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Research article

Development of zebrafish demyelination model for evaluation of remyelination compounds and RORγt inhibitors

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ABSTRACT

RAR-related orphan receptor-γt (RORγt) directs differentiation of proinflammatory T helper 17 cells and is a potential therapeutic target for chronic autoimmune and inflammatory diseases including multiple sclerosis. In this study, zebrafish at days post fertilization treated with ethidium bromide (EB) at a concentration of 75 μM for 72 h were determined as the optimum conditions for the demyelination model development. Zebrafish motility was recorded automatically using a video-track motion detector and quantitative myelin assay was measured by FluoroMyelin staining. A well-known remyelination agent thyroxine (T4) was tested to confirm whether EB-induced motility and myelin damage could be rescued. Two RORγt lead inhibitors GSK805 and SR1001 were assessed for their therapeutic effects on remyelination, axon regeneration, motor neuron promotion and anti-inflammation. T4 significantly improved EB-induced motility dysfunction and myelin damage and promoted myelin basic protein (MBP) regeneration in the demyelinated zebrafish. GSK805 and SR1001 enhanced remyelination in a dose-dependent manner and promoted MBP regeneration. Both GSK805 and SR1001 markedly recovered EB-induced axon and motor neuron damage, and exhibited significantly inhibitory effects of neutrophil infiltration and macrophage recruitment. These results indicate that EB treatment can induce zebrafish demyelination; and the zebrafish demyelination model in combination with quantitative motility and myelin assays is a predictive, reproducible and relatively high throughput screening for rapidly *in vivo* identification of remyelination compounds and RORγt inhibitors.

1. Introduction

Multiple sclerosis (MS) which is characterized by the loss of oligodendrocytes and axon demyelination is a chronic, multifocal and relapsing-remitting disorder mostly in young adults (Compston, Kellarwood, & Wood, 2008). The myelin sheath is the membrane structure protecting, supporting and nourishing axons. As the multilayered insulating structure around the axon, the myelin sheath mediates the rapid conduction of nerve impulses (Almeida, Czopka, Ffrench-Constant, & Lyons, 2011; Czopka, Ffrench-Constant, & Lyons, 2013; D'Rozario, Monk, & Petersen, 2016). Demyelination is the most common complication of MS in which the myelin sheath around the axons gets damaged (Compston et al., 2008; Paz Soldan & Rodriguez, 2002). This damage impairs the conduction of signals in the affected nerves. Then, the reduction in conduction causes deficiencies in sensation, movement, cognition, or other functions (Compston et al., 2008; Scherer & Wrabetz, 2008).

Zebrafish has emerged as a cost-efficient model to study vertebrate myelination *in vivo* (Buckley, Marguerie, Alderton, & Franklin, 2010; Preston & Macklin, 2015) and is particularly interesting and valuable due to its remarkable ability to regenerate injured axons in the central nerve system (CNS) (Bernhardt, Tongiorgi, Anzini, & Schachner, 1996; Stuermer, Bastmeyer, Bahr, Strobel, & Paschke, 1992; Su, Liu, Chan, & Wang, 2016). Zebrafish myelin structure, myelin synthesis and gene expression are highly conserved between zebrafish and mammals (Chung et al., 2013; Gerlai, 2011; Jung et al., 2010; Fang et al., 2015). The myelin structure is formed by oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system and surrounds the axons. In zebrafish, a non-tight structure is observed at 2 days post fertilization (dpf), and the myelin sheath forms at 4 dpf. The tunica vaginalis forms a compact myelin structure at 7 dpf (Bernhardt et al., 1996). Most myelin-associated mammalian genes have

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homologies with zebrafish (Emery, 2010; Farrar, Wise, Fetcho, & Schaffer, 2011; Schweitzer, Becker, Schachner, Nave, & Werner, 2006).

Chemical methods have often been used to induce demyelination in mouse models. Ethdium bromide (EB) in the white matter of the CNS is known to act like a gliotoxin, causing local oligodendroglial and astrocytic death, leading to primary demyelination, neuroinflammation, blood-brain barrier disruption and Schwann cell invasion due to the glia limitans breakdown (Graça, Bondan, Pereira, Fernandes, & Maiorka, 2001; Kuypers, James, Enzmann, Magnuson, & Whittemore, 2013; Blakemore, 1982; Bondan, Lallo, Sinhorini, Pereira, & Graça, 2000; Bondan, Lallo, Dagli, Sanchez, & Graça, 2003). EB is a simple tool for induction of neural cell degeneration and helps researchers to study the demyelination and remyelination processes (Guazzo, 2005; Bondan et al., 2000, 2006). EB can also affect the motor and sensory systems (Goudarzvand et al., 2016). Thyroxine (T4) physiologically helps oligodendrocyte precursor cells to turn into myelinating oligodendrocytes, enhancing remyelination after myelin sheet damage (Pieragostino et al., 2013). Actually, it was demonstrated that T4 administration improves clinical course and the remyelination process in MS animal models (Dell'Acqua et al., 2012).

