Original Paper

Protective Effect of Phillyrin on Lethal LPS-Induced Neutrophil Inflammation in Zebrafish

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Key Words

Lps • Phillyrin • Neutrophils • Inflammation • Zebrafish • MyD88 • NF-κB

Abstract

Background/Aims: *Forsythia suspensa* Vahl. (Oleaceae) fruits are widely used in traditional Chinese medicine to treat pneumonia, typhoid, dysentery, ulcers and oedema. Antibacterial and anti-inflammatory activities have been reported for phillyrin (PHN), the main ingredient in *Forsythia suspensa* Vahl fruits, *in vitro*. However, the underlying mechanisms *in vivo* remain poorly defined. In this study, we discovered that PHN exerted potent anti-inflammatory effects in lethal LPS-induced neutrophil inflammation by suppressing the MyD88-dependent signalling pathway in zebrafish. **Methods:** LPS-yolk microinjection was used to induce a lethal LPS-infected zebrafish model. The effect of PHN on the survival of zebrafish challenged with lethal LPS was evaluated using survival analysis. The effect of PHN on neutrophil inflammation grading *in vivo* was assessed by tracking neutrophils with a transgenic line. The effects of PHN on neutrophil production and migration were analysed by SB⁺ cell counts during consecutive hours after modelling. Additionally, key cytokines and members of the MyD88 signalling pathway that are involved in inflammatory response were detected using quantitative RT-PCR. To assess gene expression changes during consecutive hours after modelling, the IL-1β, IL-6, TNF-α, MyD88, TRIF, ERK1/2, JNK, IκBα and NF-κB expression levels were measured. **Results:** PHN could protect zebrafish against a lethal LPS challenge in a dose-dependent manner, as indicated by decreased neutrophil infiltration, reduced tissue necrosis and increased survival rates. Up-regulated IL-1β, IL-6 and TNF-α expression also showed the same tendencies of depression by PHN. Critically, PHN significantly inhibited the LPS-induced activation of MyD88, IκBα, and NF-κB but did not affect the expression of ERK1/2 MAPKs or JNK MAPKs in LPS-stimulated zebrafish. Additionally, PHN regulated the MyD88/IκBα/NF-κB signalling pathway

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by controlling IkB α , IL-1 β , IL-6, and TNF- α expression. **Conclusion:** This study provides a rationale for the clinical application of PHN as an anti-inflammatory agent.

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Introduction

LPS, the major outer membrane constituent of all gram-negative bacteria and their main causative agent, activates a variety of immunocytes, including neutrophils, macrophages and endothelial cells [1], and contributes to the systemic change known as disseminated intravascular coagulation (DIC), systemic inflammatory response syndrome (SIRS) and endotoxic shock [2, 3]. Neutrophils are key effectors of innate immune response, and LPS has multiple effects on neutrophils, such as inhibiting their apoptosis and promoting their necrosis [4-6]. Studies have demonstrated that LPS transmembrane signalling is initiated by Toll-like receptors (TLRs), which are present in the plasma membranes and endosomes of leukocytes [7]. TLRs recognize LPS and transmit extracellular activation signals inside the cell via the myeloid differentiation protein 88 (MyD88)-dependent pathway or the TIR domain-containing adaptor inducing IFN- β (TRIF)-dependent pathway. The MyD88-dependent response occurs upon TLR dimerization, and its primary effect is activating the nuclear factor κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways, which include extracellular signal-regulated kinases1/2 (ERK1/2) and c-Jun NH2-terminal kinase (JNK). The MyD88 response produces numerous inflammatory cytokines, including interleukin-1 β (IL-1 β), interleukin 6 (IL-6), and tumour necrosis factor- α (TNF- α). The TRIF-dependent pathway leads to the IFN regulatory factor 3/7 (IRF3/7)-mediated expression of type I IFNs (IFN- α and IFN- β) to produce antiviral responses and IFN-inducible chemokines [8-11].

Zebrafish (*Danio rerio*) hold considerable promise as a drug screening model. Their short generation time, low cost, high clutch-size at the embryonic and larval stages, and optical transparency permit the visualization of pathogens and lesions in real time [12]. Additionally, the zebrafish immune system is remarkably similar to that of mammals [13-15]. The main reason for using zebrafish as a vertebrate model for studying and screening antiendotoxic drugs is that they serve as an advanced tool for genomic and large-scale transgenic line analysis [16]. For example, the biological behaviour of larvae neutrophils can be detected in real time using the MPO:GFP transgenic line [17], whereas such detection is unachievable with vertebrate species.

