvin 2015; Friedman and Dingledine 2011; Vezzani and Granata 2005). Proinflammatory cytokines activate NF-κB, which in turn trigger inflammation in the brain (Ali et al. 2018; Han et al. 2018; Liu et al. 2017). During inflammatory responses, glia cells express chemokines to recruit immune cells, such as macrophages and neutrophils, into the brain (Fabene et al. 2008). Meanwhile, seizures also cause blood–brain barrier (BBB) leakage and alter the permeability of BBB, which may contribute to the migration of macrophages and neutrophils (Roch et al. 2002; van Vliet et al. 2015). In our study, BBR&Ds showed their ability to inhibit the expression of proinflammatory cytokines and reverse the recruitment of immune cells, which is possibly contribute to seizure suppression (Fig. 9). These results are consistent with the previous findings that indomethacin, an anti-inflammatory agent also has therapeutic effect on seizure suppression (Barbalho et al. 2016; Vieira et al. 2014). Although it has been reported that BBR can penetrate through the BBB and accumulate in hippocampus (Wang et al. 2005), BBB penetrating ability of BBR-D1 and BBR-D2 needs confirmation.

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Author Contributions MJ, KCL, and AS conceived the project and designed the experiments. MJ, BYZ, LZW, XNJ, and SSZ performed the experiments and analyzed the data. MJ wrote the manuscript. AS helped in the revision.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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