Fish and Shellfish Immunology 87 (2019) 395–400

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Anti-inflammatory effect of a novel synthetic compound 1-((4-fluorophenyl)thio)isoquinoline in RAW264.7 macrophages and a zebrafish model

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ARTICLE INFO

Keywords:
Zebrafish
Anti-Inflammation
RAW264.7
Neutrophil

ABSTRACT

The compound, 1-((4-fluorophenyl)thio)isoquinoline (FPTQ), is a synthetic isoquinoline derivative. To test the anti-inflammatory effect of FPTQ, we used neutrophil-specific transgenic zebrafish *Tg(mpx::EGFP)*^{i114} line and lipopolysaccharide (LPS)-stimulated RAW264.7 cells. We also used two different methods, involving tail transection and LPS stimulation in the zebrafish model. Neutrophils translocation in the zebrafish tail-transected model was inhibited by FPTQ. Neutrophil aggregation was also inhibited by FPTQ in the LPS-stimulated zebrafish model. Decreased mRNA expression of the pro-inflammatory cytokine genes, *interleukin-1β* (*il-1β*) and *interleukin-6 (il-6)*, was found in zebrafish larvae injected with FPTQ. Additionally, production of nitric oxide was inhibited by FPTQ in RAW264.7 macrophage cells treated with LPS. Moreover, the mRNA expression of *Il-1β* and *Il-6* suppressed by FPTQ treatment in RAW264.7 macrophage cells, and an enzyme immunoassay showed that FPTQ suppressed the secretion of IL-1β and IL-6 in RAW264.7 cells. These results demonstrate that FPTQ reduced inflammatory responses and, therefore, suggest that it may be effective as an anti-inflammatory agent.

1. Introduction

The inflammatory response is a dynamic defense process, that reduces the tissue injury caused by pathogens, infections, and irritants [1,2]. Various inflammatory cells, such as macrophages, neutrophils, eosinophils, and mononuclear phagocytes, are involved in the inflammatory process. Neutrophils are the first responders and play an important role in eliminating the infection. When an inflammatory reaction is initiated in damaged tissue, neutrophils in the blood migrate to the inflamed area [3]. It was previously known that an interaction between macrophages and neutrophils is occurred in response to tissue damage in zebrafish [4]. Phagocytosis by these macrophages decreases the inflammation. The dead phagocytic macrophages are released through the lymphatic vessels, and immune cells are removed from the inflammation site [5], thereby promoting normalization of tissue and secretion of various mediators that inhibit the inflammatory response [6]. Macrophages are important immune cells that play a role in a variety of disease processes and are involved in the pathogenesis of autoimmune diseases, infection, and inflammatory disorders [7]. It is well known that macrophages secrete inflammatory mediators, such as nitric oxide (NO) and pro-inflammatory cytokines [tumor necrosis factor-α (*TNF-α*), interleukin-1β (*Il-1β*), and interleukin-6 (*Il-6*)] in response to the bacterial endotoxin, lipopolysaccharide (LPS) [8,9].

The zebrafish (*Danio rerio*) is a small freshwater fish that has been employed as a useful vertebrate model organism because of its many advantages, such as its small size, low cost, and transparency [10,11]. Recently, zebrafish have also been used in toxicology and drug discovery studies. Due to transparency of the zebrafish embryo, it is possible to visualize organs, as well as fluorescence within cells, using a microscope when investigating oxidative stress and inflammation [12–14]. Furthermore, zebrafish have an immune system similar to that of humans [15], although the site of maturation differs from human. The immune cells of zebrafish include neutrophils, macrophages, T cells, and B cells [16,17]. The zebrafish can access these immune

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https://doi.org/10.1016/j.fsi.2019.01.030
Received 14 August 2018; Received in revised form 27 December 2018; Accepted 19 January 2019
Available online 25 January 2019
1050-4648/

system can be assessed in real-time because the embryos are transparent and can express fluorescent proteins in specific cells [3]. Therefore, the zebrafish is a useful animal model to characterize the human immune system.