Forsythia suspensa Vahl (Oleaceae), a climbing plant, is widely distributed throughout China, Korea and Japan. The fruit of this plant is a well-known traditional Chinese medicine (TCM) named 'Lianqiao' in Chinese. More than 40 Chinese medicinal preparations containing *F. suspensa* are listed in the Chinese Pharmacopoeia [18]. The crude drug has been widely used as antipyretic, antidotal and anti-inflammatory agents for the treatment of infections, including pneumonia, typhoid, dysentery, ulcers and oedema [19, 20]. Moreover, crude extracts have been shown to suppress vomiting; resist hepatic injury; inhibit elastase activity; alleviate hypersensitivity; and exhibit diuretic, antibacterial, antiviral, anti-inflammatory, antioxidant and analgesic effects [21-24]. Phenylethanol glycosides, lignans and flavonoids were proven to be responsible for

the various biological activities of the herb, and they were all isolated from the title plant [25]. Phillyrin (PHN) (Fig. 1), a lignin, plays important roles in the antioxidant, antibacterial, anti-obesity and anti-inflammatory effects of Lianqiao [26-28]. Recently, an *in vitro* study reported that lignans from the flowers of *O. fragrans*

Fig. 1. Chemical structure of phillyrin (PHN).

var. aurantiacus could inhibit the release of nitric oxide in RAW 264.7 macrophages [29]. However, the underlying *in vivo* mechanisms remain poorly defined.

In this study, we first found that PHN exerted antiendotoxin inflammatory effects via diminishing the expression of Myd88, which led to inhibition of the I κ B α /NF- κ B pathway but not of the ERK1/2 MAPKs or JNK MAPKs pathways, in zebrafish. Our research provides scientific evidence for the therapeutic application of PHN.

Materials and Methods

Materials

1-phenyl-2-thiourea (PTU), LPS (*E. coli*, 055: B5, L2880, total impurities <3% protein), and Sudan Black B (SB) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA); Bortezomib (PS341) was purchased from Ben Venue Laboratories Inc. (USA); RNAlater[®] solution was obtained from Ambion Inc. (Austin, TX, USA). RNAiso Plus, the PrimeScript[®] RT Reagent kit and the SYBR Green PCR Core Reagent kit were purchased from TaKaRa Biotechnology Co. (Japan). The PCR primers were purchased from Invitrogen (USA). PHN was purchased from the Guangdong Institute for Drug Control (HPLC >98%, China). The other chemicals and reagents used were obtained in the highest grade commercially available.

Zebrafish embryo and larvae maintenance

Zebrafish embryos were maintained and raised according to the protocol described by Westerfield [30]. The transgenic line Tg (MPO:GFP) [17] was kindly provided by the Key Laboratory of Zebrafish Modeling and Drug Screening for Human Diseases Institute at Southern Medical University (Guangzhou, China). All the experimental protocols and animals used in this research were approved by the Ethical Committee of Southern Medical University.

Acute inflammatory model and drug administration

Microinjection (Narshige PLI-100) was performed in a 2-nl volume per larva (except for the normal control). Three days post-fertilization (dpf), zebrafish yolks were injected with 0.5 mg/ml LPS to induce the lethal endotoxin-infected model [31]. Phosphate-buffered saline (PBS) was used as the negative control. Before administration to determine PHN effects, PHN (1.0, 3.3, 10.0 μ g/ml), dexamethasone (DEX, 5.0 μ g/ml) (positive control) and PS341 (a I κ B α degradation inhibitor, 10.0 μ g/ml) were dissolved in 0.1% dimethyl sulfoxide (DMSO) in egg water at the required concentrations. After recovering from anaesthesia (0.02% tricaine), larvae from each group were moved to a 6-well plate. The treatment groups were exposed to PHN, DEX or PS341. All the groups were maintained in a final volume of 5 ml at 28.5 °C. The fish were evaluated for mortality and disease symptoms for 96 h post-injection (hpi).