RAR-related orphan receptor-γt (ROR-γt) induces differentiation of proinflammatory T helper 17 (Th17) cells and is a potential therapeutic target for chronic autoimmune and inflammatory diseases including MS (Gaffen, Jain, Garg, & Cua, 2014; Ivanov et al., 2006; Korn, Bettelli, Oukka, & Kuchroo, 2009; Withers et al., 2016). It has been shown that the genetic deficiency of RORγt in mice severely impaired Th17 cell differentiation and conferred resistance to experimental allergic encephalomyelitis (Wang et al., 2015, 2012). As a nuclear receptor with a ligand-binding pocket, RORγt is considered to be an attractive pharmacologic target for the treatment of Th17-cell-mediated immune disorders. Indeed, several small molecular compounds that can inhibit the function of RORγt, including GSK805 (Wang et al., 2015; Withers et al., 2016; Xiao et al., 2014) and SR1001 (Solt et al., 2011), have been discovered.

The majority of work undertaken so far using zebrafish as a model has focused on investigating the mechanisms of remyelination and oligodendrocyte precursor cell (OPC) differentiation, using toxin-induced demyelination models and genetic cell ablation models (Burrows et al., 2019). In this study, we have taken advantage of larval zebrafish to develop a relative high-throughput demyelination model for drug screening and efficacy assessment. To validate the zebrafish demyelination models for future RORγt compounds screen and evaluation, two RORγt lead inhibitors GSK805 and SR1001 were selected for dose-response observations of remyelination, myelin basic protein (MBP) and axon regeneration, motor neuron promotion and anti-inflammation assessment. Our results indicate that zebrafish demyelination-associated motility assay in combination with quantitative myelin image assay is highly valuable for rapidly *in vivo* identification of remyelination compounds and RORγt inhibitors.

2. Materials and methods

2.1. Zebrafish care and maintenance

Three lines of zebrafish were used in this study: wild-type AB line, motor neuron green-fluorescent-protein (GFP) transgenic zebrafish and neutrophil GFP transgenic zebrafish. Zebrafish were housed in a light and temperature controlled aquaculture facility with a standard 14: 10 h (h) light/dark photoperiod and fed with live brine shrimp twice daily and dry flake once a day. Four to five pairs of zebrafish were set up for natural mating every time. On average, 200–300 embryos were generated. Embryos were maintained at 28 °C in fish water (0.2% Instant Ocean Salt in deionized water, pH 6.9–7.2, conductivity 480–510 $mS.cm^{-1}$ and hardness 53.7–71.6 $mg.l^{-1}$ $CaCO_3$). The embryos were washed and staged at 6 and 24 hpf (hours post fertilization) (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). The zebrafish facility at Hunter Biotechnology, Inc. is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International (Zhou, Xu, Guo, & Li, 2015).

2.2. Chemicals and compounds

EB (lot #: H150104) and T4 (lot #: H150104) were purchased from Aladdin company, Shanghai, China. GSK805 and SR1001 were kindly provided by Professor Yong-Hui Wang at Fudan University, Shanghai, China. FluoroMyelin (lot #: F34651) was bought from Molecular Probes, USA. Mouse monoclonal antibody against sea urchin acetylated-tubulin (α-AT) (lot #: T6793) and mouse monoclonal anti-MBP antibody (lot #: SAB5300427) was bought from Sigma-Aldrich, Rhodamine RedTM-X-conjugated affiniPure goat anti-mouse IgG + IgM (H + L) (Lot #: 63076) from Jackson ImmunoResearch and DyLight 488 AffiniPure Goat Anti-Mouse IgG (H + L) from Emmett technology co. LTD. All other agents were bought from Sigma-Aldrich.

2.3. Determination of no observed adverse effect level (NOAEL)

To determine NOAEL of a testing compound, 5 dpf zebrafish were treated with a testing compound for 24 h and mortality and toxicity were recorded at the end of treatment. In the initial tests, five concentrations (0.1, 1, 10, 100, and 500 mg/L for soaking drugs) were used for each drug. If a NOAEL could not be found from the initial tests, additional concentrations within the range of 0.01–2000 mg/L were tested. The NOAEL of a test compound was defined as a maximum concentration or maximum dose that did not induce any visually observable adverse effect on zebrafish and was determined under a dissecting stereomicroscope by a well-trained zebrafish toxicologist (Yang et al., 2015; Zhou et al., 2015).

2.4. Zebrafish demyelination model development

To optimize EB treatment concentration and treatment time period, thirty 2 dpf zebrafish were distributed into 6-well plates (Nest Biotech., Shanghai, China) in 3 mL fresh fish water (McGrath & Li, 2008; Westerfield, 1999). Based on the pilot study, zebrafish were treated with 0.1 μM, 1 μM, 10 μM, 25 μM, 50 μM, 75 μM and 100 μM EB for 48 h and 72 h, respectively, to induce demyelination. After EB treatment, 10 zebrafish from each group were loaded into 96-well plates and the motility was evaluated by using a video-track motion detector (ViewPoint, France). All experiments consisted of 60 min containing 3 light and dark phases (10 min each). The total distance traveled by individual zebrafish was recorded automatically and motility change percentage was calculated (Huang et al., 2016). The resting zebrafish were subject to stain with a myelin-specific live fluorescent dye FluoroMyelin. Ten zebrafish from each group were randomly selected and images were acquired using a fluorescent microscope. Quantitative image analysis was performed using image-based morphometric analysis and demyelination percentage was calculated. Based on the qualitative and quantitative results of motility and myelination image assay, the optimal EB treatment concentration and treatment time period were selected.