FPTQ is one of derivatives of 1-isoquinolin-1-ylsulfanylisoquinoline that has been selected as a candidate in anti-melanogenic screening system using zebrafish larvae. In this study, we examined its anti-inflammatory activities *in vitro* using RAW264.7 cells and *in vivo* using a *Tg(mpx:EGFP)*^{i114} zebrafish line, which labels neutrophils with the enhanced green fluorescent protein (EGFP).

2. Materials and methods

2.1. Zebrafish maintenance

Zebrafish were maintained under standard conditions as previously described [18]. All experimental protocols involving zebrafish were approved by the Animal Care and Use Committee of the Korea Research Institute of Chemical Technology. Adult fish were maintained in a recirculating tank rack system with a 14-h light/10-h dark cycle at 28 °C in a certificated zebrafish facility (Techniplast; ZebTEC, West Chester, PA, USA). The *Tg(mpx:EGFP)*^{i114} zebrafish line was used to observe green fluorescent protein in neutrophils. Embryos were obtained by incrossing *Tg(mpx:EGFP)*^{i114}. Embryos were maintained with a 14-h light/10-h dark cycle at 28 °C in an incubator (HB-302M; HANBAEK, Gwangju, Republic of Korea).

2.2. Measurement of FPTQ concentration by LC-MS/MS

At three days post-fertilization (dpf), zebrafish larvae were exposed to 5, 10, and 20 μM FPTQ for 0, 0.15, 0.5, and 4 h. Twenty-larvae were pooled and washed three times with phosphate-buffered saline (PBS). All samples were frozen in liquid nitrogen before homogenization. The homogenized samples were agitated for 10 min and centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new tube and concentrated using a speedvac (CVE-3100: EYELA, Tokyo, Japan). Analyses were performed using an Agilent 1260 series high-pressure liquid chromatography system and Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Separation was performed on a Phenomenex C_{18} column (Kinetex, 50 × 2.1 mm, 2.6 μm, C_{18}, 100 A; Phenomenex, Madrid, CA, USA) using a mobile phase composed of 10 mM ammonium formate and acetonitrile at a flow rate of 0.3 mL/min. The analytical run time was 3 min, and the injection volume was 5 μL. All measurements were performed in triplicate.

2.3. Induction of inflammation in zebrafish larvae by tail amputation

Inflammation was induced in zebrafish larvae by tail transection as previously described [19]. *Tg(mpx:EGFP)*^{i114} transgenic zebrafish larvae at 3 dpf were anesthetized using 0.02% tricaine and the lower part of the tail was amputated using a scalpel [20,21]. After injury, the larvae were exposed to various concentrations of FPTQ in a 24-well plate; 10 larvae occupied each well, which contained 1 mL of egg water. The zebrafish larvae were incubated at 28.5 °C. Four hours later, the larvae were anesthetized by transferring to 24-well plates containing 0.02% tricaine ready for visualization. Images of the larvae were obtained using a Lionheart FX automated microscope (Bio-Tek, Winooske, VT, USA).

2.4. LPS-induced inflammation by microinjection into zebrafish larvae

Zebrafish larvae were divided into experimental and control groups. The recruitment of neutrophils was evaluated *in vivo* using *Tg (mpx:EGFP)*^{i114} transgenic zebrafish larvae (3 dpf) with fluorescently marked cell populations. Microinjection was performed as previously described [22]. Larvae were anesthetized using 0.02% tricaine. LPS (Sigma-Aldrich. Co., St Louis, MO, USA) of 1 mg/mL was injected into the yolk, followed by immediate was this group also subsequently treated with FPTQ. A negative control group was injected with PBS. Microinjection (PV830 Pneumatic PicoPump; World Precision Instruments, Sarasota, FL, USA) was performed with a volume of 2 nL per larva. Treatments were performed in triplicate (10 larvae per group). A Lionheart FX automated microscope (Bio-Tek) was used to obtain fluorescent images of the larvae.

2.5. Cell culture

The RAW264.7 mouse macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea) and cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone), and 100 units/mL penicillin-streptomycin solution (Hyclone) at 37 °C in a humidified chamber containing 95% air and 5% CO_2.