Inflammation assessment criteria

The transgenic Tg (MPO:GFP) line was used so that neutrophils could be detected by green fluorescence in real time. Neutrophil recruitment was evaluated by Tg (MPO:GFP) with fluorescently marked cell populations *in vivo* after injecting the yolks with LPS. The neutrophil recruitment grade was assessed at 12 and 24 hpi, and the assessment criteria were as follows: Grade 1 = normal (zero or several neutrophils, no necrosis); Grade 2 = neutrophils scattered throughout the yolk, no necrosis; Grade 3 = neutrophil recruitment to the LPS site, no necrosis; Grade 4 = bulk infiltration of neutrophils and neutrophil adhesion, no necrosis; Grade 5 = yolk deformation and necrosis.

SB Staining

Zebrafish larva yolks injected with 0.5 mg/ml LPS at 3 dpf were collected at 1, 3, 6, 12, and 24 hpi for SB staining. Negative control, positive control and PHN treatment larvae were sampled at identical time points. Samples included 10 larvae per time point per treatment. SB staining was performed according to the protocol described by Guyader et al. (2008)[32]. Under a stereomicroscope, SB⁺ cells in yolks and abdominal aortas (AOs) were counted after staining.

Real-time quantitative PCR

Zebrafish larva yolks injected with 0.5 mg/ml LPS at 3 dpf were collected at 6, 12, 18, and 24 hpi for the extraction of total RNA. Larvae from the other groups were sampled at identical time points. Samples

included 30 larvae per time point per treatment. Larvae were stored in RNA stabilization solution at 4 °C until samples from all the time points were collected. Total RNA was extracted using RNAiso Plus according to the manufacturer's instructions. The RNAs were then quantified by UV spectrophotometry (Thermo, NanoDrop 2000). Transcribed cDNAs were then amplified using the Prime Script® RT reagent kit according to the manufacturer's instructions. Real-time quantitative PCR was performed according to the manufacturer's instructions (SYBR Green PCR Core Reagent kit). The primer sequences used were as follows:

β-actin 130 bps {F} 5'-ATGGATGAGGAAATCGCTG-3',
{R} 5'-ATGCCAACCATCACTCCCTG-3';
TNF-α 139 bps {F} 5'-GCTGGATCTTCAAAGTCGGGTGTA-3',
{R} 5'-TGTGAGTCTCAGCACACTTCCATC-3';
IL-1β 124 bps {F} 5'-TGGACTTCGACGACAAAATG-3',
{R} 5'-GTTCACTTCACGCTCTTGGATG-3';
IL-6 136 bps {F} 5'-AGACCGCTGCCTGTCTAAAA-3',
{R} 5'-TTTGATGTCGTTTACCAGGA-3';
MyD88 116 bps {F} 5'-GAGGATGGTGGTGGTCATCT-3',
{R} 5'-CGACAGGGATTAGCCGTTTA-3';
TRIF 141 bps {F} 5'-TGGGTCAGTTTCCAAGTTCC-3',
{R} 5'-CCACCTTCTGCCATTGTTTT-3';
ERK1/2 124 bps {F} 5'-CCTGAACATGACCACACTGG-3',
{R} 5'-TGCATCCACAGACCAAATA-3';
JNK 176 bps {F} 5'-CAAACCTCTGCCAGGTCATT-3',
{R} 5'-GCCGAAATCCAAAATCTTCA-3';
IκBa 184 bps {F} 5'-TTTCGGAGGAGATGGAGAGA-3',
{R} 5'-CTGTTTCAGGTACGGGTCGTT-3'; and
NF-κB 154 bps {F} 5'-GAGCCCTTTGTGCAAGAGAC-3',
{R} 5'-TGGGATACGTCCTCCTGTTC-3'

The thermocycling conditions were initial denaturation at 95 °C for 10 min, followed by 95 °C for 10 s and 60 °C for 30 s. All reactions were performed in triplicate for 40 cycles using a Stratagene Mx3005P system. Relative quantitation was performed using standard curves for each primer (sequence information). The amount of mRNA was normalized to that of β-actin.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). Survival analysis was evaluated with the Kaplan-Meier method. Comparisons between curves were made with the log-rank test. Mean values were analysed by multivariate analysis of variance. Comparisons between groups were made by the LSD test. All data were analysed with the Statistical Package for the Social Sciences (SPSS; version 20.0). P-values < 0.05 were considered statistically significant.