2.5. Zebrafish demyelination model validation

2.5.1. Motility assay and FluoroMyelin staining

To test whether EB-induced demyelination-associated motility damage could be rescued, thyroxine (T4) (Vose et al., 2013) was selected for the validation of zebrafish demyelination model. Thirty 2 dpf AB strain zebrafish were distributed into 6-well plates in 3 mL fresh fish water. Zebrafish treated with EB for 72 h, followed by incubating with 0.1% DMSO for 24 h, were used as the demyelination model. After EB treatment for 72 h, EB was removed and T4 at concentrations of 1/9 NOAEL, 1/3 NOAEL and NOAEL was added for another 24 h treatment.

After T4 treatment, 10 zebrafish from each group were loaded into 96-well plates and the motility was evaluated by using the video-track motion detector as described above (Huang et al., 2016).

The resting zebrafish were subject to stain with FluoroMyelin (Monsma & Brown, 2012). Ten zebrafish from each group were randomly selected and images were acquired using a fluorescent microscope. Quantitative image analysis was performed using image-based morphometric analysis.

2.5.2. Whole mount anti-MBP immunostainging

T4 effect on MBP at a concentration of NOAEL was further confirmed by whole mount anti-MBP immunostainging. After treatment, zebrafish were fixed in 0.4% paraformaldehyde for 1 h and washed with 0.1 M phosphate buffered saline (PBS) for 2–3 h. The preparations were then permeabilized for 30 min in 4% triton-X 100 containing 2% bovine serum albumin (BSA) and 10% goat serum. Zebrafish were incubated for 48 h at 4 °C in the mouse monoclonal anti-MBP antibody at a dilution of 1:250. Zebrafish were then washed for 2–3 h in PBS and incubated in the secondary antibody for 4 h at room temperature. The secondary antiserum was goat anti-mouse IgG (H + L) coupled to DyLight 488 (1:1000). Preparations were then washed for 7 h in PBS-Tween. Ten zebrafish from each group were randomly selected and images were acquired under a stereo-fluorescent microscope (Rothschild, Francescatto, & Tombes, 2016).

2.6. Compound effect assessments

2.6.1. Dose-response assay of remyelination

Dose-response study was carried out to assess the remyelination effect of GSK805 and SR1001. Thirty 2 dpf AB strain zebrafish were distributed into 6-well plates in 3 mL fresh fish water. Zebrafish treatment with EB for 72 h then incubation with 0.1% DMSO for 24 h were used as the demyelination model. The positive control for this assay was 0.9 μM of T4. After treatment for 72 h, EB was removed and test compounds at their respective concentrations of 1/9 NOAEL, 1/3 NOAEL and NOAEL were added for a 24 h treatment. After treatment, quantitative myelin image assay was performed as described above. The remyelination effects of GSK805 and SR1001 were further confirmed by whole mount anti-MBP immunostainging.

2.6.2. Whole mount anti-acetylated tubulin immunostaining

In order to assess the effect of GSK805 and SR1001 on axon regeneration, whole mount anti-acetylated tubulin immunostaining was performed. After treatment, zebrafish were fixed, permeabilized, and incubated for 48 h at 4 °C with mouse monoclonal antibody against α-AT antibody at a dilution of 1:100. Zebrafish were then washed and incubated in Rhodamine RedTM-X-conjugated affiniPure goat anti-mouse IgG + IgM (H + L) antibody at a dilution of 1:800. After washing, 10 zebrafish from each group were randomly selected and images were acquired (Rothschild et al., 2016).

2.6.3. Promotion of peripheral motor neuron

Motor neuron transgenic zebrafish were used to assess the effect of GSK805 and SR1001 on motor neuron. After treatment, ten 2 dpf motor neuron transgenic strain zebrafish from each group were randomly selected and images were acquired using a fluorescent microscope. Quantitative image analysis was performed using image-based morphometric analysis (Zhou, Guo, Zhang, & Li, 2014).

2.6.4. Reduction of neutrophil infiltration

Zebrafish have established the primary lateral line system by 3 dpf. Addition of copper sulfate to the water rapidly destroys hair cells of the lateral line system by inducing oxidative stress followed by cell death (d'Alençon et al., 2010). Zebrafish neutrophil inflammation model was developed by directly delivering copper sulfate to neutrophil transgenic zebrafish (Yang et al., 2014, b). to assess the effect of GSK805 and SR1001 on inflammation. Thirty 3 dpf neutrophil transgenic zebrafish were distributed into 6-well plates in 3 mL fresh fish water. Zebrafish treated with 10 μM copper sulfate for 2 h. After pre-treatment of testing compounds at their respective concentrations of 1/9NOAEL, 1/3 NOAEL and NOAEL for 1 h, copper sulfate was added for another 2 h treatment. After treatment, ten zebrafish from each group were randomly selected, images were acquired and quantitative image analysis was performed.