2.6. NO inhibition assay

NO production was assayed by measuring the accumulation of nitrite and nitrate according to a spectrophotometric method [23]. RAW264.7 cells (0.2×10^5) were seeded in each well of a 96-well plate. The cells were treated with 1 μg/mL of LPS and various concentrations of compound. The plates were incubated for an additional 24 h at 37 °C in a humidified chamber containing 95% air and 5% CO_2. To measure the nitrite and nitrate concentrations in the medium, 100 μL of the culture supernatant was collected, mixed with standard Griess reagent (Sigma-Aldrich. Co., St Louis, MO, USA), and incubated at 37 °C for 15 min to form a purple azo dye. The absorption at 540 nm was determined using a microplate reader (M1000pro; TECAN, Mannedorf, Switzerland).

2.7. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from RAW264.7 cells or zebrafish larvae using the TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of the isolated RNA were measured using a NanoQuant plate™ (TECAN) in a microplate reader (TECAN). The reactions were conducted in a MicroAmp™ Fast Optical 96-Well Reaction Plate sealed with MicroAmp™ optical adhesive film (Life Technologies, Carlsbad, CA, USA). The primer pairs used were as follows: mouse *Il-6*, (forward) 5′-GATGCTACCAAACTGGATATAATC-3′ and (reverse) 5′-GGTCCTTAGCCACTCCTTCTGTG-3′; mouse *Il-1β*, (forward) 5′—CCT TCACTTTTGAAGAAGA-3′ and (reverse) 5′-GAGGTGCTGATGTACCAG TTG-3′; mouse *GAPDH*, (forward) 5′-GAGAACTTTGGCATTGTGG-3′ and (reverse) 5′-ATGCAGGGATGATGTTCTG-3′; zebrafish *il-6*, (forward) 5′-GATTTGTGTGGGAGAGGG-3′ and (reverse) 5′-CAGGAGTTGT GTCAAGGT-3′; zebrafish *il-1β*, (forward) 5′-TGGCTGACCTGTTCT CTG-3′ and (reverse) 5′-CGATCTCCTGTTGGACAC-3′; and *β-actin* (forward) 5′-GCGAGAAGATGACCCAGA-3′ and (reverse) 5′-ATCACGATGC CAGTGGTA-3′. A reverse transcription-quantitative polymerase chain reaction was performed using Verso™ SYBR Green 1-Step qRT-PCR Low ROX Mix (Thermo Scientific, Waltham, MA, USA) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Expression levels were analyzed using the ΔΔCT method normalized to *GAPDH* (mouse) and *β-actin* (zebrafish).

2.8. Measurement of the amount of cytokines

Cell-free supernatants were collected to determine cytokine levels using commercial ELISA kits. A mouse IL-6 and IL-1β ELISA kit (Elabscience, Wu Han, Hubei Province, China) was used according to the manufacturer's instructions. All assays were performed in triplicate.

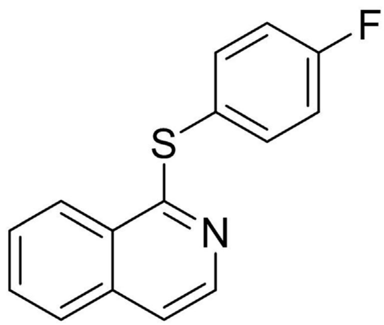

Fig. 1. The structure of FPTQ. FPTQ is a synthetic isoquinoline derivative.

The concentration of each protein was calculated from the standard curve.

2.9. Statistical analysis

All data were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and expressed as the mean ± standard deviation (SD). Statistical analyses were performed with GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA) using an unpaired *t*-test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Inhibition of neutrophil migration by FPTQ in zebrafish larvae

As shown in Fig. 1, FPTQ is a small molecule with an isoquinoline structure. We determined the anti-inflammatory effect of FPTQ using the *Tg(mpx:EGFP)*^{i114} zebrafish model. Experiments were carried out using two different methods, tail amputation and tail wounding. Dexamethasone was used as a positive control for the anti-inflammatory effect [24,25]. All treatments were performed immediately after tail amputation. The inhibitory effect of FPTQ on neutrophil migration was confirmed using the tail amputation method. Recruitment of neutrophils to the cut site was increased following tail amputation. Zebrafish larvae were exposed to 0.1% dimethyl sulfoxide (DMSO), 100 μM dexamethasone, and different doses of FPTQ. Neutrophil recruitment was observed 4 h after tail amputation (Fig. 2A–J). The results showed that the number of neutrophils migrating to the amputation site decreased in a dose-dependent manner after FPTQ treatment (Fig. 2K). In the tailfin wound method, the number of neutrophils collecting at the wound site also decreased in a dose-dependent manner (Supplementary Fig. 1).