Results

Effects of PHN on the survival of zebrafish challenged with lethal LPS

To test whether PHN could protect zebrafish from a lethal challenge with LPS, the mortality rates of zebrafish treated with different doses of PHN were analysed. All of the zebrafish challenged with 0.5 mg/ml LPS died within 48 hpi (Fig. 2). In contrast, the zebrafish treated with PHN at concentrations of 1, 3.3, and 10 µg/ml died later, and a dose-dependent increase in survival was observed. All the zebrafish survival rates in the PHN treatment groups were greater than 24% within 48 hpi. Treatment with 1 µg/ml PHN did not increase zebrafish survival but did delay time of death ($P < 0.0001$), as 26.1% of the zebrafish survived through 48 hpi, while no zebrafish were alive at 96 hpi. However, 3.3 µg/ml PHN not only delayed the zebrafish time of death but also decreased the number of deaths ($P < 0.0001$); the survival rate increased to 17% at 96 hpi. The zebrafish administered 10 µg/ml PHN or 5 µg/ml DEX exhibited significantly increased survival, with 65% and 53% survival observed at 48 hpi, respectively. By 96 hpi, their survival rates were still greater than 30%. Meanwhile,

Fig. 2. Protective effects of PHN and DEX on zebrafish challenged by LPS. Three hundred and fifty larvae were randomly divided into 7 groups ($n = 50$). Survival of zebrafish larvae exposed to the indicated doses of PHN after a 0.5 mg/ml injection of LPS into their yolks at 3 dpf was assessed. The negative and positive controls were PBS and 5 $\mu\text{g/ml}$ DEX, respectively. The mortalities of the zebrafish were observed for 96 h after administration. ** $P < 0.001$ relative to the LPS group; ## $P < 0.001$ between the 1 $\mu\text{g/ml}$ PHN treatment group and the 10 and 3.3 $\mu\text{g/ml}$ PHN treatment groups; + $P < 0.05$ between the 10 and 3.3 $\mu\text{g/ml}$ PHN treatment groups. Each curve represents data pooled from three independent experiments.

the median survival time among the PHN concentrations (1, 3.3, and 10 $\mu\text{g/ml}$) were 40 ± 1.2 , 48 ± 3.9 , and 67 ± 7.8 h, respectively; LPS and 5 $\mu\text{g/ml}$ DEX were 34 ± 1.2 and 65 ± 10.8 h, respectively. PHN could markedly extend the median survival time of zebrafish, and the protection provided by 10 $\mu\text{g/ml}$ PHN was much better than that provided by 1 and 3.3 $\mu\text{g/ml}$ PHN ($P < 0.05$). The above results confirmed that suitable doses of PHN could provide significant protection to zebrafish challenged with lethal LPS. We used 10 $\mu\text{g/ml}$ PHN as our immersion concentration and focused on inflammation grading, neutrophil activation, pro-inflammatory cytokine expression and the inflammatory signalling pathway in zebrafish larvae at different time points after treatment with PHN.

Effects of PHN on inflammation grading in zebrafish yolks *in vivo*

To determine the effects of PHN on LPS-mediated neutrophil recruitment in inflammatory response *in vivo*, inflammation grading was evaluated at 12 and 24 hpi using a Tg (mpo:GFP) line. At 3.5 dpf, neutrophils mainly appeared in the PBI, and both the head stroma and epidermis expressed only a few of these cells [32]. Yolks with severe neutrophil infiltration were observed 12 h after being injected with LPS (Fig. 3-B), but only a small number of sparsely distributed neutrophils were observed in PHN-treated larvae (Fig. 3-D). The PBS negative control and DEX-treated group produced similar results (Fig. 3-A, C). At 24 hpi, tracking neutrophils *in vivo* showed severe yolk deformation, necrosis, and only a few neutrophils remaining in the LPS group (Fig. 3-B'). In contrast, the zebrafish administered PHN exhibited only some neutrophil recruitment in their yolks, and no necrosis was observed (Fig. 3-D', C'). Meanwhile, treatment with PHN markedly decreased the inflammation grading of larvae compared with that of the LPS group at the abovementioned time points (Fig. 3-E, F, $n = 20$, $P < 0.001$). No neutrophils or necrosis were found in the zebrafish yolks of the PBS group at 24 hpi (Fig. 3-A', F). These results showed that PHN could significantly reduce the infiltration of neutrophils and decrease the extent of necrosis induced by LPS.