2.6.5. Reduction of macrophage recruitment

Lipopolysaccharide (LPS), an endotoxin molecule, has been used to induce inflammatory responses (Yang et al., 2014, b). Thirty 3 dpf AB strain zebrafish were distributed into 6-well plates in 3 mL fresh fish water. Zebrafish intravenously injected with 0.5 mg/mL LPS for 2 h were used as the macrophage inflammation model (Yang et al., 2014, b). After GSK805 and SR1001 pre-treatment at their respective concentrations of 1/9NOAEL, 1/3 NOAEL and NOAEL for 1 h, LPS was injected for another 2 h treatment. After treatment, zebrafish were stained with 2.5 μg/mL neutral red for 16 h. and the effects of a tested compound on the inflammation were quantified based on the image analyses.

2.7. Statistical analysis

One-way ANOVA followed by the Dunnett's test was used to compare differences among groups. All statistical analyses were performed using the SPSS 16.0 software (SPSS, USA), and $p < .05$ was considered statistically significant. For quantitative analysis, all data were presented as mean ± SEM, and results were statistically compared between drug-treated and vehicle-treated zebrafish groups.

3. Results

3.1. Zebrafish demyelination model

To develop a zebrafish demyelination model for drug screening and efficacy assessment, the optimal concentration and treatment time period of the demyelination inducer EB were first determined based on the motility assay (Fig. 1a) and FluoroMyelin staining (Fig. 1b). Zebrafish at 2 dpf were treated with 0.1, 1, 10, 25, 50, 75 and 100 μM EB for 24 h to 48 h or treated with 0.1 μM EB for 72 h did not result in the motility distance decrease and demyelination ($p > .05$, data not shown). As shown in Fig. 1c, Various degrees of motility distance reduction and demyelination were observed in all zebrafish treated with 0.1, 1, 10, 25, 50, 75 and 100 μM of EB for 72 h. Dose-dependent motility distance decrease and demyelination were found, of which treatment with 75 μM of EB for 72 h induced motility distance reduction and demyelination in 100% zebrafish without visually observable other tissue and organ toxic phenotypes (Chen et al., 2014) and the distance recovery and remyelination was markedly observed when the zebrafish were treated with a well-known remyelination agent T4 (Fig. 1a, Fig. 1b). Treatment with 100 μM of EB for 72 h induced significantly reduced motility and demyelination ($p < .05$ and $p < .01$) that could not be rescued by T4 treatment ($p > .05$, data not shown). The motility distance reduction was 11–70% and demyelination was 2–79% for zebrafish treated with EB at concentrations from 0.1 μM to 100 μM (Fig. 1c). Based on these results, zebrafish treated with 75 μM of EB for 72 h was selected as the optimum treatment concentration and treatment period for subsequent demyelination model development.

To determine whether the zebrafish response to demyelination therapeutics is similar to that of mammalian model systems, we treated zebrafish with 75 μM of EB for 72 h, then EB was removed and T4 at concentrations of 1/9 NOAEL, 1/3 NOAEL and NOAEL was added for another 24 h treatment. As expected, after a 24 h treatment, T4 at concentrations of NOAEL significantly increased the motility distance (Fig. 1a) and myelin staining intensity (Fig. 1b), implying that