3.2. Inhibition of neutrophil migration in the LPS-induced inflammation zebrafish model

It has been reported that LPS stimulates host defense mechanisms to activate immune cells and induce metabolic changes and sepsis [26]. Phenotypic abnormalities and yolk necrosis or larvae death were observed in zebrafish larvae with LPS injected into their yolks. To test the anti-inflammatory effect of FPTQ, we injected 2 nL of 1 mg/mL LPS solution into the yolks of Tg*(mpx:EGFP)*^{i114} zebrafish larvae and exposed the zebrafish larvae immediately to FPTQ treatment. The fluorescent neutrophils were observed 4 h after yolk injection (Fig. 3A–F). We counted the number of neutrophils in the yolk area and this number was significantly decreased by treatment with 20 μM FPTQ (Fig. 3G), demonstrating that FPTQ had an anti-inflammatory effect during the early phase of inflammation.

3.3. Effect of FPTQ on the expression of il-1β and il-6 mRNA in LPS-injected zebrafish

IL-1β and IL-6 are important pro-inflammatory cytokines in innate immune response systems that initially release cytokines when exposed to pathogens. It has been reported that zebrafish *il-1β* is one of the pro-inflammatory cytokines involved in infection, tissue regeneration, and various inflammatory diseases [27]. Zebrafish *il-6* is produced by various cells, such as macrophages, endothelial cells, and fibroblasts, during processes such as differentiation and activation of specific cells [28]. Thus, understanding the role of these cytokines in zebrafish may reveal their function in mammals.

To confirm the anti-inflammatory effect of FPTQ, the expression levels of *il-1β* and *il-6*, which are genes for major cytokines known to be involved in the inflammatory response, were measured using RT-qPCR. Total RNA was extracted from zebrafish larvae which were injected using the same protocol as in Fig. 3. The mRNA level of *il-1β* in larvae injected with LPS increased approximately 1.5-fold compared to PBS injection. The LPS-induced expression of *il-1β* was reduced to approximately 38.9% of the LPS-induced level by co-treatment with 20 μM FPTQ (Fig. 4A). The mRNA level of *il-6* in larvae injected with LPS was increased by approximately 9-fold compared to PBS injection. Inhibition by 20 μM FPTQ reduced mRNA levels to approximately 61.7% of the LPS-induced level (Fig. 4B). Taken together, these results indicate that the early innate immune response in zebrafish was suppressed by FPTQ treatment.

3.4. Anti-oxidant and anti-inflammatory effects of FPTQ in mammalian cells

NO, which is known to be a precursor of the immune response, is induced by external stimuli [29]. We investigated the effects of FPTQ on NO production and inflammation at the cellular level. RAW264.7 macrophage cells were treated with various concentrations (0.5, 1, 5, and 10 μM) of FPTQ. Significant cytotoxicity was not observed at these concentrations (Supplementary Fig. 2). NO production induced by LPS treatment was significantly inhibited at > 1 μM FPTQ, and 10 μM FPTQ treatment caused a 31% anti-oxidant effect (Fig. 5A).

To evaluate the anti-inflammatory effects of FPTQ at the cellular level, *Il-6* and *Il-1β* mRNA levels were quantified after 24 h. Inflammation was induced by LPS treatment in RAW264.7 cells, which were then treated with 1, 10, or 20 μM FPTQ. The dramatic increase in the expression levels of *Il-1β* and *Il-6* caused by LPS treatment was decreased by FPTQ treatment (Fig. 5B and C). At a concentration of 10 μM, FPTQ caused a 27% and 44% reduction in the mRNA expression of *Il-1β* and *Il-6*, respectively.