Fig. 3. PHN inhibits neutrophil recruitment and decreases the yolk necrosis induced by LPS; Tg (mpo:GFP) marks neutrophils (A-D'). Fluorescence imaging of 3-dpf Tg (mpo:GFP) live larvae ($n = 20$) at 12 hpi (A, B, C, D) and 24 hpi (A', B', C', D') using the GFP channel. The yellow arrow in B indicates the neutrophil, while the red arrow in B' indicates yolk necrosis. The negative control was PBS (A/A'), and the positive control was 5 $\mu\text{g}/\text{ml}$ DEX (C/C'). The LPS model group (B/B') and the 10 $\mu\text{g}/\text{ml}$ PHN treatment group (D/D') are shown. E and F show the inflammatory grading scatter diagrams in each group at 12 and 24 hpi, respectively. Grade 1 = normal (zero or several leucocytes, no necrosis); Grade 2 = leucocytes scattered in the yolk, no necrosis; Grade 3 = leucocyte recruitment to the LPS site, no necrosis; Grade 4 = bulk infiltration of leucocytes and leucocyte adhesion, no necrosis; Grade 5 = yolk deformation and necrosis. The scatter diagrams represent data pooled from three independent experiments.

Effects of PHN on neutrophil production and migration

Previous studies have demonstrated that SB can be used to detect the presence of neutrophils with dark colour [32, 33]. Here, to quantify the activation or migration of neutrophils and to determine the significance of the treatment group, migration quantity and directionality were calculated from the tracks of SB⁺ cells (Fig. 4-A, red arrow) migrating towards or away from the injection point. As expected, an intense increase in the total number of SB⁺ cells in the LPS group compared with that in the PBS group was observed from 1 to 12 h (Fig. 4-B, n = 10, P<0.0001). However, at 24 hpi, the total number of SB⁺ cells was sharply decreased, with obvious deformation and necrosis in the yolks observed (smaller than that of the PBS group, P<0.0001). The total number of SB⁺ cells in the PHN-treated group was obviously less than that in the LPS group from 1 to 12 h, which was similar to that in the PBS group. In the yolk sacs, SB⁺ cells (Fig. 4-C) in the LPS group accumulated with time, as they peaked at 6 hpi (NO. = 179.7 ± 11.7) and then declined. A sharp decrease in the number of SB⁺ cells at the injection site was observed by 24 hpi (NO. = 30.4 ± 1.7). In the AOs (Fig. 4-D), SB⁺ cells in the LPS group continuously decreased for up to 24 hpi (NO. = 39.1 ± 2.8) after peaking at 3 hpi (NO. = 104.6 ± 6.4). In contrast, the number of cells in the yolks peaked at 6 hpi, but few SB⁺ cells remained at 12 and 24 hpi in the PBS group (Fig. 4-C). The number of cells in the AOs consecutively increased after 6 h (Fig. 4-D). Notably, treatment with PHN remarkably decreased the number of yolk cells at the corresponding time points, except at 24 hpi. While the SB⁺ cell counts in the PHN group was greater than that in the LPS group at 24 hpi, necrosis was not observed (Fig. 4-C, P<0.0001). Meanwhile, the number of SB⁺ cells in the AOs declined consecutively after peaking at 3 hpi. Similar results were observed in the DEX group. The above results demonstrated that PHN could markedly diminish neutrophil production and inhibit neutrophil migration and activation from 1 h to 24 h when stimulated by LPS.

Fig. 4. PHN inhibits neutrophil production and migration. (A) SB staining to label neutrophils at 3 dpf. The red arrow shows the neutrophil (n = 10). (B) Linear chart revealing the total number of SB⁺ cells in zebrafish from 1 to 24 h after treatment with 5 µg/ml DEX and 10 µg/ml PHN; ** P<0.001 compared to the LPS group. (C) Linear chart revealing the number of SB⁺ cells in the yolk sac from 1 to 24 h after treatment with DEX and PHN; * P<0.001 compared to the LPS group. (D) Linear chart revealing the number of neutrophils in the AOs from 1 to 24 h after treatment with DEX and PHN. Negative controls were performed using PBS; * P<0.05 and ** P<0.001 compared to the LPS group.