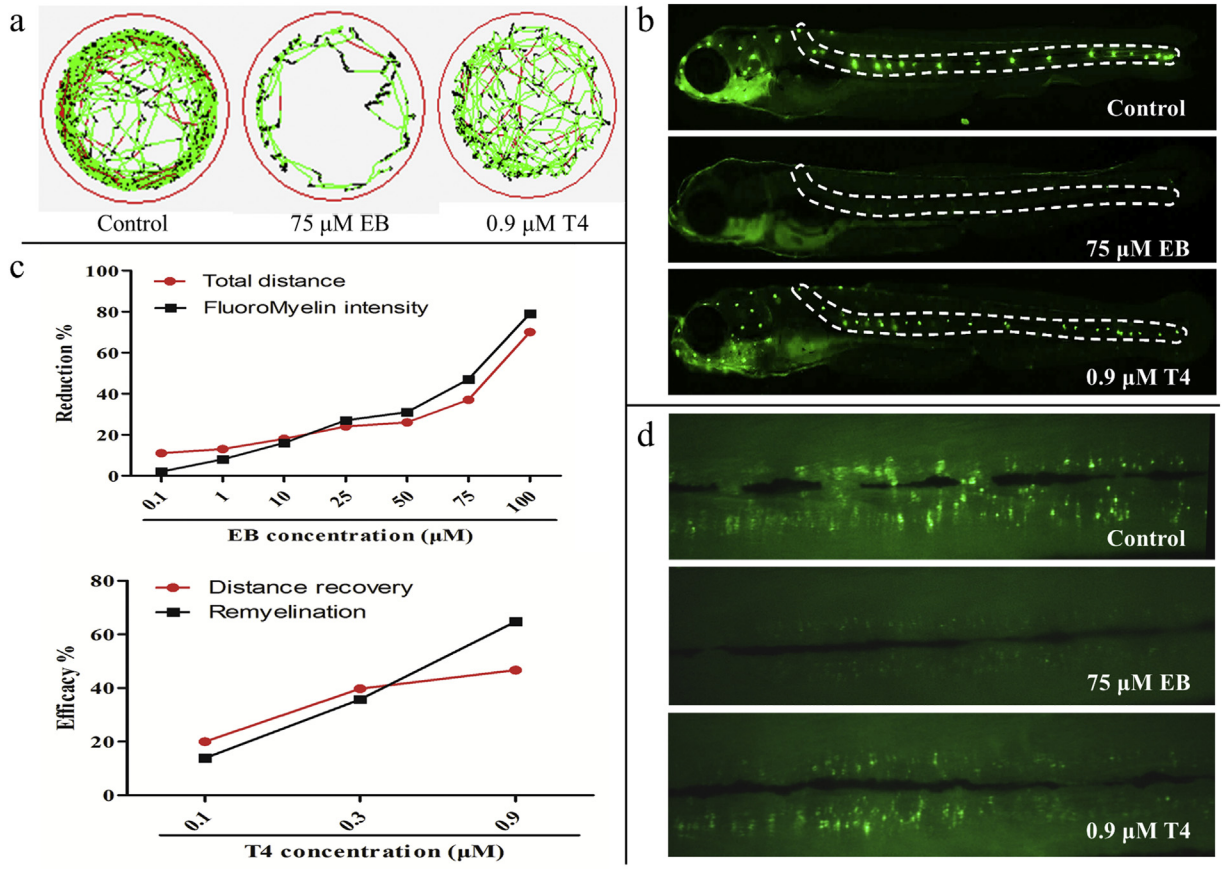

Fig. 1. Development and validation of zebrafish demyelination model. Motility was significantly reduced in 6 dpf zebrafish treated with 75 μM EB for 72 h that could be rescued by treatment with 0.8 μM T4 for 24 h (a); Severely demyelination was observed in 6 dpf zebrafish treated with EB and stained with FluoroMyelin, and the demyelination could be markedly recovered after T4 treatment (b); EB induced dose-dependent motility reduction in zebrafish that could be rescued by T4 treatment in a dose-dependent manner (c); and.
Treatment with 75 μM EB for 72 h resulted in zebrafish MBP loss, and MBP regeneration was observed after T4 treatment, measured by whole mount anti-MBP immunostainging (d).

remyelination occurred in the demyelinated zebrafish. The motility distance recovery was 20%, 40%, 47% and remyelination was 14%, 36%, 65% for the demyelinated zebrafish treated with T4 at concentrations of 1/9 NOAEL($p > .05$), 1/3 NOAEL($p < .01$) and NOAEL ($p < .001$), respectively (Fig. 1c). The effect of T4 on the remyelination at a concentration of NOAEL was further confirmed by whole mount anti-MBP immunostainging (Fig. 1d).

3.2. Effect of RORγt inhibitor compounds

3.2.1. Dose-response of remyelination

Subsequent dose-response of remyelination study was carried out to assess the remyelination effect of *RORγt inhibitors* GSK805 and SR1001. We found that GSK805 and SR1001 promoted remyelination *in vivo* in the demyelinated zebrafish in a dose-dependent manner. Demyelination was successfully induced in zebrafish treated with EB only, and remyelination was markedly observed in the demyelinated zebrafish treated with GSK805 and SR1001. The remyelination recovery was 25%, 54%, 61% for GSK805 and 77%, 63%, 30% for SR1001 at concentrations of 1/9 NOAEL, 1/3 NOAEL and NOAEL (Fig. 2d, Table 1) ($p < .05$, $p < .01$, and $p < .001$).

3.2.2. MBP, axon and motor neuron promotion

As shown in Fig. 2a, b and c, MBP, axon and motor neuron were damaged in the zebrafish treated with EB, and significantly regeneration of MBP, axon and motor neuron were observed after the zebrafish treated with GSK805 and SR1001. The regeneration of MBP was 6%, 29%, 45% for GSK805 and 50%, 32%, 19% for SR1001 at concentrations of 1/9 NOAEL, 1/3 NOAEL and NOAEL, respectively; the regeneration of axon was 27%, 38%, 49% for GSK805 and 44%, 27%, 14% for SR1001 at concentrations of 1/9 NOAEL, 1/3 NOAEL and NOAEL, respectively; and the regeneration of motor neuron was 19%, 37%, 43% for GSK805 and 45%, 23%, 7% for SR1001 at concentrations of 1/9 NOAEL, 1/3 NOAEL and NOAEL, respectively (Fig. 2d, Table 1) ($p < .05$, $p < .01$, or $p < .001$).

3.2.3. Anti-inflammatory effect

As shown in Fig. 3a, the control zebrafish showed the normal distribution of fluorescent neutrophil cells, sparsely localized in the ventral trunk and tail; whereas the zebrafish exposed to copper sulphate showed a general dispersal of fluorescent neutrophils, suggestive of active migration from their initial location to a few clusters along the horizontal midline of the trunk and tail; Quantification data showed that there was a significant difference ($p < .05$) in the number of neutrophils localized to the lateral line in copper-treated zebrafish as compared to untreated control. GSK805 and SR1001 exhibited statistically significant inhibition of neutrophil infiltration at most tested concentrations ($p < .05$, and $p < .001$) (Table 1). The anti-inflammatory effect on neutrophils was 8%, 25%, 21% for GSK805 and 15%, 12%, 2% for SR1001 at concentrations of 1/9 NOAEL, 1/3 NOAEL and NOAEL, respectively.