3.5. Effect of FPTQ on the secretion of IL-1β and IL-6 proteins by LPS-stimulated RAW264.7 cells

A mouse enzyme-linked immunosorbent assay (ELISA) kit was used to measure the pro-inflammatory cytokine production in supernatants from LPS-treated RAW264.7 macrophages as described in the materials and methods. To determine whether FPTQ suppressed the production of pro-inflammatory cytokines, such as IL-1β and IL-6, RAW264.7 macrophages were incubated with 1 or 10 μM FPTQ. As shown in Fig. 6, compared to vehicle treatment, LPS induced a significant increase in IL-1β and IL-6 production. In contrast, 10 μM FPTQ significantly reduced the LPS-induced production of IL-1β and IL-6 compared to LPS treatment. The secretion of IL-1β and IL-6 proteins was inhibited by approximately 52% and 74%, respectively (Fig. 6A and B).

3.6. Measurement of FPTQ absorbed by zebrafish larvae

The concentration of FPTQ in zebrafish larvae was investigated to confirm that FPTQ entered into the zebrafish larvae. Larvae at 3 dpf

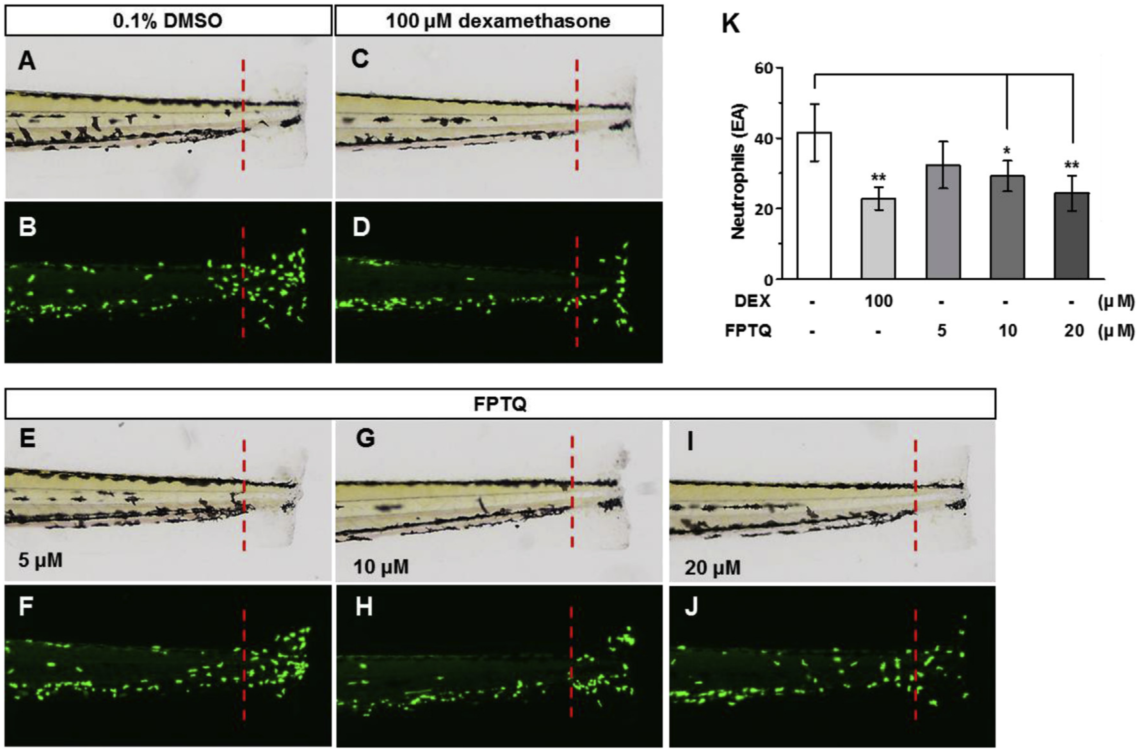

Fig. 2. Inhibitory effect of FPTQ on neutrophil migration toward the amputation site. Fluorescence microscopy was used to reveal neutrophils in *Tg (mpx:EGFP)*^{i114} larvae. The number of neutrophils from the red line to the transection site was counted. Neutrophils were detected at the wound site 4 h after tail amputation and treatment. (A–B) Control larvae were treated with 0.1% DMSO. (C–D) The positive control was treated with 100 μM dexamethasone. (E–J) Treatment with FPTQ at concentrations of 5 μM, 10 μM, and 20 μM. (K) The number of neutrophils was counted at the wound site. n > 10 larvae, $^{*}P < 0.05$, $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

