Ponatinib-induced ischemic stroke in larval zebrafish for drug screening

Xiao-Yu Zhu, Bo Xia, Ting Ye, Ming-Zhu Dai, Hua Yang, Chun-Qi Li, Ping Li

PII: S0014-2999(20)30384-8

DOI: https://doi.org/10.1016/j.ejphar.2020.173292

Reference: EJP 173292

To appear in: *European Journal of Pharmacology*

Received Date: 14 October 2019

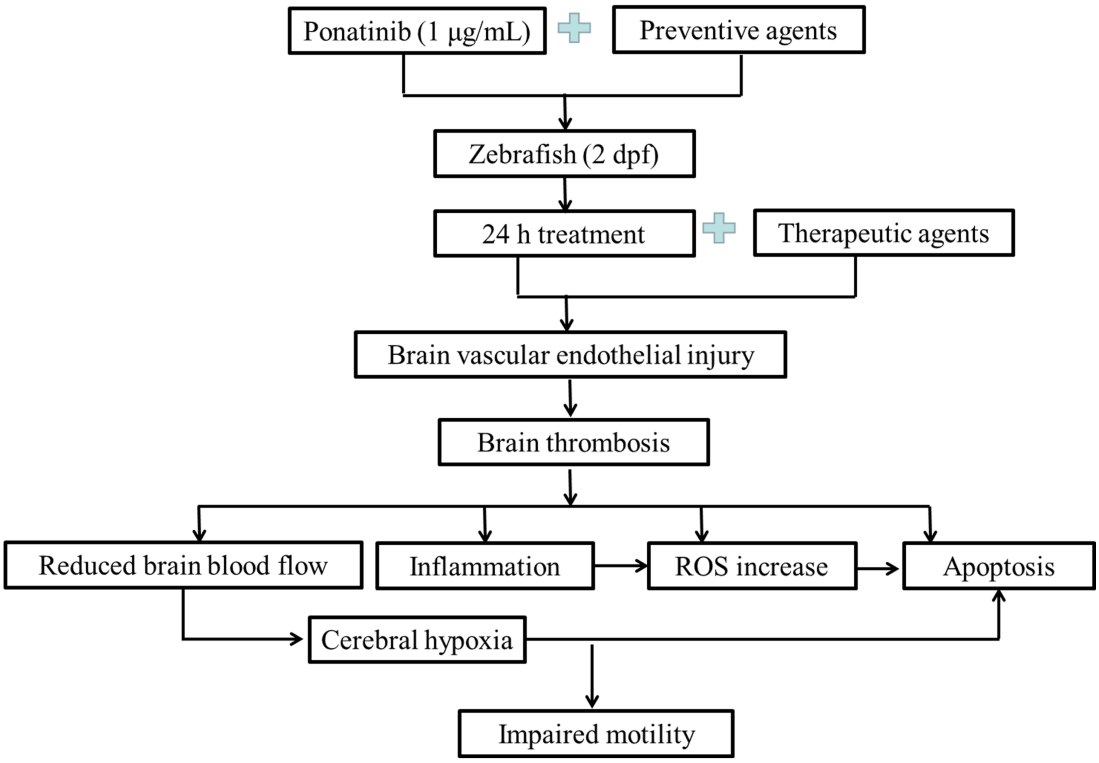
Revised Date: 16 May 2020

Accepted Date: 17 June 2020

Please cite this article as: Zhu, X.-Y., Xia, B., Ye, T., Dai, M.-Z., Yang, H., Li, C.-Q., Li, P., Ponatinib-induced ischemic stroke in larval zebrafish for drug screening, *European Journal of Pharmacology* (2020), doi: https://doi.org/10.1016/j.ejphar.2020.173292.

Credit Author Statement

Ping Li, Hua Yang and Chun-Qi Li designed the research; Xiao-Yu Zhu, Bo Xia, and Ming-Zhu Dai performed the research; Xiao-Yu Zhu, Bo Xia and Ming-Zhu Dai analyzed the data; Xiao-Yu Zhu and Chun-Qi Li wrote the paper. We thank Rick Li at Boston Latin School, Massachusetts, USA for editing this manuscript.


Ponatinib-induced ischemic stroke in zebrafish
Ponatinib (1 μg/mL)
Preventive agents
Zebrafish (2 dpf)
24 h treatment
Therapeutic agents
Brain vascular endothelial injury
Brain thrombosis
Reduced brain blood flow
Inflammation
ROS increase
Apoptosis
Cerebral hypoxia
Impaired motility

Ponatinib-induced ischemic stroke in larval zebrafish for drug screening

Xiao-Yu Zhu [a, b], Bo Xia [b], Ting Ye [b], Ming-Zhu Dai [b], Hua Yang [a], Chun-Qi Li [b*], Ping Li [a*]

[a] *State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, Jiangsu Province 210009, P. R. China*

[b] *Hunter Biotechnology, Inc, F1A, building 5, No. 88 Jiangling Road, Binjiang Zone, Hangzhou City, Zhejiang Province 310051, P. R. China*

** Corresponding author: State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing city, Jiangsu Province 210009, China. Tel: + 862583271379; fax:+ 862583271379. E-mail address: liping2004@126.com (P. Li).*

** Co-Corresponding author: Hunter Biotechnology, Inc., F1A, building 5, No. 88 Jiangling Road, Binjiang, Hangzhou, Zhejiang Province 311231, P. R., China. Tel: + 86 571 83782172; E-mail: jackli@zhunter.com (C. Q. Li).*

ABSTRACT

Conventional mammalian ischemic stroke models for drug screening are technically challenging, laborious and time-consuming. In this study, using Ponatinib as an inducer, we developed and characterized a zebrafish ischemic stroke model. This zebrafish ischemic stroke had the cerebral vascular endothelial injury, thrombosis, reduced blood flow, inflammation and apoptosis as well as the reduced motility. The zebrafish ischemic stroke model was validated with 6 known human therapeutic drugs of ischemic stroke (Aspirin, Clopidogrel, Naoxintong capsules, Edaravone, Xingnaojing injection, Shuxuening injection). The mRNA levels of the neovascularization-related gene (*vegfaa*) and vascular endothelial growth factor receptor gene (*VEGFR*), neurodevelopment related genes (*mbp* and *α1-tubulin*), brain-derived neurotrophic factor (*BDNF*) and glial cell derived neurotrophic factor (*GDNF*) were significantly downregulated; whereas apoptosis-related genes (*caspase-3*, *caspase-7*, *caspase-9* and *bax/bcl-2*), and inflammatory factor genes (*IL-1β*, *IL-6*, *IL-10*, *TNF-α* and *NF-κB*) were remarkably upregulated in the model. These results suggest that the pathophysiology of Ponatinib-induced zebrafish ischemic stroke is similar to that of human ischemic stroke patients and this whole animal model could be used to study the complex cellular and molecular pathogenesis of ischemic stroke and to rapidly identify therapeutic agents.

Keywords: Zebrafish; Ponatinib; Ischemic stroke; Animal model; Drug screening

1. Introduction

According to the World Health Organization, ischemic stroke has always been the second leading cause of death worldwide and a major contributor to disability (Pandian et al., 2018; Chandra et al., 2017; Li et al., 2017). Ischemic stroke occurs when a vessel supplying blood to the brain is obstructed and it accounts for about 87 % of all strokes (Pandian et al., 2018; Christoph et al., 2015). The success of preclinical stroke research in developing new therapeutics might rely-at least in part-on the selection of the appropriate stroke model to use (Christoph et al., 2015).

Numerous studies have demonstrated that the complex situation of ischemic stroke cannot be modeled in an *in vitro* system with single cells or pieces of brain tissue with the absence of intact blood vessels and blood flow as well as the lack of infiltration of leukocytes (Sommer et al., 2017). Mimicking all aspects of human stroke in one animal model is not feasible because ischemic stroke in humans is a heterogeneous disorder with a complex pathophysiology. While animal models have helped us better understand the pathophysiology of ischemic brain damage, the use of animal models has very limited translational success in ischemic stroke therapies (Papoutsoglou et al., 2012). There is a need to develop alternative animal models for understanding the complex pathophysiology of ischemic stroke and, particularly for the purpose of drug screening (Papoutsoglou et al., 2012).

Alternative models such as mechanical occlusions of middle cerebral artery are often technically challenging: the use of invasive procedures as well as unstable effects from treatments are major limitations (Traystman et al., 2003). Although these limitations do not make the models less important or valuable, studies performed on alternative models are necessary to provide more objective results. Zebrafish could be useful in investigating ischemic brain damage due to its transparency, easy observation (Papoutsoglou et al., 2012; Yu et al., 2016) and the requirement for optomotor response while swimming (Springer et al., 1977), which is helpful for qualitative and quantitative evaluation of the therapeutic effect of drugs on sequelae of stroke. Several initial studies have shown that adult zebrafish can be induced to

develop hypoxic-ischemic cerebral damage (Yu et al., 2011; Yu et al., 2013; Braga et al., 2013). Photothrombosis could also induce brain injury in adult zebrafish although the zebrafish were usually likely to die in a short time due to its end-arterial occlusive nature (Yu et al., 2016).

Ponatinib was initially approved as a breakthrough multi-targeted tyrosine-kinase inhibitor for the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia (Sadovnik et al., 2014; Zirm et al., 2012). Ponatinib was withdrawn from the market (latter remarked with a black box warning of the vascular toxicity and thrombus formation et al.) based on emerging data indicating approximately 48% adverse vascular events and 24% arterial and venous thrombosis leading to myocardial infarction, stroke, limb ischemia, and vascular stenosis (Prasad and Mailankody, 2014). Later it was confirmed that Ponatinib affected angiogenesis on the intersegmental and subintestinal vessels in the zebrafish and in human umbilical vein endothelial cells via blocking VEGFR signaling pathway and damaging vascular endothelial cells (Ai et al., 2018). Hamadi et al reported that Ponatinib increased *ex vivo* thrombus formation and this effect was partially related to calcium channel activation and TxA2 generation (Hamadi et al., 2019).

In the current report, we have developed and validated a larval zebrafish ischemic stroke model induced by Ponatinib under an optimized exposure concentration and treatment period. The pathophysiology of this ischemic stroke animal model demonstrated high similarities with human ischemic stroke and could be used for the disease study and for the drug screening.

2. Materials and methods

2.1. Zebrafish care and maintenance

Two lines of zebrafish were used in this study: Tg (MPO::GFP) transgenic zebrafish for inflammation analyses and Albino strain zebrafish for all other studies. An early study has confirmed that Albino strain zebrafish are mutant in *slc45a2*

(Tsetskhladze et al, 2016). Zebrafish care and maintenance procedures were reported in our previous publications (Zhu et al., 2016). Briefly, the adult zebrafish were housed in a temperature-controlled and light-controlled aquaculture facility with a standard 14: 10 h light/dark photoperiod and fed with live brine shrimp and dry flakes. The zebrafish were paired for natural mating every day and the 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 $CaCO_3$) and washed and staged at 6 and 24 hpf (hours post fertilization). This study was approved by the IACUC (Institutional Animal Care and Use Committee) at Hunter Biotechnology, Inc. and the IACUC approval number was 001458. The zebrafish facility and the laboratory at Hunter Biotechnology, Inc. are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International, and by the China National Accreditation Service for Conformity Assessment (CNAS).