Fig. 5. PHN reduces the expression of the proinflammatory cytokines TNF- α , IL-1 β , IL-6. Zebrafish larvae injected with 0.5 mg/ml LPS at 3 dpf were sampled at 6, 12, 18, and 24 hpi to measure TNF- α , IL-6 and IL-1 β mRNA using RQ-PCR (A-C). (A) Group comparison of IL-1 β expression at the same time; * $P < 0.05$ and ** $P < 0.001$ compared to the LPS group. (B) Group comparison of IL-6 expression at the same time; * $P < 0.05$ and ** $P < 0.001$ compared to the LPS group. (C) Group comparison of TNF- α expression at the same time; * $P < 0.05$ and ** $P < 0.001$ compared to the LPS group. # $P < 0.05$ and ## $P < 0.001$ compared to the control group.

Fig. 6. PHN inhibits LPS-induced inflammation by suppressing MyD88/NF- κ B signalling. Zebrafish larvae injected with 0.5 mg/ml LPS at 3 dpf were sampled at 6, 12, 18, and 24 hpi to measure MyD88, TRIF, I κ B α , NF- κ B ERK and JNK mRNA using RQ-PCR (A-F). Group comparison of MyD88 (A), TRIF (B), I κ B α (C), NF- κ B (D), ERK (E) and JNK (F) expression at the same time; * $P < 0.05$ and ** $P < 0.001$ compared to the LPS group; # $P < 0.05$ and ## $P < 0.001$ compared to the control group.

Effects of PHN on the mRNA expression of IL-1 β , IL-6, and TNF- α

Because LPS-treated leukocytes can produce the pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α , which contribute to inflammation [11, 34], we detected the expression of these cytokines. As shown in Fig. 5, IL-1 β , IL-6, and TNF- α expression was markedly increased in LPS-infected larvae from 6 to 24 hpi compared with PBS-injected larvae, but the PBS-injected larvae expression levels and changes were different at these time points (Fig. 5). IL-1 β showed an immediate response to LPS infection, as its expression markedly increased

Fig. 7. The inhibitory effects of PHN and SP341 on the LPS-activated MyD88/NF- κ B pathway. Zebrafish were pretreated for 12 h with PHN or PS341 after they were injected with LPS for 0.5 h. The mRNA expression levels of $\text{IkB}\alpha$ (A), JNK (B), ERK1/2 (C), $\text{IL-1}\beta$ (D), IL-6 (E) and $\text{TNF-}\alpha$ (F) were then determined; * $P < 0.05$ and ** $P < 0.001$ compared between the indicated groups; n.s. indicates no statistically significant difference.

(30-fold) at 18 hpi. PHN could significantly reduce the $\text{IL-1}\beta$ expression up-regulated by LPS from 6 to 18 hpi (Fig. 5-A, $P < 0.05$) but could not decrease $\text{IL-1}\beta$ expression at 24 hpi. After LPS stimulation, IL-6 was remarkably expressed in zebrafish in a time-dependent manner from 6 to 24 hpi and was inhibited in the presence of PHN (Fig. 5-B, $P < 0.05$). Meanwhile, the up-regulated $\text{TNF-}\alpha$ expression levels (Fig. 5-C) were smaller than those of $\text{IL-1}\beta$ and IL-6 at each corresponding time point. Similar to $\text{IL-1}\beta$, $\text{TNF-}\alpha$ expression up-regulated by LPS was significantly suppressed by PHN before 18 hpi ($P < 0.001$) but was not suppressed at 24 hpi ($P > 0.05$). The same results were observed in the DEX treatment group ($P < 0.05$). Although the up-regulated levels of $\text{IL-1}\beta$, IL-6 and $\text{TNF-}\alpha$ varied from 6 to 18 h, they showed the same PHN inhibition tendency during LPS-induced inflammation.

PHN inhibits LPS-induced inflammation by suppressing MyD88/NF- κ B signalling

TLR signalling is divided into two distinct signalling pathways in mammals, the MyD88-dependent and the TRIF-dependent pathway. The MyD88-dependent pathway leads to the expression of pro-inflammatory genes, such as TNF , $\text{IL-1}\beta$, IL-6, through the activation of NF- κ B, while the TRIF-dependent pathway does not. Therefore, we hypothesized that the anti-inflammatory effects of PHN are potentially associated with the MyD88-dependent pathway in zebrafish. To confirm this hypothesis, we observed MyD88 and TRIF expression. Our results showed that stimulating zebrafish with LPS resulted in increased MyD88 expression from 6 to 24 hpi, which was obviously prevented by PHN (Fig. 6A, $P < 0.05$). By contrast, LPS and PHN did not affect the expression of TRIF (Fig. 6B, $P > 0.05$).