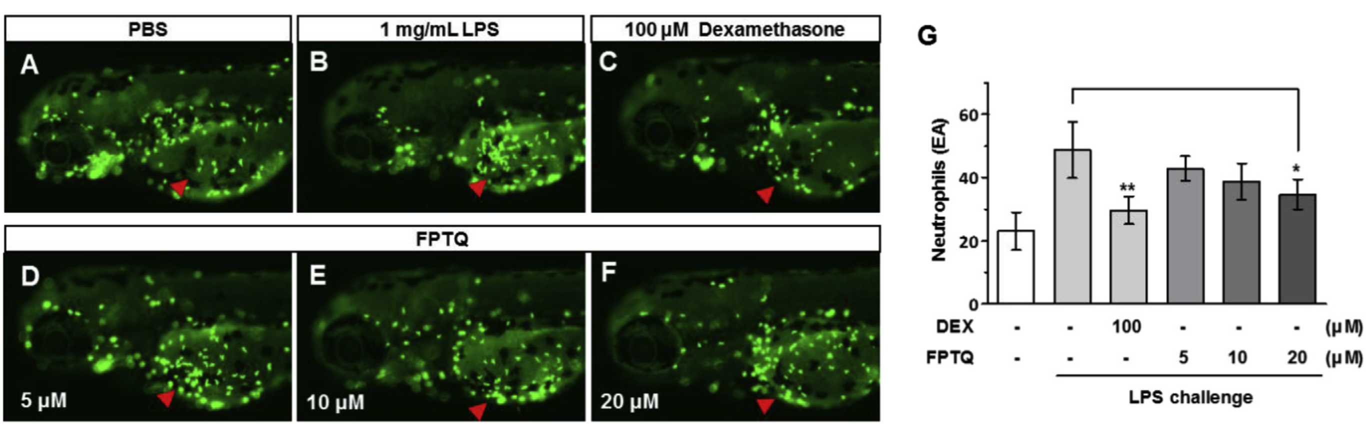

Fig. 3. Inhibition of neutrophil aggregation in response to LPS-induced inflammation in zebrafish larvae. All larvae except those of the PBS group were injected with 1 mg/mL LPS. The injection volume was 2 nL. Neutrophils were detected at 4 h after injection with PBS (A), and 4 h after injection with 1 mg/mL LPS (B). (C) The positive control was treated with 100 μM dexamethasone. (D–F) FPTQ was used at a concentrations of 5 μM, 10 μM, and 20 μM. (G) The number of neutrophils was counted in the yolk and statistically analyzed. n > 20 larvae, $^{*}P < 0.05$, $^{**}P < 0.01$.

were treated with 5, 10, or 20 μM FPTQ for 0, 0.15, 0.5, 1, and 4 h. The amount of FPTQ in the whole-body continuously increased up to 0.5 h after FPTQ exposure and reached a constant value after 4 h (Fig. 7). The amount of FPTQ absorbed by the zebrafish larvae was thought to be sufficient to produce an anti-inflammatory effect.

4. Discussion

We previously reported the anti-melanogenic effects of the compound, KDZ-001 [30]. Melanocytes are melanin-producing cells found in the skin, hair follicles, and eyes of humans. Melanin is produced from tyrosine via the 3,4 dihydroxyphenylalanine (DOPA) pathway [31]. When exposed to stimuli, melanin is synthesized in the melanosome by the action of tyrosinase. The amount of melanin produced in melanocytes is genetically determined and affected by various factors, such as inflammation, aging, and exposure to UV light [32]. FPTQ is a derivative of KDZ-003 with anti-melanogenic effects. Therefore, we anticipated that FPTQ would have antioxidant or anti-inflammatory effects leading to anti-melanogenic effects. We tried to use a zebrafish model to test for anti-inflammatory effects of FPTQ.