2.2. Chemicals

Ponatinib (lot #: HY-12047) was bought from MedChemExpress (St. Monmouth Junction, USA). O-dianisidine (lot #: 119-90-4), Aspirin (lot #: G1220028), Clopidogrel (lot #: R-4411-3G), Phorbol-12-myristate-13-acetate (PFA, lot #: 16561-29-8) and Edaravone (lot #: K1820098) were bought from Sigma-Aldrich (St. Louis, USA). Naoxintong capsules (lot #: 1802100) were purchased from Shanxi Buchang Pharmaceutical Co., Ltd (Shanxi, China). Xingnaojing injection (lot #: 180704) was purchased from Wuxi Jimin Credible Shanhe Pharmaceutical Co., Ltd (Jiangsu, China). Shuxuening injection (lot #: 1705271) was bought from Shanxi Zhengdong Taisheng Pharmaceutical Co., Ltd (Shanxi, China). Drug stock solutions were prepared in either 100% dimethyl sulfoxide (DMSO) or 0.9% sodium chloride, and serial dilutions were made before each experiment. Zebrafish treated with 0.1% DMSO or 0.9% sodium chloride served as a vehicle control. Untreated zebrafish were used to confirm that the vehicle solvents did not have an adverse effect on the zebrafish.

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

Two days post fertilization (dpf) zebrafish were treated with a testing drug for 24 h and mortality and toxicity were recorded at the end of treatment. In the initial tests, 5 concentrations (0.1, 1, 10, 100, and 500 mg/L for soaking drugs) or doses (0.1, 1, 10, 100, and 500 ng/zebrafish for microinjected drugs) were used for each drug. If a NOAEL could not be found from these initial tests, additional concentrations or doses within the range of 0.01–2000 mg/L or 0.01–2000 ng/zebrafish were tested. The NOAEL of a test drug was defined as a maximum concentration or maximum dose that did not induce any observable adverse effect on zebrafish and was determined under a dissecting stereomicroscope by two qualified zebrafish experts (Zhu et al., 2016).

2.4. Circulation microinjection

The zebrafish were anesthetized with 0.03% tricaine (Sigma, USA) and loaded on a customized microplate designed specifically for microinjection. Two injectable drugs (Shuxuening injection and Xingnaojing injection) were diluted in 0.9% sodium chloride at proper concentrations and loaded into a pulled glass capillary (World Precision Instruments, USA) that was drawn on an electrode puller (NARISHIGE, Japan) and then trimmed to form a needle with a resulting internal diameter of approximately 15 micron and the outer diameter of approximately 18 micron. The microneedle was attached to an air driven Cell Tram (NARISHIGE, Japan). The tip of the needle was inserted into the circulation of zebrafish under a dissecting stereomicroscope and the pulse time was controlled to deliver 10 nL of the drug solution into the circulation through the glass capillary (Zhu et al., 2016). Zebrafish injected with 10 nL 0.9% sodium chloride served as a vehicle control and untreated zebrafish were used to confirm that the vehicle solvent did not have an adverse effect on the zebrafish.

2.5. Zebrafish ischemic stroke model development

2.5.1. Ponatinib concentration and treatment period optimization

The zebrafish exhibit functional platelets and coagulation factors by 36 hpf

(Weyand et al., 2014; Gregory et al., 2002; Khandekar et al., 2012) and therefore we chose 2 dpf zebrafish as an appropriate stage to start Ponatinib treatment for the cerebral ischemia model development. To optimize Ponatinib treatment concentration and treatment time period, thirty zebrafish at 2 dpf were distributed into 6-well plates (Nest Biotech., Shanghai, China) in 3 ml fresh fish water. Zebrafish were treated with 0.3 μg/ml, 1 μg/ml and 3 μg/ml Ponatinib for 12 h, 24 h and 48 h, respectively, to induce cerebral thrombosis. The highest tested concentration (3 μg/ml) used in this investigation was the maximum non-lethal concentration (MNLC) of Ponatinib in the zebrafish identified in the pilot study. Zebrafish treated with 0.1% DMSO served as a vehicle control. Untreated zebrafish were used to confirm vehicle solvent did not have adverse effect on zebrafish.

2.5.2. Cerebral thrombosis assessment

To measure the cerebral thrombosis induced by Ponatinib, zebrafish were stained with o-dianisidine using a method reported and patented previously by our group (Zhu et al., 2016; China patent number: 201110126427.2) to quantify the thrombus in the head area of zebrafish. After Ponatinib treatment, zebrafish from each group without fixture were incubated in 0.6 mg/ml o-dianisidine staining solution (Sigma, USA) with 10 mM sodium acetate and 4% ethanol for 15 min in the dark at 28°C and then washed for 3 times with 100% DMSO. Thiry zebrafish from each group were observed and photographed under a stereomicroscope (Nikon, SMZ645, Tokyo, Japan) to count the number of those zebrafish with the cerebral thrombosis and calculate the cerebral thrombus incidence. Quantitative image analyses were performed by measuring the area of cerebral thrombosis using NIS-Elements D3.10 image analysis software (Nikon, Japan) and the data were expressed as mean ± S.E.M.

2.5.3 Histopathological examination

To confirm Ponatinib-induced cerebral thrombosis, zebrafish histopathology was performed. After treatment, the zebrafish were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline for 4 h at 4℃, dehydrated in graded series of ethanol

solutions before paraffin embedding. The embedded zebrafish were longitudinally sectioned at 5 μm and stained with hematoxylin and eosin (H & E). Thirty zebrafish were used for each group. Histological images were obtained using a histological microscope (Leica, German) and pathological diagnosis was completed by a certified pathologist.

2.5.4. Brain blood flow measurement

Zebralab Blood Flow System (Viewpoint, France) was used to record the zebrafish brain blood flow videos for 10 zebrafish per group and the videos were analyzed by the ZebraBlood™ software (v1.3.2, ViewPoint, Lyon, France), which works by detecting changes in pixel density and combining them with vessel diameter to generate a flow rate in nL/s for every frame (Parker et al., 2014). The brain blood flow (O) was quantified based on the data derived from the recored videos as reported before (Zhu et al., 2018). The relative blood flow was calculated based on the following formula: The relative blood flow (%)= ($O_{Ponatinib}/O_{Control}$)×100%.

2.5.5. Cerebral vascular angiography

Cerebral vascular angiography was performed through injecting 0.02 micron red fluorospheres (Molecular Probes, Eugene, OR) into zebrafish brain artery (Craig et al., 2012). Prior to injection 2% solution of bovine serum albumin (BSA) was prepared in fish water, followed by filter sterilization. Fluorospheres were diluted at a ratio of 1:1 in BSA solution to a total volume of 100 μL. The larval zebrafish were anesthetized with Tricane and laid on their sides on an agarose ramp. After loading beads from the back of a sharp thin pulled capillary, fluorospheres were injected into the brain herringbone suture with the picopump using approximately 80 msec as the pulse time.

2.5.6. Motility impairment assessment

At the end of treatment, 10 zebrafish from each group were loaded into 96-well plates, 1 fish per well, The zebrafish were acclimated in the 96-well plate at 28 ℃ for 10 min before the motility recording. All experiments consisted of 60 min containing 3 cycles of a light/dark phase (10 min light and 10min dark each). Total distance and speed of the zebrafish movement to light-dark and dark-light cycles were recorded

and analyzed by viewpoint behavior analyzer (Zebralab V3, ViewPoint Life Sciences Co., Ltd.) (Huang et al., 2016). The reduced percentage rate of total movement distance (D) in the zebrafish treated with 1 μg/ml Ponatinib was calculated based on the following formula: the reduced percentage rate of total distance = $(1-D_{Ponatinib}/D_{Control})\times100\%$.

2.6. Drug effects on zebrafish ischemic stroke

Six known human ischemic stroke therapeutic drugs (Aspirin, Clopidogrel, Naoxintong capsules, Edaravone, Xingnaojing injection, Shuxuening injection) were selected for assessing drug effects on the zebrafish stroke. Thirty 2 dpf Albino zebrafish were distributed into 6-well plates in 3 ml fresh fish water. Zebrafish were cotreated with 1 μg/ml Ponatinib and a testing drug for 24 h at a serial concentrations or dosages as indicated in Table 1. Zebrafish treated with 1 μg/ml Ponatinib for 24 h were used as the ischemic stroke model. Zebrafish treated with 0.1% DMSO or 0.9% sodium chloride were used as vehicle controls. Untreated control zebrafish group was also included. After treatment, zebrafish cerebral thrombosis was quantified using a method as described above and the cerebral thrombosis incidence (I) was counted. Quantitative image analysis was performed by measuring the area (A) of cerebral thrombosis using NIS-Elements D3.10 image analysis software (Nikon, Japan) and the relative area of cerebral thrombosis of a testing drug was calculated based on the following formula: the relative area of cerebral thrombosis (%) $=A_{Drug}/A_{Model}\times100\%$.

Urokinase, known as urokinase-type plasminogen activator (uPA) (Merino et al., 2018), was selected for assessing its therapeutic effect on the zebrafish stroke. Plasminogen is an inactive form (zymogen) of the serine protease plasmin. Activation of plasmin triggers a proteolytic cascade that, depending on the physiological environment, participates in thrombolysis or extracellular matrix degradation (Degryse & Bernard, 2011). Thirty Albino zebrafish at 2 dpf were distributed into six-well plates in 3 ml fresh fish water. Zebrafish were first treated with 1 μg/ml Ponatinib for 24 h, followed by treatment with 100 μg/ml urokinase for another 24 h. Zebrafish treated with 1 μg/ml Ponatinib for 24 h were used as thrombosis model.

After treatment, zebrafish thrombus was quantified as above.

2.7. Mechanisms of Ponatinib-induced ischemic stroke

2.7.1. Nitric oxide assay

The nitric oxide (NO) levels in Ponatinib-treated zebrafish were measured using a NO specific fluorescent probe dye, diaminofluorophore 4-amino-5-methylanino-2, 7-difluroﬂuroescein diacetate (DAF-FM DA). Transformation of DAF-FM DA by NO generates highly fluorescent triazole derivatives. Ponatinib-treated zebrafish were transferred into 96-well plates and incubated with DAF-FM DA solution (5 μM) for 1 h in the dark at 28°C. After incubation, 10 zebrafish from each group were randomly selected using a plastic disposable pipette that was positioned in the center of a container and picked up zebrafish whoever entered the pipette and observed under a stereo fluorescence microscope. Nikon NIS-Elements D 3.10 Advanced Image Processing Software was used to capture and analyze the images. The fluorescence signal (S) was measured and the relative NO level was calculated based on the formula below: the relative NO level (%)= $(S_{Ponatinib}/S_{Control})\times 100\%$.