Since MyD88 has been found to activate NF- κ B, a critical transcriptional regulator for inflammatory-related gene coding in response to LPS [35], we examined the effects of PHN on the LPS-induced activation of NF- κ B and $\text{IkB}\alpha$, whose degradation and phosphorylation result in the release and transfer of NF- κ B into the nucleus [36]. As shown in Fig. 6C, after LPS

stimulation, the mRNA I κ B α expression levels were remarkably decreased from 6 to 24 hpi in zebrafish ($P < 0.05$), while NF- κ B expression significantly increased in a time-dependent manner (Fig. 6D, $P < 0.05$). However, PHN treatment prevented this phenomenon (Fig. 6C-D). These results suggest that the anti-inflammatory effect of PHN is associated with the MyD88/I κ B α /NF- κ B signalling pathway.

Recently, some studies have indicated that the LPS-induced production of inflammatory cytokines is strongly affected by MAPKs, which include ERK1/2 and JNK [37-39]. We thus determined the effects of PHN on the LPS-induced activation of MAPKs in zebrafish. We found that the expression levels of ERK1/2 (Fig. 6E) and JNK (Fig. 6F) were up-regulated by LPS ($P < 0.05$); however, PHN did not affect this up-regulation from 6 to 24 hpi (Fig. 6E-F, $P > 0.05$). These findings demonstrate that while PHN attenuates LPS-induced inflammation in zebrafish, this effect is likely not associated with the ERK1/2 and JNK signalling pathways.

Comparison of the inhibitory effects of PHN and signalling inhibitors on the LPS-activated MyD88/NF- κ B pathway

Since we observed that PHN suppressed the LPS-triggered activation of NF- κ B, we next determined the relative contribution of the anti-inflammatory activity of PHN to inhibiting these signalling pathways. We used the pharmaceutical inhibitor PS341 to specifically block I κ B α degradation. Zebrafish were pretreated for 12 h with PHN or PS341 after they were injected with LPS for 0.5 h. The mRNA expression levels of I κ B α , JNK, ERK1/2, IL-1 β , IL-6 and TNF- α were then determined. As shown in Fig. 7A-C, PS341 inhibited only the phosphorylation of I κ B α and increased the expression of I κ B α , demonstrating the specificity of the inhibitor we used in our experiments. Regarding IL-1 β , IL-6 and TNF- α production, PS341 significantly reduced the LPS-induced up-regulation of these cytokines compared to that of the LPS-stimulated group. However, when compared to the PHN-treated group, PS341 could not exhibit the same inhibition (Fig. 7D-F, $P < 0.05$).

Taken together, these findings demonstrate that PHN inhibits LPS-induced lethal neutrophil inflammation in zebrafish by suppressing the MyD88/I κ B α /NF- κ B pathway.

Discussion

Cells or rodents are traditionally used to study and screen antiendotoxic drugs [40-42]. However, drugs that have antiendotoxic effects *in vitro* may be ineffective *in vivo*. Conversely, drugs that are effective only *in vivo* can sometimes be unidentifiable with *in vitro* screens. Rodent screening models also have many shortcomings, as they are time consuming, strenuous and costly. Due to unique advantages in biology, genomics, and genetics, as well as high conservativity in the disease signal transduction pathway, drug screens in zebrafish embryos and larvae hold considerable promise in filling the gap between cell and rodent models [16, 34, 43]. In the present study, we show a simple, quick and practical method for screening and evaluating antiendotoxic agents using zebrafish embryos. Previous studies found that PHN could depress inflammation *in vitro* (28). Herein, we showed that PHN suppressed LPS-stimulated inflammation *in vivo*.

Kaplan-Meier survival curves are usually used to represent the efficacy of new antiendotoxic drugs. Cumulative survival from a curve is expressed with the drug stimulus or action time. The detection of different drug challenge doses on equivalent doses of LPS, as well as the formation of actual dose-response curves, provides valuable statistics for the antiendotoxic drug being studied. Thus, the clinical efficacy of the experimental therapy can be tested [44]. Here, utilization of zebrafish cumulative survival as a hard, primary end-point for evaluating the efficacy of PHN was very useful. We analysed the survival of zebrafish treated with different doses of PHN for 96 hpi. The data presented in Fig. 2 indicate that PHN could prolong the survival time of zebrafish and increased their survival rate in a dose-dependent manner compared to the LPS group. These results could provide essential information for evaluating PHN efficacy in a pre-clinical trial.