In the LPS-injected zebrafish, significantly increased the recruitment of macrophages to the vein was shown, as compared with PBS-injected control (Fig. 3b) ($p < .05$). However, the treatment with GSK805 and

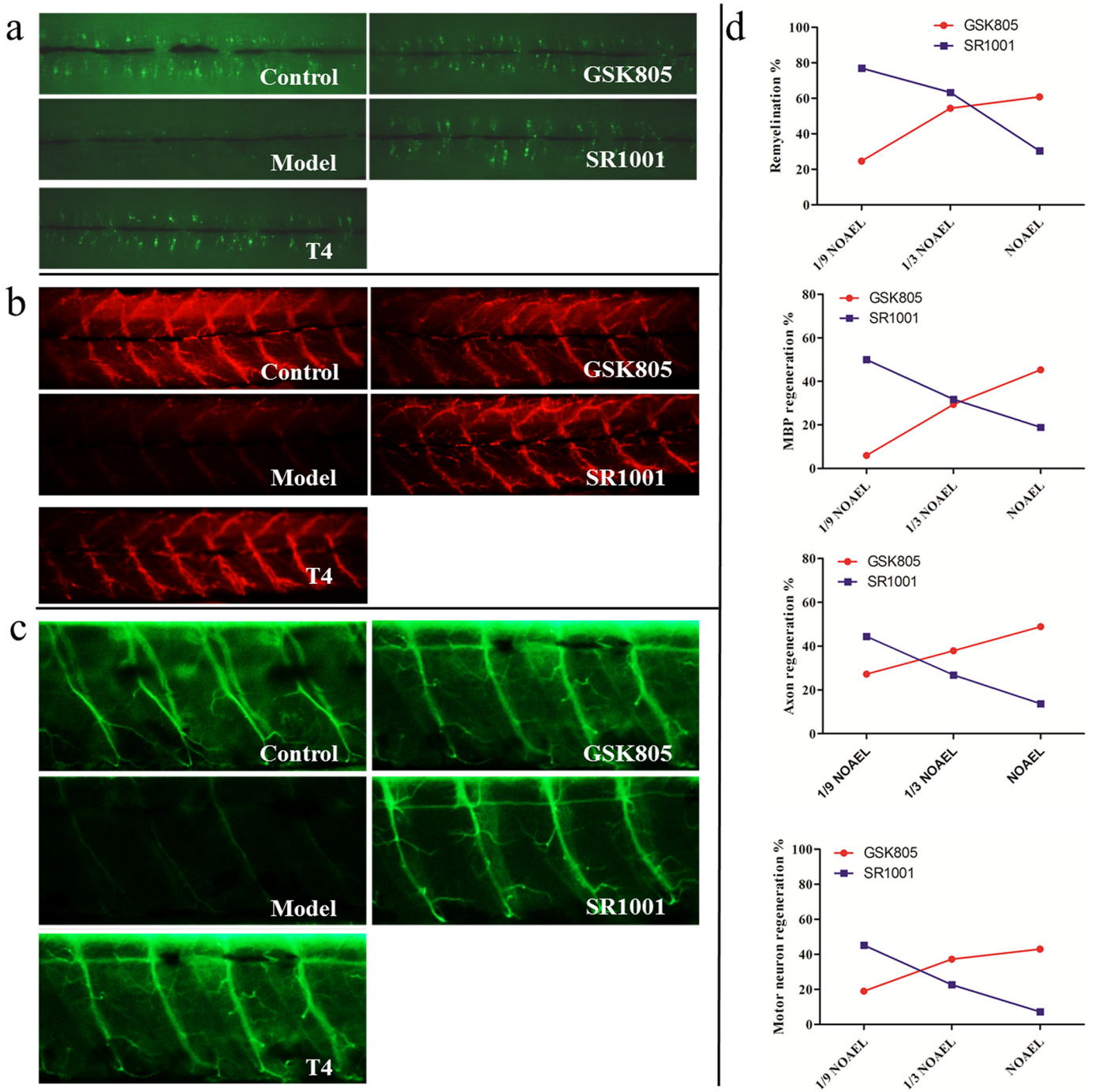

Fig. 2. GSK805 and SR1001 promote myelin, MBP, axon and motor regeneration. MBP loss (a), axon (b) and motor neuron (c) damage were found in zebrafish treated with EB (Model), that could be rescued by treatment with GSK805 and SR1001. Quantitative analysis of remyelination MBP and axon and motor regeneration (d).

SR1001 had significantly reduced macrophage recruitment ($p < .05$). Treatment with a positive control drug, indomethacin (80 μM), caused a similar reduction in macrophage recruitment. As indicated in Fig. 3b and Table 1, the anti-inflammatory effect on macrophages was 27%, 30%, 40% for GSK805 and 38%, 42%, 55% for SR1001 at concentrations of 1/9 NOAEL, 1/3 NOAEL and NOAEL, respectively.