2.7.2. Brain apoptosis assessment

The brain apoptosis was measured by staining the zebrafish with an apoptotic cells specific living fluorescent dye acridine orange (Zhu et al., 2019). In brief, the zebrafish were stained with 2.5 μg/ml acridine orange for 30 min and then washed for 3 times in fish water. The apoptotic cells displayed yellow-green fluorescent signal (S) in the brain under a stereofluorescent microscope. Nikon NIS-Elements D 3.10 Advanced Image Processing Software was used to capture and quantify the images as reported before by our team (Li et al., 2006). The induction % of brain apoptosis in the zebrafish treated with Ponatinib was calculated based on the following formula: percentage of the apoptosis induction = $(S_{Ponatinib}/S_{Control}-1)\times 100\%$ (Zhu et al., 2019).

2.7.3. Brain inflammation quantification

Tg(MPO::GFP) transgenic zebrafish that express neutrophil specific fluorescence were used for the brain inflammation quantification in the ischemic stroke model. Tg(MPO::GFP) transgenic zebrafish at 2 dpf were treated with Ponatinib at

concentrations of 0.3, 1 and 3 μg/ml for 24 h and then 10 zebrafish from each group were randomly selected using a plastic disposable pipette for image acquisition under a stereofluorescence microscope. Quantitative image analysis was performed using image-based morphometric analysis as described above. The brain inflammation percentage rate in zebrafish treated with Ponatinib was calculated based on the formula below: percentage of the brain inflammation = $(S_{Ponatinib}/S_{Control}-1) \times 100\%$.

2.7.4. Reactive oxygen species (ROS) assay

An oxidation specific and sensitive probe, 5-(and 6-)-chloromethyl-20, 70-dichloro-dihydrofluoresceindiacetate (CM-H2DCFDA, Life Technologies, Carlsbad, CA) was applied to measure zebrafish reactive oxygen species levels. Zebrafish treated with 10 ng/ml Phorbol-12-myristate-13-acetate (PFA) for 24 h were used as a positive control (Hermann et al., 2004). The treated zebrafish were incubated with 0.5 mg/ml CM-H2DCFDA for 1 h in dark at 28°C. After washed for 3 times using fish water, the zebrafish were transferred into a 96-well microplate, 1 zebrafish per well, and reactive oxygen species was measured at 488 nm under a multimode microplate reader (Zhu et al., 2019).

2.7.5. Gene expression analyses

Quantitative PCR was performed to measure the gene expression levels of neovascularization-related gene (*vegfaa),* vascular endothelial growth factor receptor gene (*VEGFR),* neurodevelopment related genes (*mbp* and *a1-tubulin*), brain-derived neurotrophic factor (*BDNF*), glial cell derived neurotrophic factor (*GDNF*), apoptosis-related genes (*caspase-3*, *caspase-7*, *caspase-9, bax* and *bcl-2*), and inflammatory factor genes (*IL-1β*, *IL-6*, *IL-10*, *TNF-a* and *NF-κB*) in the zebrafish ischemic stroke. The qPCR primers used in this study were listed in Table 2. After Ponatinib treatment, total RNA was extracted from fifty homogenized zebrafish per group using Trizol reagent (Invitrogen Life Technologies), and 3 replicates of RNA extracted from the pools of zebrafish were analyzed. The quality of RNA samples was evaluated using the methods recommended by the NanoDrop 2000 (Thermo Scientific). About 2 μg total RNA of each sample was used for cDNA synthesis using

FastQuant RT Kit (With gDNase) (Tiangen) and qPCR amplifications were carried out with a CFX Connect detection system (Biorad) using the iTaq Universal SYBR Green Supermix (Biorad) in which there were three technical or biological replicates. The PCR protocol was optimized as the following: 2 min at 95°C, 40 cycles of 5 s each at 95°C, and 30 s at 60°C. Melting curve analysis was performed to check the specificity of the primers. The gene expression data was normalized against β-actin level and relative quantification of each gene mRNA level among groups was calculated using the $2^{-\triangle\triangle Ct}$ method (Sharif et al., 2016).

2.8. Statistical analysis

All the data were first checked whether obey normal distribution equal variance. One-way ANOVA followed by the Dunnett's test was used to compare differences among the groups. All statistical analyses were performed using the SPSS 16.0 software (SPSS, USA), and $P < 0.05$ was considered statistically significant. For quantitative analyses, all data were presented as mean ± S.E.M., and results were statistically compared between the Ponatinib-treated and the control zebrafish groups. All experiments were repeated for at least 3 times to confirm their reproducibility.

3. Results

3.1. Zebrafish ischemic stroke model development

3.1.1. Ponatinib concentration and treatment period optimization

We found that treatment with 0.3 μg/ml Ponatinib for 12 h to 48 h did not induce obvious cerebral thrombosis in zebrafish at all. Various degrees of cardiovascular toxicity including pericardial edema, bradycardia or no blood circulation was observed in all zebrafish treated with 3 μg/ml Ponatinib for 12 h to 48 h. Time-dependent cerebral thrombus formation was demonstrated in the zebrafish treated with 1 μg/ml Ponatinib, of which treatment for 24 h induced cerebral thrombosis in 100% zebrafish without observable other toxicity (Fig. 1A); and the thrombus formation was markedly reduced when the zebrafish were co-treated with a well-known antithrombotic drug Aspirin (Fig. 1B). Treatment with 1 μg/ml Ponatinib

for 48 h induced severe thrombosis with obvious cardiovascular toxicity.

In quantitative analysis of thrombosis, time-dependent increases of the cerebral thrombosis incidence and thrombus area were demonstrated in the zebrafish treated with 1 μg/ml Ponatinib for up to 24 h (data not shown). Thrombus formation was confirmed by histopathological examination in the zebrafish treated with 1 μg/ml Ponatinib for 24 h (Fig. 1C). Based on these results, Ponatinib treatment at 1 μg/ml for 24 h was selected as the optimum treatment concentration and treatment period for the development and validation of the zebrafish ischemic stroke model.

3.1.2. Ponatinib induced brain blood flow reduction

Brain blood flow dynamics was detected based on analysis of the motion of erythrocytes within tracking area by the ZebraBlood™ software (Fig. 2A, Fig. 2B). Ponatinib treatment resulted in a concentration-dependent decrease of brain blood flow, and within a few following days, the circulation ceased and the zebrafish died (data not shown). The relative brain blood flow was 96%, 34% and 2% of control group in the zebrafish treated with Ponatinib at concentrations of 0.3, 1 and 3 μg/ml, respectively. Statistically significant brain blood flow reductions were found in the zebrafish treated with Ponatinib at 1 μg/ml ($P < 0.001$) and 3 μg/ml ($P < 0.001$). Ponatinib-induced brain blood flow reductions were further confirmed by cerebral vascular angiography. As indicated in Fig. 2C, control zebrafish brain showed relatively regular rich blood flow (Movie 1), whereas Ponatinib-treated zebrafish demonstrated a markedly reduced brain blood flow and completely or partly blood vessel blockage (Movie 2).

3.1.3. Ponatinib induced motility impairment

Locomotion activities of the ischemic stroke zebrafish were evaluated under alternating cycles of light-dark stimulation. As indicated in Fig. 2D, zebrafish movement distance decreased significantly in a Ponatinib dose-dependent manner and the reduction percentage rates of total distance were (10 ± 3.9)%, (80 ± 1.3)% and (99.5 ± 0.1)% in the zebrafish exposed to Ponatinib at concentrations of 0.3 μg/ml ($P > 0.05$), 1 μg/ml ($P < 0.001$) and 3 μg/ml ($P < 0.001$), respectively, as

compared with control zebrafish.

3.2. Drugs effects on the zebrafish ischemic stroke

To determine whether the model zebrafish response to ischemic stroke therapeutic drugs was similar to the response of mammalian model systems, we cotreated the zebrafish with 1 μg/ml Ponatinib and each of 6 known human drugs (Aspirin, Clopidogrel, Naoxintong capsules, Edaravone, Xingnaojing injection, Shuxuening injection) currently used in clinical practice. As indicated in Table 1, NOAEL in the ischemic stroke zebrafish was 100 μg/ml for Aspirin, 15 μg/ml for Clopidogrel, 62.5 μg/ml for Naoxintong capsules, 25 μg/ml for Edaravone, 10 ng for Xingnaojing injection, and 35 ng for Shuxuening injection, respectively.

As expected, after a 24 h co-treatment, human ischemic stroke therapeutic drugs Aspirin, Clopidogrel, Naoxintong capsules, Edaravone, Xingnaojing injection, Shuxuening injection significantly reduced the cerebral thrombosis incidence and the area of cerebral thrombosis as compared with the model group zebrafish (Fig. 3), implying that human therapeutics were effective on the ischemic stroke zebrafish. As compared with the ischemic stroke model zebrafish, the relative area of cerebral thrombus was reduced to 7 - 55% for Aspirin, 45 - 98% for Clopidogrel, 48 - 86% for Naoxintong capsules, 19 - 37% for Edaravone, 15 - 44% for Xingnaojing injection, and 9 - 30% for Shuxuening injection, respectively. Statistically significant thereapeutic effects on the zebrafish ischemic stroke were observed at 3.125 - 62.5 μg/ml ($P < 0.01$ & $P < 0.001$) for Aspirin; 1.88 - 15 μg/ml ($P < 0.05$ &$P < 0.001$) for Clopidogrel; 3.9 - 62.5 μg/ml ($P < 0.05$, $P < 0.01$ & $P < 0.001$) for Naoxintong capsules; 1.56 - 25 μg/ml ($P < 0.05$, $P < 0.01$ & $P < 0.001$) for Edaravone; 0.08 - 10 ng ($P < 0.001$) for Xingnaojing injection; and 2.2 - 35 ng ($P < 0.001$) for Shuxuening injection (Table 1).

Furthermore, we used 1 μg/ml Ponatinib to induce zebrafish cerebral thrombosis first and then treated the cerebral thrombosis zebrafish with urokinase to evaluate whether or not it could resolve the thrombosis. As compared with the ischemic stroke model zebrafish, urokinase significantly reduced the cerebral thrombosis incidence

and the relative area of cerebral thrombus was reduced to 30% for urokinase. Statistically significant therapeutic effects on the zebrafish cerebral thrombosis were observed at 100 μg/ml ($P < 0.001$) for urokinase.

3.3. Ponatinib induced cerebral vascular endothelial injury

The vascular endothelial-related genes *VEGFR* and *vegfaa* were downregulated in the zebrafish treated with 1 μg/ml Ponatinib, 0.07-fold and 0.58-fold declinations relative to the control zebrafish, respectively ($P < 0.001$) (Fig. 4A).

As shown in Fig. 4B, the fluorescent intensity of NO staining in the zebrafish treated with 1 μg/ml Ponatinib was significantly attenuated as compared with the control zebrafish, and the relative NO levels were 92%, 66% and 6% of control group in the zebrafish treated with Ponatinib at concentrations of 0.3 μg/ml, 1 μg/ml and 3 μg/ml, respectively. Statistically significant differences ($P < 0.001$) were found in the zebrafish treated with Ponatinib at 1 μg/ml and 3 μg/ml (Fig. 4C).