Neutrophils play pivotal roles in the LPS stimulation of inflammatory diseases. During early periods of inflammation, neutrophils circulating in the blood are the first and most abundant leukocytes to arrive at the inflammation point to eliminate the causative organism and to resist infection [45]. However, overactive neutrophils have been shown to aggravate inflammatory tissue injury, while reduced neutrophil excessive exudation was shown to attenuate inflammation [46-48]. Some reports have also shown that neutrophil recruitment is markedly defective in the advanced stages of severe infectious diseases, and patients with low neutrophil levels (high neutrophil necrosis) have an increased risk for these diseases [49-51]. Here, decreased neutrophil (MPO:GFP/SB⁺ cells) recruitment to the yolk (injection point) and a decreased total number of neutrophils were observed in the PHN-treated group before 12 hpi. Moreover, a sharp reduction in the total number of neutrophils and obvious necrosis was observed at 24 hpi, while PHN treatment prevented this phenomenon. This result was similar to previous research [52] reporting that PHN could enhance immunological functions in mice. Neutrophil necrosis and apoptosis are known to be related to immunoregulation function. The PHN-dependent enhancement of immunological function might significantly inhibit the sharp reduction of neutrophils in the advanced stages of inflammatory diseases.

In response to the LPS stimulus, a series of intracellular signalling cascades are initiated, which ultimately lead to the activation of neutrophils and their release of pro-inflammatory cytokines. MyD88, the first responsive intracellular signalling molecule, has been implicated as a key modulator in the inflammatory signalling cascades triggered by LPS, including the NF- κ B and MARK pathways [8]. As shown in our results, PHN obviously inhibited the LPS-induced expression of MyD88, but not of TRIF, indicating that PHN could suppress LPS-induced inflammation in the MyD88-dependent pathway.

The nuclear transcription factor NF- κ B is a potential positive regulator that stimulates the release of pro-inflammatory cytokines during inflammation [53]. Our present data indicated that PHN suppressed the degradation of I κ B α and the transactivational activity of NF- κ B in LPS-stimulated zebrafish models to extents similar to that of the proteasome inhibitor PS341. We also observed that PHN apparently attenuated the LPS-triggered expression of TNF- α , IL-6 and IL-1, suggesting that PHN inhibits LPS-induced lethal inflammation *in vivo* by suppressing the MyD88/I κ B α /NF- κ B signalling pathway.

The MARK signalling pathway plays pivotal roles in immune and inflammatory responses. In particular, some evidence has shown that the ERK1/2 MAPK and JNK MAPK signalling pathways are related to leukocyte migration and pro-inflammatory cytokine expression [10, 37]. Previous studies revealed that the inhibition of ERK, as well as the inhibition of pro-inflammatory cytokines and chemokines, using specific inhibitors effectively attenuated acute lung injury by reducing lung neutrophilia and hyperpermeability [54]. PHN also significantly inhibited ERK and p38 pathway activation in acute lung injury [55]. However,

Fig. 8. The speculated anti-inflammatory network of PHN. $\perp$ indicates an inhibitory effect. PHN exerts anti-inflammatory effects and suppresses the expression of MyD88, I κ B α and NF- κ B, as well as the release of inflammatory factors induced by LPS, by suppressing the MyD88-dependent inflammatory pathway *in vivo*.

in this study, we determined only the effects of PHN on the expression of JNK and ERK1/2 to show that no statistically significant differences existed in JNK and ERK1/2 activation. Further investigating the effects of PHN on the activity of MAPK subunits, such as p38 MAPK, in the future will be worthwhile.

In summary, we first found that PHN exhibits significant anti-inflammatory effects against zebrafish stimulated with LPS by reducing neutrophil infiltration, attenuating necrosis and inhibiting proinflammatory cytokine expression. We elucidated that the underlying mechanism involves the suppression of MyD88-NF- κ B signalling (Fig. 8). Our study provides a rationale for the clinical application of PHN as an anti-inflammatory agent.

Disclosure Statement

The authors declare no conflicts of interest.

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