4. Discussion

In this study, we have optimized EB treatment concentration and treatment time period to develop a live zebrafish demyelination model with quantitative motility assay and myelin image analysis. Zebrafish at 2 dpf were treated with EB at a concentration of 75 μM for a time period of 72 h were determined as the optimum conditions for the zebrafish demyelination model development. The total distance traveled by individual zebrafish was recorded automatically by using a video-track motion detector (Huang et al., 2016) and quantitative myelin image analysis was performed using FluoroMyelin staining (Monsma and Brown, 2012) in the zebrafish spinal cord. We found that larval zebrafish treated with EB under these optimum conditions developed demyelination in the spinal cord with movement dysfunction, similar to the pathophysiology observed in human demyelination patients (Paz

Table 1
Efficacy of tested compounds on demyelinating and inflammatory zebrafish.

Group	Concentration (μM)	Efficacy %					
		Myelin	MBP	Axon	Motor neuron	Neutrophil	Macrophage
T4	0.8	51***	38***	29*	37*	–	–
Indomethacin	80	–	–	–	–	85***	89**
GSK805	1/9 NOAEL	25	6	27*	19	8*	27
	1/3 NOAEL	54***	29***	38***	37*	25***	30*
	NOAEL	61***	45***	49***	43*	21***	40*
SR1001	1/9 NOAEL	77***	50***	44**	45*	15***	38
	1/3 NOAEL	63***	32***	27*	23*	12***	42*
	NOAEL	30	19**	14	7	2	55*

* $p < .05$
** $p < .01$
*** $p < .001$.

Soldan and Rodriguez, 2002), indicating that EB could induce demyelination in larval zebrafish. This zebrafish demyelination model is stable and highly reproducible with CV values of intra-experiments, inter-experiments and daily-to-daily variations ≤25% (data not shown). To test whether EB-induced demyelination-associated motility damage and demyelination could be rescued, T4 was chosen (Vose et al., 2013). As expected, T4 significantly improved motility and myelin staining intensity of the demyelinated zebrafish as compared with untreated demyelination model zebrafish, implying remyelination was going on in the demyelinated zebrafish treated with T4. Whole mount immunostainging also suggested that T4 could promote MBP regeneration, further supporting that demyelination-associated motility assay in combination with FluoroMyelin staining in the EB-induced demyelinated zebrafish is a reliable array of methods for *in vivo* remyelinating drug assessment.

In a comparison between the new discoveries made in this report and the existing models, lysophosphatidylcholine (LPC) (Burrows et al., 2019) has been used to induce demyelination in adult zebrafish at 4–7 months of age (Münzel et al., 2014), as has been performed in murine models. Unfortunately, this zebrafish demylination model requires surgery and a relatively long time for recovery making its applicability for drug screening limited. A genetic inducible demyelination model has also been generated by the ablation of oligodendrocytes and thus removal of myelin. This was generated using a nitroreductase/metronidazole system expressed under the control of the *mbp* and *sox10* promoter (Chung et al., 2013). This system allows the inducible ablation of the entire oligodendrocyte population and CNS demyelination occurs within 48 h of metronidazole administration. This genetic model