3.4. Ponatinib induced cerebral cell death

There were no observable brain apoptosis in the control zebrafish, but marked apoptosis in the brain area were found in the zebrafish treated with 1 μg/ml Ponatinib (Fig. 5A). In consistence with the brain cell death, significant upregulations of the *caspase-3*, *caspase-7*, *caspase-9* and *bax* gene expression were shown in the zebrafish treated with 1 μg/ml of Ponatinib, 1.55-, 1.59-, 2.74- and 2.47-fold increases relative to the control group ($P = 0.0488$, 0.0422, 0.0014 and 0.0007). Expression of *caspase-8* and *bcl-2 genes* had no change, but *bcl-2/bax* ratio was decreased in the ischemic stroke zebrafish ($P = 0.0004$) (Fig. 5B). Nerve growth factors and associated protein genes *BDNF*, *GDNF*, *mbp* and *a1-tubulin* were 0.08-, 0.63-, 0.30- and 0.59-fold lower in the ischemic stroke zebrafish as compared with control group ($P = 0.0023$, 0.0096, 0.0122 and 0.0359) (Fig. 5C).

3.5. Ponatinib induced cerebral inflammation

As indicated in Fig. 6A, the control zebrafish showed the normal distribution of fluorescent neutrophils, mostly localized in the intracerebral vessels; whereas the zebrafish exposed to 1 μg/ml of Ponatinib demonstrated a general dispersal of fluorescent neutrophils, suggesting the active migration of neutrophils from their

initial location to a few clusters in the brain. Quantitative analysis showed a significant difference in the number of neutrophils between 1 μg/ml Ponatinib-treated zebrafish and the control zebrafish ($P < 0.001$). The gene expression levels of the inflammatory factors *IL-1β, IL-6, IL-10, TNF-α and NF-κB* in Ponatinib-induced ischemic stroke zebrafish were 1.20-, 1.39-, 1.61-, 3.24- and 1.58-folds higher than control zebrafish ($P = 0.0167$, 0.0437, 0.0024, 0.0154 and 0.0030) (Fig. 6B).

3.6. Ponatinib induced reactive oxygen species production

Ponatinib treatment resulted in increased reactive oxygen species production and reactive oxygen species levels relative to control zebrafiash were 105%, 251% and 564%, respectively, in the zebrafish treated with Ponatinib at concentrations of 0.3, 1 and 3 μg/ml. Reactive oxygen species level of the zebrafish treated with 10 ng/ml PFA relative to control was 485%. Statistically significant differences ($P < 0.001$) were found in the zebrafish treated with Ponatinib at 1 and 3 μg/ml and PFA (Fig. 6C).

4. Discussion

In the present study, we have developed and characterized an alternative ischemic stroke animal model in the larval zebrafish treated with a vascular endothelial cell-damaging drug Ponatinib (Ai et al., 2018; Guilhot et al., 2019) under the optimum treatment concentration and the exposure time period. This *in vivo* zebrafish model demonstrated the brain vascular endothelial injury, brain thrombosis, reduced brain blood flow, brain inflammation, brain apoptosis, and impaired motility, closely similar to the pathophysiology of the ischemic stroke patients.

In the clinical trials and subsequently extensivel uses, serious arterial thrombosis and venous thromboembolism leading to fatal myocardial infarction and stroke occurred in Ponatinib-treated patients (Medeirosa et al., 2018), postulating that Ponatinib may have a powerful effect on cardiovascular, cerebrovascular and peripheral vascular systems (Lafiti et al., 2019; Medeirosa et al., 2018;

Gover-Proaktor et al., 2019). In the pilot studies, we found that Ponatinib treatment on the later stage of larval zebrafish at 5 dpf for 24 h at higher concentrations ranging from 2 to 8 μg/ml induced peripheral vascular thrombosis in abdomental artery and inhibited peripheral angiogenesis without cerebral thrombosis (data not published). These preliminary results suggested that Ponatinib-induced target organ vascular toxicity could be dose- and exposure time-dependent and could be also related to larval zebrafish development stages. In order to establish a zebrafish ischemic stroke model without marked toxicity in other organs, we extensively assessed and optimized zebrafish treatment stages, Ponatinib treatment concentrations and treatment time periods, and finally identified 2 dpf zebrafish for a 24-h treatment at 1 μg/ml concentration of Ponatinib as the optimum conditions for the development of the zebrafish ischemic stroke model.

The brain specific thrombosis formation was supported by the following evidences: 1) the whole body of images of o-dianisidine staining indicated the brain-specific thrombus formation (Fig. 1A); 2) the histopathology confirmed the brain thrombosis (Fig 1C); 3) the angiography showed the brain blood flow reduction and cerebral blood vessel blockage (Fig. 2C); 4) the brain-specific apoptosis was demonstrated in Fig. 5A; and 5) the brain-specific inflammation was indicated in Fig. 6A.

The most common cause of ischemic stroke is the thrombosis, leading to reduction of the cerebral blood flow. This blood flow decrease and the resulting absence of oxygen, glucose and nutrients, induce a cascade of events that lead to the brain cell death and functional impairments (Mehta et al., 2007). If brain blood flow is not restored in a short time, the neurons in the penumbra enter apoptosis (Endres et al., 2008). Here Ponatinib treatment on 2 dpf zebrafish at 1 μg/ml resulted in a concentration-dependent brain blood flow reduction with the brain blood vessel blockage. If without anti-thrombosis treatment, within a few following days, Ponatinib-treated zebrafish circulation ceased and the zebrafish died.

Several mechanisms have been demonstrated to be involved in cerebral ischemic injury including neuronal apoptosis, inflammatory responses, oxidative stress and

mitochondrial dysfunction (Pandya et al., 2011). Here we characterized the pathophysiology of Ponatinib-induced zebrafish ischemic stroke and found the vascular endothelial injury, thrombosis, inflammation, apoptosis and dysfunctional movement in the ischemic stroke zebrafish. In addition, reactive oxygen species production was increased in the model zebrafish. In supporting these findings, the mRNA levels of the neovascularization-related gene (*vegfaa)* and vascular endothelial growth factor receptor gene (*VEGFR),* neurodevelopment related genes (*mbp* and *a1-tubulin*), brain-derived neurotrophic factor (*BDNF*) and glial cell derived neurotrophic factor (*GDNF*) were significantly downregulated; whereas apoptosis-related genes (*caspase-3*, *caspase-7*, *caspase-9* and *bax*/*bcl-2*), and inflammatory factor genes (*IL-1β*, *IL-6*, *IL-10*, *TNF-α* and *NF-κB*) were upregulated.

Cerebral hypoxia which forms as a consequence of ischemia is well known to induce NO and VEGF production, leading to angiogenesis. However, there were a number of reports confirmed that Ponatinib treatment resulted in the “narrowed” blood vessels in the zebrafish and human umbilical vein endothelial cells via blocking VEGFR signaling pathway (Ai et al., 2018); and that Ponatinib damages NO- and VEGF-producing vascular endothelial cells (Ai et al., 2018), thus probably leading to reduced NO and VEGF production. We postulate that the reduced NO and VEGF could occur at early stages of ischemic stroke and may increase at later stages of the pathogenesis following hypoxia. In addition, free radicals were increased and played important roles in the pathogenesis of the ischemic stroke (Rodrigo et al., 2013; Sun et al., 2018; Naritomi and Moriwaki, 2013; Imai et al., 2019), causing a vast array of injuries mediated by many different pathways (Facchinetti et al., 1998; Lapchak and Araujo, 2003; Cherubini et al., 2005). We postulated reactive oxygen species increase was a stroke sequel and the elevated reactive oxygen species further aggravated stroke damage.

Six known human ischemia stroke therapeutic drugs Aspirin (thromboxane A2 inhibitor) (Lekstrom et al, 1991), Clopidogrel (adenosine diphosphate [ADP] inhibitor) (Plosker et al, 2007), Naoxintong capsules (Xu et al, 2015), Edaravone

(Zheng et al, 2016), Xingnaojing injection (Zhang et al, 2018), Shuxuening injection (Lyu et al, 2018) were chose to assess their therapeutic effects on the Ponatinib-induced zebrafish ischemic stroke. All these human ischemia stroke therapeutics except Edaravone are classic anti-clotting drugs and consistently reduced the zebrafish cerebral thrombosis, implying that this zebrafish model could be used for screening and assessing the preventive and therapeutic agents of the ischemic stroke.

Edaravone's therapeutic effect is thought to be via improving survival of neurons under situations of oxidative stress such as following reperfusion injury in stroke patients (Lapchak et al, 2009). Some reports have suggested that free radicals are important factors affecting both coagulation and thrombolysis systems in ischemic lesions (Rodrigo et al, 2013; Li et al, 2016). Previous studies have found that Edaravone can prevent vascular endothelial cell injury (Li et al, 2013) and augment NO release from vascular cells and platelets, leading to suppression of platelet thrombus formation (Yamashita et al, 2006). Thus Edaravone's therapeutic effects on the ischemic stroke displayed in this study could be through both reducing oxidative stress and inhibiting thrombosis. Additionally, we could not exclude any possible drug-drug interactions between Ponatinib and a therapeutic drug when treated together in this study although no deposits and no color changes were found in the treatment solution.

In comparison with conventional animal models, this novel and innovative larval zebrafish ischemic stroke model could be highly predictive, relatively high-throughput and cost-efficient and may speed up the drug discovery process for the ischemic stroke. Further model validations and a pilot study using this model to screening preventive and therapeutic agents are in progress in our laboratory.

5. Conclusions

The larval zebrafish ischemic stroke model developed and optimized in this study was a living and pathophysiology-associated whole animal assay and this model

could be used for rapid *in vivo* screening and assessment of the preventive and therapeutic agents of the ischemic stroke.

Funding

This work was sponsored in part by the National Natural Science Foundation of China (No. 81730104).

Author contribution

Ping Li, Hua Yang and Chun-Qi Li designed the research; Xiao-Yu Zhu, Bo Xia, and Ming-Zhu Dai performed the research; Xiao-Yu Zhu, Bo Xia and Ming-Zhu Dai analyzed the data; Xiao-Yu Zhu and Chun-Qi Li wrote the paper. We thank Rick Li at Boston Latin School, Massachusetts, USA for editing this manuscript.

Declaration of competing interest

Xiao-Yu Zhu is a Ph.D. student at State Key Laboratory of Natural Medicines of the China Pharmaceutical University and the research and development director at Hunter Biotechnology, Inc; Bo Xia, Ting Ye, Ming-Zhu Dai and Chun-Qi Li are employees of the Hunter Biotechnology, Inc; Hunter Biotechnology, Inc is a zebrafish-based biotechnology company specializing in developing and commercializing novel and innovative models and assays for drug, health product and food industries.

References

Ai, N., Chong, C., Chen, W., Hu, Z., Su, H., Chen, G., Wong, Q.W.L., Wei, G., 2018. Ponatinib exerts anti-angiogenic effects in the zebrafish and human umbilical vein endothelial cells via blocking VEGFR signaling pathway. Oncotarget 9, 31958–31970.

Binder, D.K., 2004. The role of BDNF in epilepsy and other diseases of the mature nervous system. Adv. Exp. Med. Biol. 548, 34–56.

Braga, M.M., Rico, E.P., Córdova, S.D., Pinto, C.B., Blaser, R.E., Dias, R.D.,

Rosemberg, D.B., Oliveira, D.L., Souza, D.O., 2013. Evaluation of spontaneous recovery of behavioral and brain injury profiles in zebrafish after hypoxia. Behav. Brain Res. 253, 145–151.