Prior to testing for a possible anti-inflammatory effect, zebrafish larvae (3 dpf) were exposed to FPTQ at 10–100 μM for 24 h in order to assess the toxic effects of FPTQ. When zebrafish larvae were exposed to 50 μM or 100 μM FPTQ, various toxicities such as edema, tail bending,

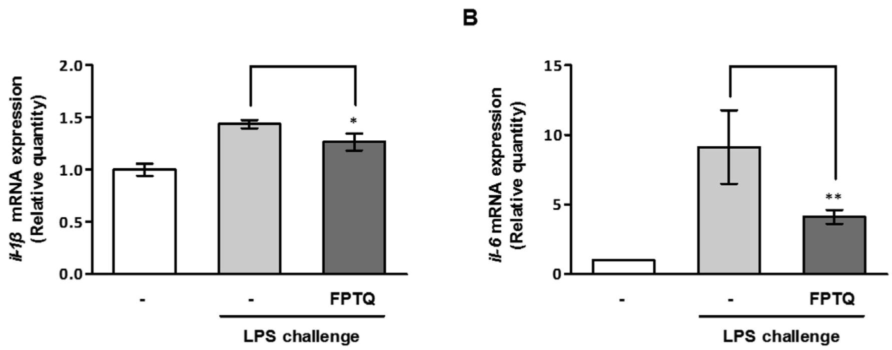

Fig. 4. Expression of *il-1β* and *il-6* mRNA in LPS-injected zebrafish. Total RNA was extracted from zebrafish larvae. The mRNA expression levels of *il-1β* (A) and *il-6* (B) were analyzed by RT-qPCR. *P < 0.05, **P < 0.01.

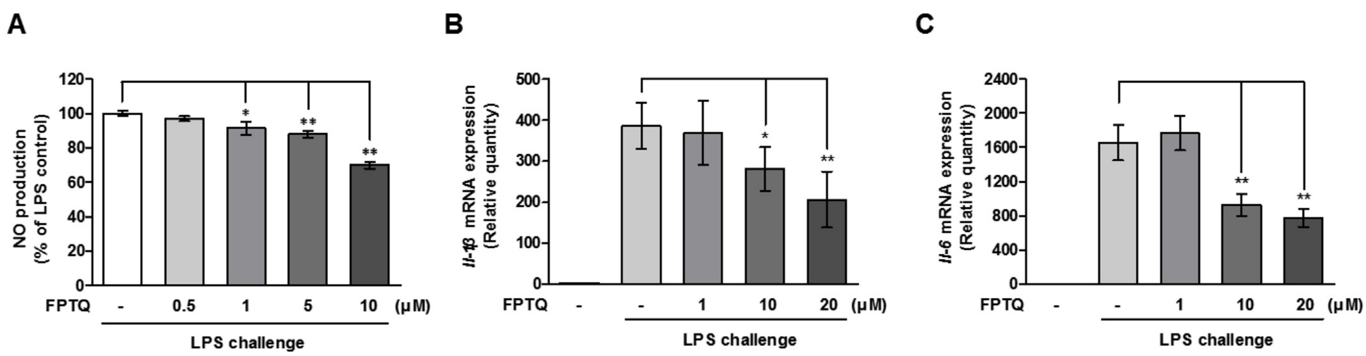

Fig. 5. Anti-oxidant and anti-inflammatory effects of FPTQ in RAW264.7 macrophage cells. (A) The inhibitory effect on NO production. (B) The mRNA expression levels of *Il-1β* and (C) *Il-6* were analyzed by RT-qPCR. *P < 0.05, **P < 0.01.