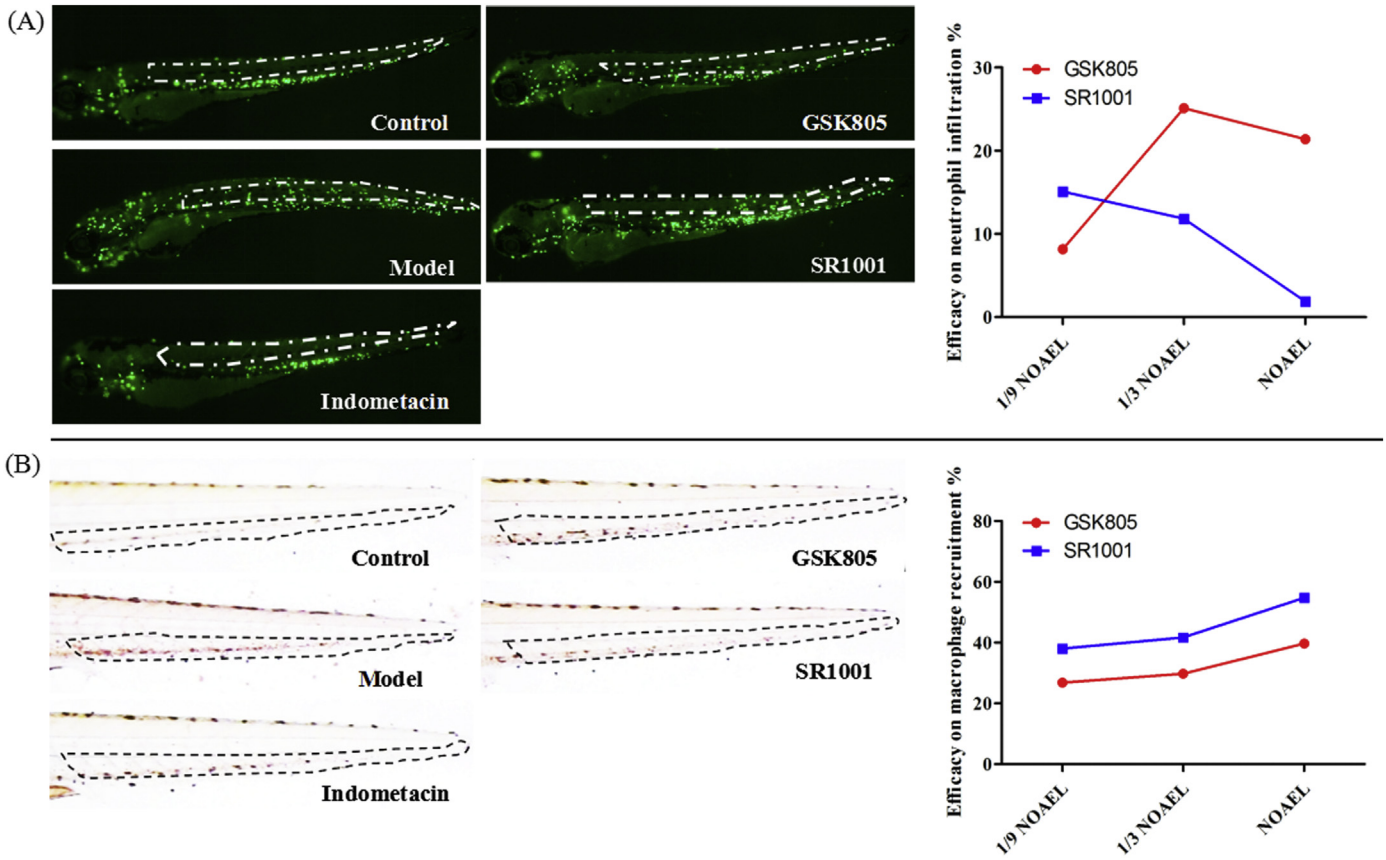

Fig. 3. Anti-inflammatory efficacy of GSK805 and SR1001 in inflammatory zebrafish models. Qualitative and quantitative analysis of GSK805 and SR1001 on neutrophil infiltration (a); Qualitative and quantitative analysis of GSK805 and SR1001 on macrophages recruitment (b).

is advantageous in screening for therapeutics that promote remyelination, but questions remain over its applicability as a disease model, especially as complete ablation of the oligodendrocyte population is not a common MS mechanism (Burrows et al., 2019). More recently, an experimental autoimmune encephalomyelitis (EAE) zebrafish model has been developed for developing rapid screens *in vivo* by subcutaneously injecting with $MOG_{35–55}$ near the spinal vertebrae (Kulkarni et al., 2017). The advantage of the zebrafish as an EAE model is how rapidly the clinical symptoms are induced with a detectable disease phenotype at 3 days post immunisation and significant impairment at 7 days post fertilization. Further work is required to validate and understand this model as MOG has not currently been identified in the zebrafish genome, so it is imperative to identify what is driving the autoimmunity (Burrows et al., 2019).

To validate the demyelinated zebrafish model for future RORγt inhibitor screen and evaluation, two RORγt lead inhibitors GSK805 (Wang et al., 2015; Withers et al., 2016; Xiao et al., 2014) and SR1001 (Solt et al., 2011) were selected for dose-response assessment of the remyelination and MBP promotion. We found that GSK805 and SR1001 promoted remyelination and MBP regeneration *in vivo* in a dose-dependent manner in this zebrafish model. In order to assess the effects of GSK805 and SR1001 on axon and motor neuron promotion, whole mount anti-acetylated tubulin immunostaining was performed and motor neuron transgenic zebrafish was used (Rothschild et al., 2016). We found that GSK805 and SR1001 could rescue EB-induced axon and motor neuron damage in the demyeliated zebrafish. We further assessed the inflammatory effect of GSK805 and SR1001 on two zebrafish inflammation models induced by directly delivering copper sulfate to neutrophil transgenic zebrafish and intravenously injecting LPS (Yang et al., 2014, b), respectively. We demonstrated that GSK805 and SR1001 exhibited significant inhibition of neutrophil infiltration and macrophages recruitment. These results suggest that EB-induced zebrafish demyelination model is probably suitable for screening and assessing RORγt inhibitors.

The zebrafish as a model organism offer opportunities for rapid *in vivo* drug discovery and development. Larval zebrafish demyelination model developed in this study was a live and pathophysiology-associated whole animal assay. This model is a useful tool for whole animal demyelination studies and for screening remyelination compounds and RORγt inhibitors. The use of zebrafish as an alternative animal model for remyelination agents and RORγt inhibitors screening could save time, decrease costs, and speed up drug discovery process. Extended studies are in progress to define histopathology, biochemical and gene expression profiles in this zebrafish model. We are also further validating this model using more FDA-approved MS therapeutics and newly synthesized RORγt inhibitors to develop this model into a higher throughput assay or even an automatic assay system. We believe that the power of the zebrafish models and assays could be better utilized to advance the basic and translational research and drug discovery and development.

5. Conclusions

This study developed and validated a zebrafish demyelination model that could be used for *in vivo* screening and efficacy assessment of remyelination compounds and RORγt inhibitors. This conventional zebrafish demyelination model could speed up therapeutic drug research and development for autoimmune diseases including multiple sclerosis.

Authors' contributions

Xiao-Yu Zhu, Yonghui Wang, Lei Wang and Chun-Qi Li designed the studies; Xiao-Yu Zhu, Sheng-Ya Guo and Bo Xia performed the experiments; Xiao-Yu Zhu and Sheng-Ya Guo analyzed the data; Xiao-Yu Zhu and Chun-Qi Li wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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