Chandra, A., Stone, C.R., Du, X., Li, W.A., Huber, M., Bremer, R., Geng, X., Ding, Y., 2017. The cerebral circulation and cerebrovascular disease III: Stroke. Brain Circ. 3, 66–77.

Chen, S.D., Wu, C.L., Hwang, W.C., Yang, D.I., 2017. More insight into BDNF against neurodegeneration: anti-apoptosis, anti-oxidation, and suppression of autophagy. Int. J. Mol. Sci. 18, 545.

Cherubini, A., Ruggiero, C., Polidori, M.C., Mecocci, P., 2005. Potential markers of oxidative stress in stroke. Free Radic Biol. Med. 39, 841–852.

Christoph, K., Felix, F., Michael, S., 2015. Animal models of ischemic stroke and their application in clinical research. Drug Des. Devel. Ther. 9, 3445–3454.

Craig, M.P., Gilday, S.D., Dabiri, D., Hove, J.R., 2012. An optimized method for delivering flow tracer particles to intravital fluid environments in the developing zebrafish 9, 108–119.

Degryse, Bernard, 2011. The Urokinase Receptor System as Strategic Therapeutic Target: Challenges for the 21st Century. Curr. Pharm. Des. 17, 1872–1873.

Duarte, E.P., Curcio, M., Canzoniero, L.M., Duarte, C.B., 2012. Neuroprotection by GDNF in the ischemic brain. Growth Factors 30, 242–257.

Endres, M., Engelhardt, B., Koistinaho, J., Lindvall, O., Meairs, S., Mohr, J.P., Planas, A., Rothwell, N., Schwaninger, M., Schwab, M.E., Vivien, D., Wieloch, T., Dirnagl, U., 2008. Improving outcome after stroke: Overcoming the translational roadblock. Cerebrovasc Dis. 25, 268–278.

Facchinetti, F., Dawson, V.L., Dawson, T.M., 1998. Free radicals as mediators of neuronal injury. Cell Mol. Neurobiol. 18, 667–682.

Fumagalli, F., Racagni, G., Riva, M.A., 2006. Shedding light into the role of BDNF in the pharmacotherapy of Parkinson's disease. Pharmacogenomics J. 6, 95–104.

Gover-Proaktor, A., Granot, G., Pasmanik-Chor, M., Pasvolsky, O., Shapira, S., Raz,

O., Raanani, P., Leader, A., 2019. Bosutinib, dasatinib, imatinib, nilotinib, and ponatinib differentially affect the vascular molecular pathways and functionality of human endothelial cells. Leuk. Lymphoma. 60, 189–199.

Gregory, M., Hanumanthaiah, R., Jagadeeswaran, P., 2002. Genetic analysis of hemostasis and thrombosis using vascular occlusion. Blood Cells Mol. Dis. 29, 286–295.

Guilhot, F., 2019. Ponatinib and platelets a conflict in CML. Blood 133, 1520–1521.

Hamadi, A., Grigg, A.P., Dobie, G., Burbury, K.L., Schwarer, A.P., Kwa, F.A., Jackson, D.E., 2019. Ponatinib tyrosine kinase inhibitor induces a thrombo-inflammatory response. Thromb. Haemost. 119, 1112–1123.

Hermann, A.C., Millard, P.J., Blake, S.L., Kim, C.H., 2004. Development of a respiratory burst assay using zebrafish kidneys and embryos. J. Immunol. Methods 292, 119–129.

Huang, W., Li, C., Shen, Z., Zhu, X., Xia, B., Li, C., 2016. Development of a Zebrafish Model for Rapid Drug Screening against Alzheimer's Disease. Journal of Pharmacy and Pharmacology 4, 162–173.

Imai, T., Iwata, S., Miyo, D., Nakamura, S., Shimazawa, M., Hara, H., 2019. A novel free radical scavenger, NSP-116, ameliorated the brain injury in both ischemic and hemorrhagic stroke models. J. Pharmacol. Sci. 141, 119–126.

Ishikawa, M., Zhang, J.H., Nanda, A., Granger, D.N., 2004. Inflammatory responses to ischemia and reperfusion in the cerebral microcirculation. Front Biosci. 9, 1339–1347.

Ji, H.J., Wang, D.M., Hu, J.F., Sun, M.N., Li, G., Li, Z.P., Wu, D.H., Liu, G., Chen, N.H., 2014. IMM-H004, a novel courmarin derivative, protects against oxygen- and glucose-deprivation/restoration-induced apoptosis in PC12 cells. Eur. J. Pharmacol. 723, 259–266.

Jin, R., Yang, G., Li, G., 2010. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells, J. Leukoc. Biol. 87, 779–789.

Khandekar, G., Kim, S., Jagadeeswaran, P., 2012. Zebrafish thrombocytes: functions

644 and origins. Adv. Hematol. 2012, 857–058.
645 Kuida, K., 2000. Caspase-9. Int. J. Biochem. Cell Biol. 32, 121–124.
646 Lapchak, P.A., Araujo, D.M., 2003. Therapeutic potential of platelet glycoprotein
647 IIb/IIIa receptor antagonists in the management of ischemic stroke. Am. J.
648 Cardiovasc. Drugs 3, 87–94.
649 Lapchak, P.A., Zivin, J.A., 2009. The lipophilic multifunctional antioxidant
650 edaravone (radicut) improves behavior following embolic strokes in rabbits: a
651 combination therapy study with tissue plasminogen activator. Exp. Neurol.
652 215, 95–100.
653 Latifi, Y., Moccetti, F., Wu, M., Xie, A., Packwood, W., Qi, Y., Ozawa, K., Shentu,
654 W., Brown, E., Shirai, T., McCarty, O.J., Ruggeri, Z., Moslehi, J., Chen, J.,
655 Druker, B.J., López, J.A., Lindner, J.R., 2019. Thrombotic microangiopathy as a
656 cause of cardiovascular toxicity from the BCR-ABL1 tyrosine kinase inhibitor
657 ponatinib. Blood 133, 1597–1606.
658 Lekstrom, J.A., Bell, W.R., 1991. Aspirin in the prevention of thrombosis. Medicine
659 (Baltimore) 70, 161–178.
660 Li, C.Q., Pang, B., Kiziltepe, T., Trudel, L.J., Engelward, B.P., Dedon, P.C., Wogan,
661 G.N., 2006. Threshold Effects of Nitric Oxide-Induced Toxicity and Cellular
662 Responses in Wild-Type and p53-Null Human Lymphoblastoid Cells. Chem.
663 Res. Toxicol. 19, 399–406.
664 Li, W.A., Geng, X., Ding, Y., 2017. Stroke is a global epidemic: new developments
665 in clinical and translational cerebrovascular diseases research. Neurol. Res. 39,
666 475–476.
667 Li, W., Xu, H., Hu, Y., He, P., Ni, Z., Xu, H., Zhang, Z., Dai, H., 2013. Edaravone
668 protected human brain microvascular endothelial cells from methylglyoxal-
669 induced injury by inhibiting AGEs/RAGE/oxidative stress. PLoS One 8,
670 e76025.
671 Li, W., Yang, S., 2016. Targeting oxidative stress for the treatment of ischemic
672 stroke: Upstream and downstream therapeutic strategies. Brain Circ. 2, 153–163.

673 Lin, B., Ginsberg, M.D., Busto, R., Dietrich, W.D., 1998. Sequential analysis of
674 subacute and chronic neuronal, astrocytic and microglial alterations after
675 transient global ischemia in rats. Acta Neuropathologica 95, 511–523.
676 Lyu, M., Cui, Y., Zhao, T., Ning, Z., Ren, J., Jin, X., Fan, G., Zhu, Y., 2018.
677 Tnfrsf12a-mediated atherosclerosis signaling and inflammatory response as a
678 common protection mechanism of Shuxuening Injection against both
679 myocardial and cerebral ischemia-reperfusion injuries. Front Pharmacol. 312,
680 1–17.
681 Medeirosa, B.C., Possick, J., Fradley, M., 2018. Cardiovascular, pulmonary, and
682 metabolic toxicities complicating tyrosine kinase inhibitor therapy in chronic
683 myeloid leukemia: strategies for monitoring, detecting, and managing. Blood
684 Rev. 32, 289–299.
685 Merino, P., Diaz, A., Manrique, L.G., Cheng, L., Yepes, M., 2018. Urokinase-type
686 plasminogen activator (uPA) promotes ezrin-mediated reorganization of the
687 synaptic cytoskeleton in the ischemic brain. J. Biol. Chem. 293, 9234–9247.
688 Mehta, S.L., Manhas, N., Raghubir, R., 2007. Molecular targets in cerebral ischemia
689 for developing novel therapeutics. Brain Res. Rev. 54, 34–66.
690 Murphy, K.M., Ranganathan, V., Farnsworth, M.L., Kavallaris, M., Lock, R.B., 2000.
691 Bcl-2 inhibits bax translocation from cytosol to mitochondria during
692 drug-induced apoptosis of human tumor cells. Cell Death Differ. 7, 102–111.
693 Naritomi, H., Moriwaki, H., 2013. Prevention of post-stroke disuse muscle atrophy
694 with a free radical scavenger. Front Neurol. Neurosci. 32, 139–147.
695 Pandian, J.D., Gall, S.L., Kate, M.P., Silva, G.S., Akinyemi, R.O., Ovbiagele, B.I.,
696 Lavados, P.M., Gandhi, D.B.C., Thrift, A.G., 2018. Prevention of stroke: A
697 global perspective. Lancet 392, 1269–1278.
698 Pandya, J.D., Sullivan, P.G., Pettigrew, L.C., 2011. Focal cerebral ischemia and
699 mitochondrial dysfunction in the TNF alpha- transgenic rat. Brain Res. 1384,
700 151–160.
701 Papoutsoglou, S.E., 2012. The role of the brain in farmed fish. Rev. Aquacult. 4,

1–10.

Parker, T., Libourel, P.A., Hetheridge, M.J., Cumming, R.I., Sutcliffe, T.P., Goonesinghe, A.C., Ball, J.S., Owen, S.F., Chomis, Y., Winter, M.J., 2014. A multi-endpoint in vivo larval zebrafish (Danio rerio) model for the assessment of integrated cardiovascular function. J. Pharmacol. Toxicol. Methods 69, 30–38.

Plosker, G., Lyseng-Williamson, K., 2007. Clopidogrel: a review of its use in the prevention of thrombosis. Drugs 67, 613–646.

Prasad, V., Mailankody, S., 2014. The accelerated approval of oncologic drugs: lessons from ponatinib. Jama. 311, 353–354.

Richardson, J.S., Sethi, G., Lee, G.S., Malek, S.N., 2016. Chalepin: isolated from Rutaangustifolia L. Pers induces mitochondrial mediated apoptosis in lung carcinoma cells. BMC Complement. Altern. Med. 16, 389.

Rodrigo, R., Fernández-Gajardo, R., Gutiérrez, R., Matamala, J.M., Carrasco, R., Miranda-Merchak, A., Feuerhake, W., 2013. Oxidative stress and pathophysiology of ischemic stroke: novel therapeutic opportunities. CNS Neurol. Disord. Drug Targets 12, 698–714.