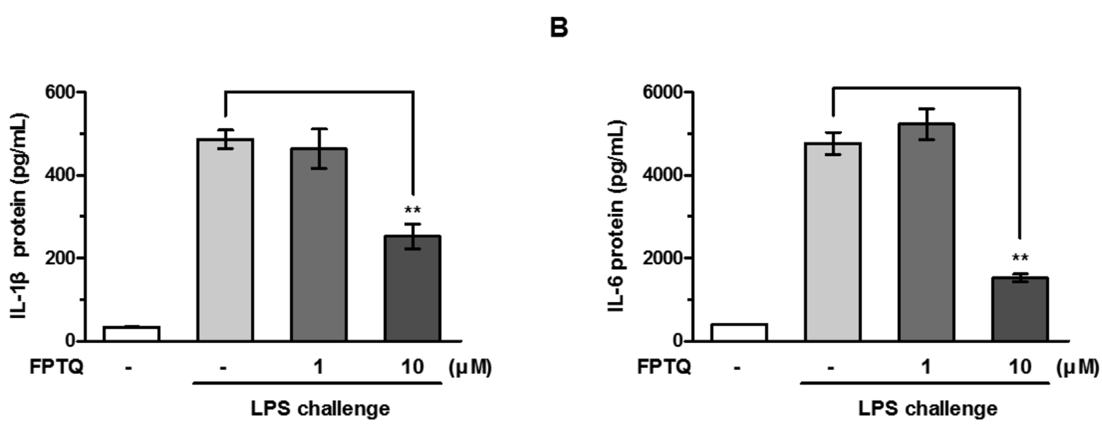

Fig. 6. Effect of FPTQ on secretion of IL-1β and IL-6 protein in LPS-stimulated RAW264.7 cells. (A) The secretion of IL-1β and (B) IL-6 was analyzed using an ELISA kit. **P < 0.01.

and heart rate, were shown early during (< 4 h) exposure. However, edema was only observed in one larvae at 8 h after exposure to 20 μM FPTQ (data not shown). Based on this result, we decided to use FPTQ up to a maximum concentration of 20 μM because zebrafish showed no toxicity within the first 8 h after treatment with this concentration. Next, Tg*(mpx:EGFP)*^{i114} zebrafish larvae were used to determine the time-point for FPTQ treatment. In previous studies, it was shown that the number of neutrophils was maximally increased between 3 and 6 h after injury of the tailfin [3,33]. When neutrophils were counted at the wound site over time, their number decreased from 4 h after injury (Supplementary Fig. 3). Therefore, we carried out all the experiment using zebrafish at 4 h after FPTQ treatment.

In zebrafish, the expression levels of *il-1β* and *il-6* were significantly reduced after treatment with 20 μM FPTQ (Fig. 4A). This concentration was slightly higher than that used to treat RAW264.7 macrophage cells. Significant reductions in the amount of secreted proteins as well as the mRNA levels *Il-1β* and *Il-6* in RAW264.7 macrophage cells were observed with 10 μM FPTQ treatment. These differences between the zebrafish model and RAW264.7 cells were thought to be due to species or test system differences. In conclusion, we demonstrated that FPTQ has anti-inflammatory effects. FPTQ inhibited the translocation of neutrophils in a *Tg*(*mpx:EGFP*) ^{i114} zebrafish line. There was decreased gene expression of the pro-inflammatory cytokine *il-6*. Furthermore, in RAW264.7 macrophage cells, FPTQ had an inhibitory effect on LPS-induced NO production and suppressed the expression of pro-inflammatory cytokines. Taken together, these results showed that FPTQ had anti-inflammatory effects and that the zebrafish model is valuable as an *in vivo* model.

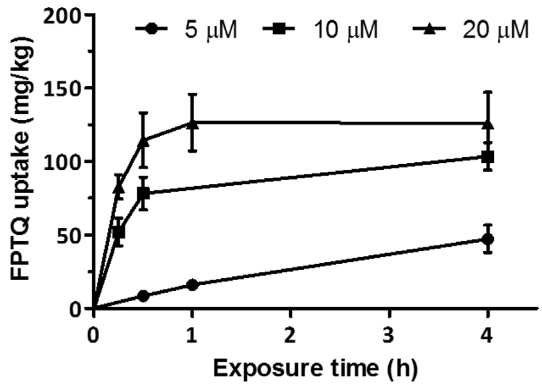

Fig. 7. Measurement of FPTQ absorbed by zebrafish larvae. FPTQ was detected in zebrafish larvae after exposure to FPTQ at concentrations of 5, 10, and 20 µM. Zebrafish larvae were then prepared for LC-MS/MS analysis.

Acknowledgements

This work was supported by a grant from the Ministry of Trade, Industry and Energy, Korea (grant no. 2018-10063396).

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.030.

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