Sadovnik, I., Lierman, E., Peter, B., Herrmann, H., Suppan, V., Stefanzl, G., Haas, O., Lion, T., Pickl, W., Cools, J., Vandenberghe, P., Valent, P., 2014. Identification of Ponatinib as a potent inhibitor of growth, migration, and activation of neoplastic eosinophils carrying FIP1L1-PDGFRA. Exp. Hematol. 42, 282–293.

Sekeljic, V., Bataveljic, D., Stamenkovic, S., Ułamek, M., Jabłoński, M., Radenovic, L., Pluta, R., Andjus, P.R., 2012. Cellular markers of neuroinflammation and neurogenesis after ischemic brain injury in the long-term survival rat model. Brain Struct. Funct. 17, 411–420.

Sharif, F., Steenbergen, P. J., Metz, J. R., Champagne, D. L., 2016. Long-lasting effects of dexamethasone on immune cells and wound healing in the zebrafish. Wound Repair Regen. 23, 855–865.

Sheikh, A.M., Malik, M., Wen, G., Chauhan, A., Chauhan, V., Gong, C.X., Liu, F., Brown, W.T., Li, X., 2010. BDNF-Akt-Bcl2 antiapoptotic signaling pathway is

731 compromised in the brain of autistic subjects. J. Neurosci. Res. 88, 2641–2647.
732 Sommer, Clemens J., 2017. Ischemic stroke: experimental models and reality. Acta.
733 Neuropathologica 133, 245–261.
734 Springer, A.D., Easter, S.S.Jr., Agranoff, B.W., 1977. The role of the optic tectum in
735 various visually mediated behaviors of goldfish. Brain Res. 128, 393–404.
736 Stanne, T.M., Aberg, N. D., Nilsson, S., Jood, K., Blomstrand, C., Andreasson, U.,
737 Blennow, K., Zetterberg, H., Isgaard, J., Svensson, J., Jern, C., 2016. Low
738 circulating acute brain-derived neurotrophic factor levels are associated with
739 poor long-term functional outcome after ischemic stroke. Stroke. 47, 1943–
740 1945.
741 Stoll, G., Jander, S., Schroeter, M., 1998. Inflammation and glial responses in
742 ischemic brain lesions. Prog. Neurobiol. 56, 149–171.
743 Sun, M.S., Jin, H., Sun, X., Huang, S., Zhang, F.L., Guo, Z.N., Yang, Y., 2018. Free
744 Radical Damage in Ischemia-Reperfusion Injury: An Obstacle in Acute
745 Ischemic Stroke after Revascularization Therapy. Oxid. Med. Cell Longev. 2018,
746 3804979.
747 Tsetskhladze, Z.R., Canfield, V.A., Ang, K.C., Wentzel, S.M., Reid, K.P., Berg, A.S.,
748 Johnson, S.L., Kawakami, K., Cheng, K.C., 2016. Functional assessment of
749 human coding mutations affecting skin pigmentation using zebrafish. PLoS One,
750 7, e47398.
751 Traystman, R.J., 2003. Animal models of focal and global cerebral ischemia. ILAR. J.
752 44, 85–95.
753 Weyand, A.C., Shavit, J.A., 2014. Zebrafish as a model system for the study of
754 hemostasis and thrombosis. Cur. Opin. Hematol. 21, 418–422.
755 Wilson, X., Gelb, A.W., 2002. Free radicals, antioxidants, and neurologic injury:
756 possible relationship to cerebral protection by anesthetics. J. Neurosurg.
757 Anesthesiol. 14, 66–79.
758 Wu, Q., Cai, H., Song, J., Chang, Q., 2017. The effects of sEH inhibitor on
759 depression-like behavior and neurogenesis in male mice. J. Neurosci. Res. 95,

2483–2492.

Xu, H., Shi, Y., Zhang, Y., Jia, Q., Li, D., Zhang, Y., Liu, F., Yang, H., 2015. Identification of key active constituents of Buchang Naoxintong capsules with therapeutic effects against ischemic stroke by using an integrative pharmacology-based approach. Mol. Biosyst. 12, 1–33.

Yamagata, K., Tagami, M., Ikeda, K., Tsumagari, S., Yamori, Y., Nara, Y., 2002. Differential regulation of glial cell line-derived neurotrophic factor (GDNF) mRNA expression during hypoxia and reoxygenation in astrocytes isolated from stroke-prone spontaneously hypertensive rats. Glia 37, 1–7.

Yamashita, T., Shoge, M., Oda, E., Yamamoto, Y., Giddings, J.C., Kashiwagi, S., Suematsu, M., Yamamoto, J., 2006. The free-radical scavenger, edaravone, augments NO release from vascular cells and platelets after laser-induced, acute endothelial injury in vivo. Platelets 17, 201–206.

Youle, R.J., Strasser, A., 2008. The bcl-2 protein family: Opposing activities that mediate cell death. Nat. Rev. Mol. Cell Biol. 9, 47–59.

Yu, X., Li, Y.V., 2011. Zebrafish as an alternative model for hypoxic-ischemic brain damage. Int. J. Physiol. Pathophysiol. Pharmacol. 3, 88–96.

Yu, X., Li, Y.V., 2013. Neuroprotective effect of zinc chelator DEDTC in a zebrafish (Danio rerio) model of hypoxic brain injury. Zebrafish. 10, 30–35.

Yu, X., Li, Y.V., 2016. Zebrafish (Danio rerio) developed as an alternative animal model for focal ischemic stroke. Acta. Neurochir. Suppl. 121, 115–119.

Zhang, F., Kang, Z., Li, W., Xiao, Z., Zhou, X., 2012. Roles of brain-derived neurotrophic factor/tropomyosin-related kinase B (BDNF/TrkB) signalling in Alzheimer's disease. J. Clin. Neurosci. 19, 946–949.

Zhang, Y.M., Qu, X.Y., Zhai, J.H., Tao, L.N., Gao, H., Song, Y.Q., Zhang, S.X., 2018. Xingnaojing Injection protects against cerebral ischemia reperfusion injury via PI3K/Akt-Mediated eNOS phosphorylation. Evid. Based Complement Alternat. Med. 2361046, 1–13.

Zheng, J., Chen, X., 2016. Edaravone offers neuroprotection for acute diabetic stroke

patients. Ir. J. Med. Sci. 185, 819–824.

Zhu, X.Y., Liu, H.C., Guo, S.Y., Xia, B., Song, R.S., Lao, Q.C., Xuan, Y.X., Li, C.Q., 2016. A Zebrafish thrombosis model for assessing antithrombotic drugs. Zebrafish. 13, 335–344.

Zhu, X.Y., Wu, S.Q., Guo, S.Y., Yang, H., Xia, B., Li, P., Li, C.Q., 2018. A zebrafish heart failure model for assessing therapeutic agents. Zebrafish. 15, 243–253.

Zhu, X.Y., Xia, B., Wu, Y.Y., Yang, H., Li, C.Q., Li, P., 2019. Fenobucarb induces heart failure and cerebral hemorrhage in zebrafish. Aquat. Toxicol. 209, 34-41.

Zirm, E., Spies-Weisshart, B., Heidel, F., Schnetzke, U., Bohmer, F.D., Hochhaus, A., Fischer, T., Scholl, S., 2012. Ponatinib may overcome resistance of FLT3-ITD harbouring additional point mutations, notably the previously refractory F691I mutation. Br. J. Haematol. 157, 483–492.

818
819
820
821
822

Figure legends

Figure 1. Characterization and optimization of Ponatinib treatment concentration and treatment time period. Control zebrafish indicated no cerebral thrombosis (Control) and the zebrafish at 2 dpf treated with 1 μg/ml Ponatinib for 24 h visually showed cerebral thrombosis (Model) (A & B). Cerebral thrombosis was markedly reduced when the zebrafish were cotreated with a well-known antithrombotic drug Aspirin (Aspirin) (B). Histopathological examination confirmed thrombus formation in the brain blood vessels of the zebrafish treated with Ponatinib for 24 h, and no thrombus in the vehicle-treated zebrafish (C). The arrow in (C) Control showed no thrombus formation, and in (C) Model indicated thrombus formation in the zebrafish brain. Scale bar = 10 μm.

834

Figure 2. Brain blood flow dynamics was measured based on the motion of erythrocytes within tracking area using the ZebraBlood™ software. Blood flow dynamics maps of control and Ponatinib-treated zebrafish were shown in (A) right and (B) right, respectively. Cerebral angiography of control and Ponatinib-treated zebrafish, confirming the reduced brain blood flow and cerebral blood vessel blockage (arrow) (C). Locomotor activities of the larval zebrafish exposed to 1 μg/ml Ponatinib for 24 h were decreased as compared with untreated zebrafish， the movement diagrams were from 4 parallel wells (D).

843

Figure 3. Reduced cerebral thrombosis in the ischemic stroke zebrafish cotreated with ischemic stroke therapeutic drugs for 24 h, including Aspirin, Clopidogrel, Naoxintong capsules, Edaravone, Xingnaojing injection, and Shuxuening injection.

Urokinase was selected for assessing its therapeutic effect on the zebrafish stroke model in which the zebrafish were first treated with 1 μg/ml Ponatinib for 24 h, followed by treatment with 100 μg/ml urokinase for another 24 h.

Figure 4. Ponatinib induced brain vascular endothelial damage. The *VEGFR* and *vegfaa* gene expressions were downrerulated in the zebrafish exposed to 1 μg/ml Ponatinib (A); Fluorescent intensity of NO staining in the zebrafish treated with 1 μg/ml Ponatinib was measured after DAF-FM DA staining under a stereo fluorescence microscope (B); and the relative NO levels in the zebrafish treated with Ponatinib at concentrations of 0.3 μg/ml, 1 μg/ml and 3 μg/ml over the control were shown (C). Data were expressed as mean ± S.E.M. Compared with control group: ***P < 0.001.

Figure 5. Apoptosis and reduced nerve growth factor gene expressions in Ponatinib-induced ischemic stroke zebrafish. There were no obvious apoptotic cells indicated in the control zebrafish, whereas considerable numbers of apoptotic cells appeared in the brain of Ponatinib-treated zebrafish (A). Statistically significant upregulated expressions of the *caspase-3*, *caspase-7*, *caspase-9* and *bax* genes were found in Ponatinib-induced ischemic stroke zebrafish; the expressions of *caspase-8* and *bcl-2 genes* had no changes, and bcl-2/bax ratio was decreased after Ponatinib treatment (B). Relative gene expression levels of *BDNF*, *GDNF*, *mbp* and *a1-tubulin* in Ponatinib-induced ischemic stroke zebrafish (C). Data were expressed as mean ± S.E.M. Compared with control group: *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 6. Inflammation, reactive oxygen species production, and the gene expression levels of the inflammatory factors in Ponatinib-induced ischemic stroke zebrafish. Fluorescent neutrophols were mainly located in blood vessels in the control zebrafish, and increased neutrophil infiltrations in the brain of Ponatinib-induced ischemic stroke zebrafish (A). Relative gene expression levels of *IL-1β*, *IL-6*, *IL-10*, *TNF-α*

and *NF-κB* in the control and Ponatinib-induced ischemic stroke zebrafish (B). Reactive oxygen species (ROS) levels relative to control group in the zebrafish treated with 0.3, 1 and 3 μg/ml Ponatinib (C). Data were expressed as mean ± S.E.M. Compared with control group: *P < 0.05, **P < 0.01, and ***P < 0.001.

Movie legends

Movie 1. Control zebrafish brain showed regular rich blood flow.

Movie 2. Zebrafish treated with Ponatinib demonstrated a markedly reduced brain blood flow and completely or partly blood vessel blockage.

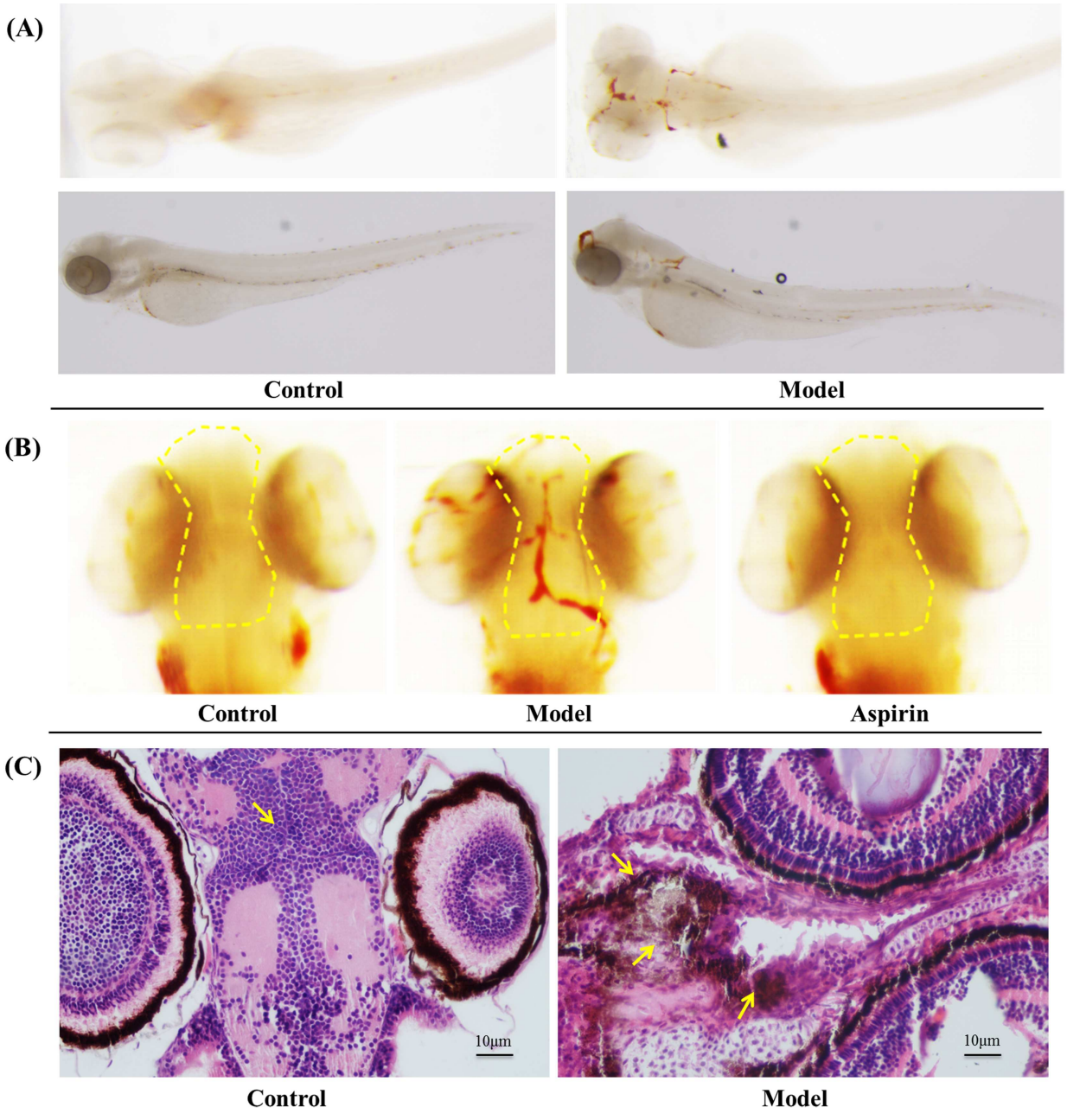
Table 1. Efficacy of antithrombotic drugs on the zebrafish ischemic stroke (mean ± S.E.M., n=10 for 3 repeats)

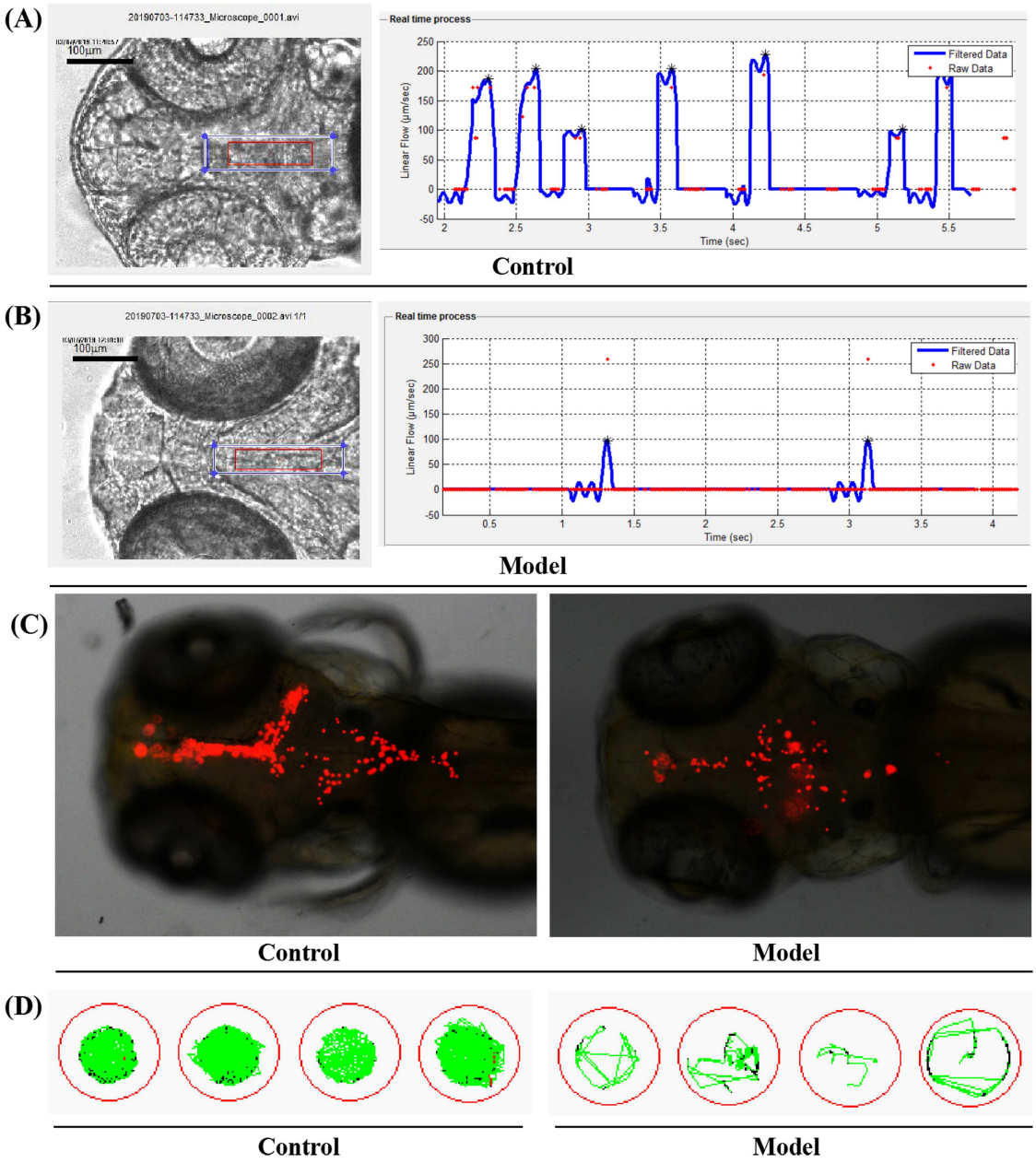
Drugs	Concentrations/Dosages	Cerebral thrombosis incidence (%)	Relative area of cerebral thrombosis (%)
Control	-	0	0
Model	-	100	100
Aspirin	3.125 μg mL^{-1}	60 [b]	55 ± 11.6
	6.25 μg mL^{-1}	60 [b]	55 ± 12.1 [a]
	12.5 μg mL^{-1}	60 [b]	50 ± 9.0 [a]
	25 μg mL^{-1}	55 [b]	36 ± 7.0 [c]
	50 μg mL^{-1}	15 [c]	7 ± 1.3 [c]
	100 μg mL^{-1}	10 [c]	7 ± 1.2 [c]
Clopidogrel	0.94 μg mL^{-1}	80	98 ± 17.8
	1.88 μg mL^{-1}	75 [a]	73 ± 15.7
	3.75 μg mL^{-1}	50 [c]	71 ± 9.2 [a]
	7.5 μg mL^{-1}	40 [c]	63 ± 14.0 [a]
	15 μg mL^{-1}	35 [c]	45 ± 8.6 [b]
Naoxintong capsules	3.9 μg mL^{-1}	75 [a]	86 ± 15.5
	7.8 μg mL^{-1}	60 [b]	68 ± 14.3 [a]
	15.625 μg mL^{-1}	55 [b]	58 ± 10.5 [b]
	31.25 μg mL^{-1}	50 [c]	48 ± 12.5 [b]
	62.5 μg mL^{-1}	45 [c]	48 ± 9.6 [b]
Edaravone	1.56 μg mL^{-1}	60 [b]	37 ± 10.6 [b]
	3.125 μg mL^{-1}	55 [b]	32 ± 7.6 [c]
	6.25 μg mL^{-1}	50 [c]	31 ± 6.8 [c]
	12.5 μg mL^{-1}	30 [c]	23 ± 6.8 [c]
	25 μg mL^{-1}	25 [c]	19 ± 9.6 [c]
Xingnaojing injection	0.78 ng	45 [c]	44 ± 12.1 [b]
	0.16 ng	45 [c]	31 ± 9.9 [c]
	0.31 ng	40 [c]	29 ± 7.5 [c]
	0.625 ng	40 [c]	29 ± 8.7 [c]
	1.25 ng	40 [c]	27 ± 8.7 [c]
	2.5 ng	35 [c]	26 ± 11.4 [c]
	5 ng	30 [c]	15 ± 4.3 [c]
	10 ng	50 [c]	25 ± 5.3 [c]
Shuxuening injection	2.2 ng	40 [c]	30 ± 9.8 [c]
	4.375 ng	25 [c]	27 ± 7.7 [c]
	8.75 ng	25 [c]	24 ± 5.5 [c]
	17.5 ng	25 [c]	19 ± 4.8 [c]
	35 ng	20 [c]	9 ± 1.8 [c]
Urokinase	100 μg mL^{-1}	50 [c]	30 ± 9.7 [c]

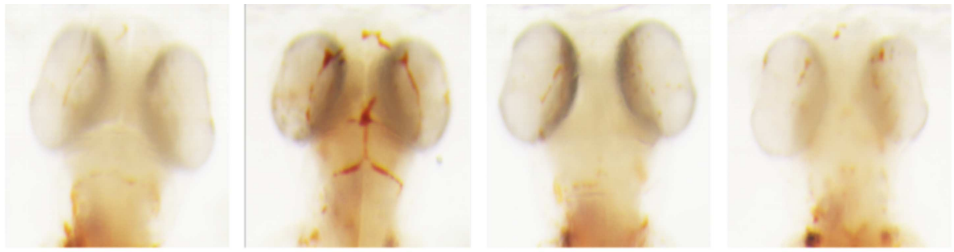
Compared with vehicle control: [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

Table 2. Sequences of primer pairs used in the real-time quantitative PCR reactions.

Gene	Forward (5'-3')	Reverse (5'-3')
β-actin	TCGAGCAGGAGATGGGAACC	CTCGTGGATACCGCAAGATTC
caspase-3	GGACATGCGGATACGGAGAC	TGCAGATGCCCCATCCTTAC
caspase-7	AAGTGGAGGATCGCAGGTTTG	TCTCTTGGTCCTCCCCTGAC
caspase-8	CTGCTCAAACGAACAGGCAC	TCCGGCAAAAGGCAGTGTAA
caspase-9	GCTTCTGTCAAAGGGGGTCT	CAGAAATGACAGGAGGGCGA
bax	GACTTGGGAGCTGCACTTCT	TCCGATCTGCTGCAAACACT
bcl-2	CACTGGATGACTGACTACCTGAA	CCTGCGAGTCCTCATTCTGTAT
VEGFR	GAGCGATGCTCCCGTTATCA	CACAACTCCACTCTCCCTGG
vegfaa	AGAGGGAGGCAGAAGCACAT	CCAAAGGGACGGTTGTAGAGT
IL-1β	GTCACACTGAGAGCCGGAAG	GCAGGCCAGGTACAGGTTAC
IL-6	GCAGTATGGGGGAACTATCCG	CTGACCCCTTCAAATGCCGT
IL-10	TTCAGGAACTCAAGCGGGAT	AAGAGCAAATCAAGCTCCCCC
TNF-α	ATCTTCAAAGTCGGGTGTATG	TGTGCCCAGTCTGTCTCC
NF-κB	GATGTTCACTGCGTTCCT	GTCTTCTGTCTCTTCCTCTG
GDNF	AGCCATCCAAGAGAGCTGTG	GTCCCGCTTCATCTGAGGTT
BDNF	TGGGTAAATCGCGACTGGTT	CTGTTGGAACATTTTCCCCTATG
mbp	AATCAGCAGGTTCTTCGGAGGAGA	AAGAAATGCACGACAGGGTTGACG
α1-tubulin	AAT CACCAATGCTTGCTTCGA GCC	TTCACGTCTTTGGGTACCACGTCA

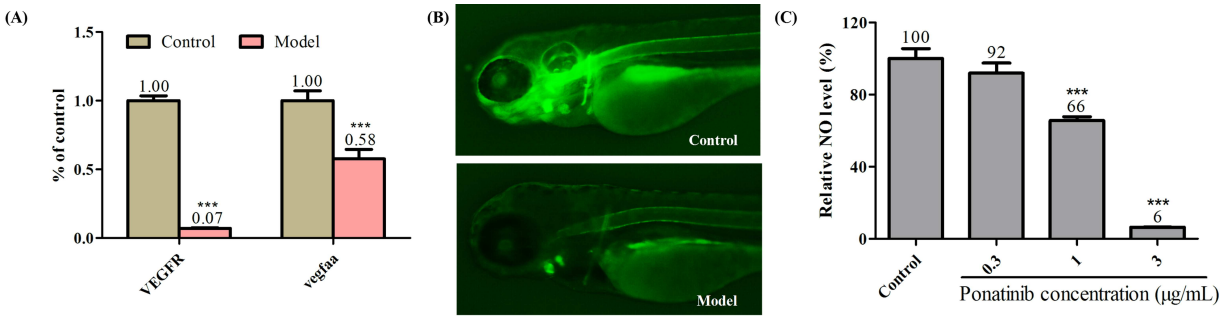

(A)
Control
Model
(B)
Control
Model
Aspirin
(C)
10μm
10μm
Control
Model

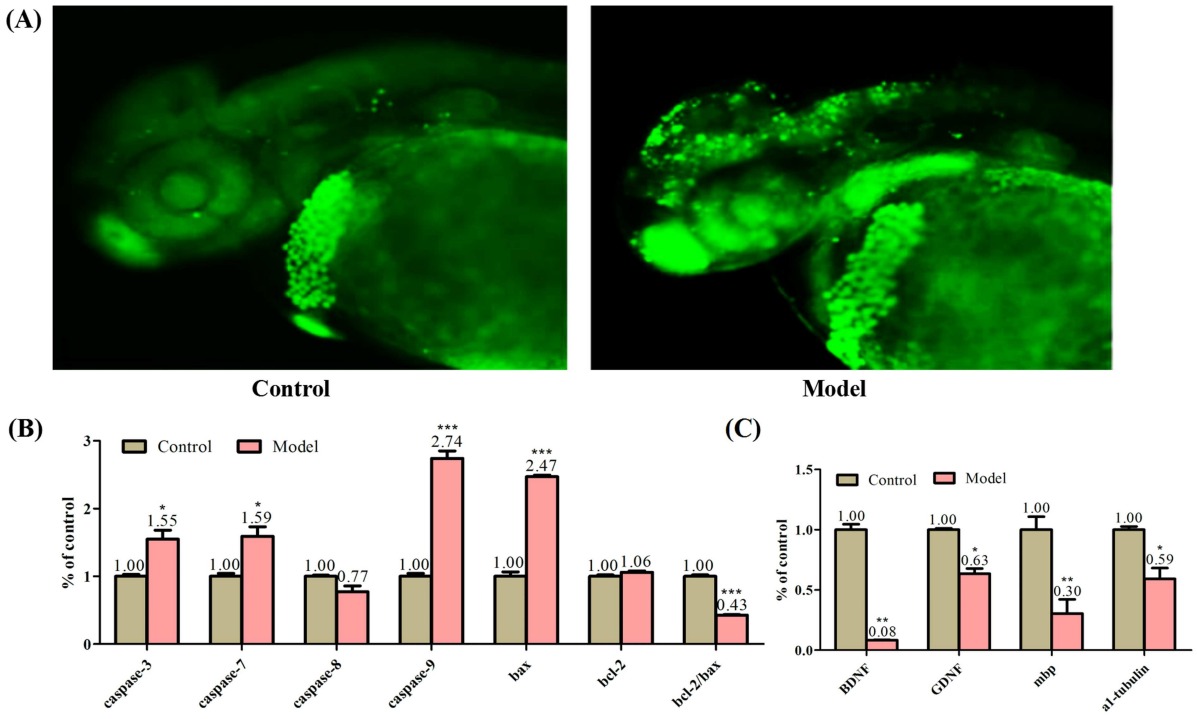

(A)
20190703-114733_Microscope_0001.avi
100μm
Real time process
Filtered Data
Raw Data
Linear Flow (μm/sec)
Time (sec)
Control
(B)
20190703-114733_Microscope_0002.avi 1/1
100μm
Real time process
Filtered Data
Raw Data
Linear Flow (μm/sec)
Time (sec)
Model
(C)
Control
Model
(D)
Control
Model

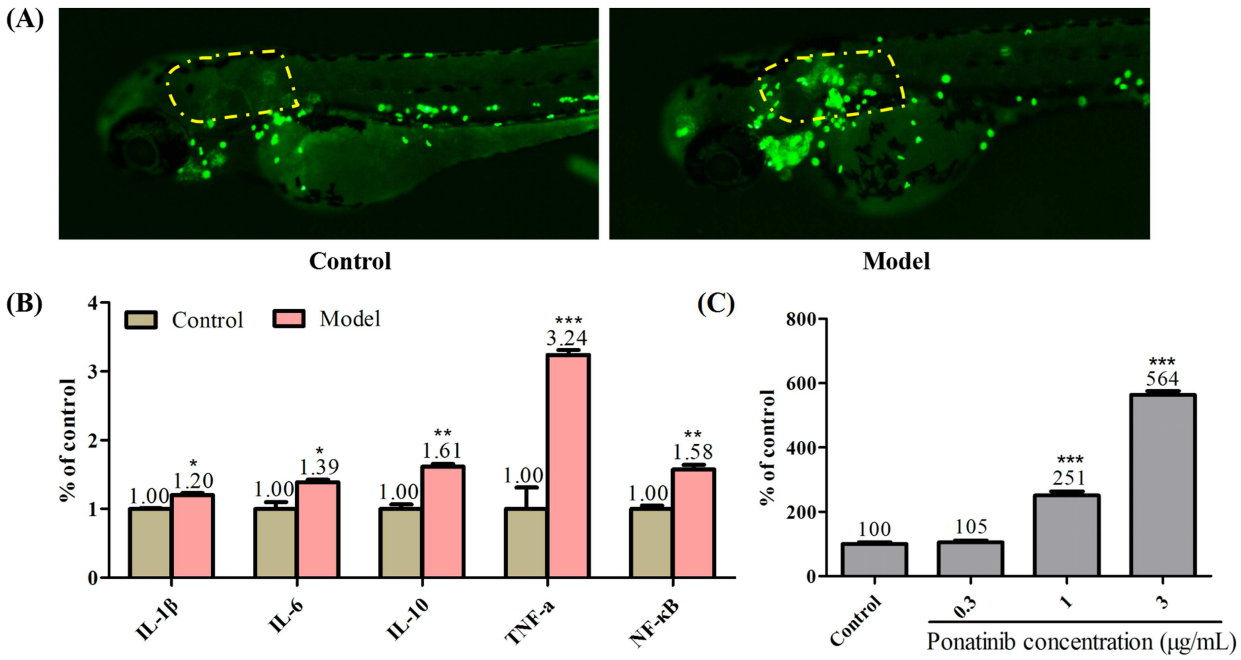


Control Model Aspirin Clopidogrel

Naoxintong capsules Edaravone Xingnaojing injection Shuxuening injection Urokinase




(A)
Control
Model
(B)
Control
Model
% of control
caspase-3
caspase-7
caspase-8
caspase-9
bax
bcl-2
bcl-2/bax
(C)
Control
Model
% of control
BDNF
GDNF
nbp
a1-tubulin



Highlights

We developed and characterized a larval zebrafish ischemic stroke model using Ponatinib as an inducer under an optimized exposure concentration and treatment period. The larval zebrafish ischemic stroke was further validated with 6 known human ischemic stroke therapeutics. The pathophysiology of this zebrafish ischemic stroke is closely similar to that of human ischemic stroke and could be used for rapidly identifying the preventive and therapeutic agents.