A Zebrafish Thrombosis Model for Assessing Antiplatelet Drugs

Yanjun Jiang, Ye Rong, Ruixue Wu, Yi Wen, and Liu Hu
Zhejiang Yangshengtang Institute of Natural Medication Co., Ltd.
Hangzhou, China
Email: {yjjiang2, yrong3, rxwu2, ywen25, lhu}@mail.yst.com.cn

***Abstract*—This study aimed to develop a larval zebrafish (Danio rerio) thrombosis model for antiplatelet drug discovery by adopting CD41-eGFP (yst101) transgenic zebrafish. In this model, the thrombocytes expressed the enhanced green fluorescent protein driven by the zebrafish CD41 promoter. Briefly, fish at 2 days post-fertilization (dpf) were treated with ponatinib for 24 h by direct soaking, resulting in the caudal vein thrombus. The transparent larvae were used, and the thrombi were clearly observed and quantitatively evaluated by the image analysis of the red blood cells stained with o-dianisidine in the caudal vein. Meanwhile, thrombocyte aggregation was also observed at the site of the caudal vein by confocal microscopy. In addition, the thrombosis-preventive effects of aspirin and tirofiban, both FDA -approved antiplatelet drugs, were demonstrated and validated. However, clopidogrel, the other human antithrombotic drug, was found to have no or little antithrombotic effect on this zebrafish-based thrombosis model, which might be due to a hepatic metabolic defect of the transgenic line used in this study.**

***Index Terms*—thrombosis, zebrafish, CD41-eGFP, antiplatelet drugs**

I. Introduction

Platelets provide the first defense line when vascular integrity is compromised. They home to the site of injury where they adhere to the damaged vessel wall, become activated, and aggregate to form a platelet plug, also known as thrombus. Thrombosis can occur in arteries or veins, and both can be hazardous to health. Arterial thrombosis was recognized as one of the underlying causes for most heart attacks and strokes. In deep veins, the venous thrombi can break off, travel to the lungs, and finally result in pulmonary embolism. Therefore, it is necessary to develop antiplatelet drugs to prevent this common and potentially life-threatening condition.

The zebrafish has become a widely used model organism because of its fecundity, morphological and physiological similarities to mammals, existence of many genomic tools, and ease with which large, phenotype-based screens can be performed. The zebrafish also provides opportunities to accelerate the process of drug discovery [1]. Ponatinib is an oral drug developed for treating chronic myeloid leukemia and Philadelphia chromosome–positive (Ph+) acute lymphoblastic leukemia. It is a multi-targeted tyrosine kinase inhibitor [2]. Receptor tyrosine kinase inhibition by ponatinib may result in the inhibition of cellular proliferation and angiogenesis, and also induce the apoptosis of vascular endothelial cells. According to relevant Food and Drug Administration (FDA) reports, severe thrombosis, vascular stenosis, and other adverse reactions occurred after the clinical use of ponatinib [3].

Aspirin can inhibit platelet cyclooxygenase. Low-dose aspirin irreversibly blocks the formation of thromboxane A2 in platelets, producing an inhibitory effect on platelet aggregation. Clopidogrel is an antagonist at the platelet P2Y12 adenosine diphosphate (ADP) receptor [4, 5]. Tirofiban is a glycoprotein IIb/IIIa receptor antagonist that inhibits platelet activation and platelet–monocyte interaction.

In the present study, a thrombosis model that used ponatinib to induce thrombosis in CD41-eGFP (yst101) transgenic zebrafish was developed to quantitatively assess the antithrombotic effect of antiplatelet agents. This model was further validated using three FDA-approved antithrombotic drugs (aspirin, tirofiban, and clopidogrel). The results indicated that the zebrafish thrombosis model developed and validated in this study was a convenient and predictive model for rapid in vitro screening and efficacy assessment of antiplatelet aggregation agents.

II. Materials and Methods

A. Zebrafish Aquaculture

The zebrafish were maintained in a light and temperature controlled aquaculture facility under a standard 14:10h light/dark photoperiod. They were fed with dry flake once and live brine shrimp twice per day.

The wild-type AB strain zebrafish were purchased from the China Zebrafish Resource Center. We established the zebrafish CD41 promoter driven enhanced GFP expression transgenic line (zfCD41-eGFP=yst101) by microinjection and the subsequent screen (describe elsewhere). This stable transgenic line was adopted for the thrombosis assessment.

Zebrafish embryos were collected and incubated in E3 medium (19.3 mM NaCl, 0.23mM KCl, 0.13mM $MgSO_4$, $7H_2O$, 0.2 mM $Cacl_2$, pH7.2) with 0.1% methylene blue

Manuscript received August 20, 2019; revised December 20, 2019.

doi: 10.18178/ijpmbs.9.1.38-42

until transfer to 1-phenyl-2-thiourea (PTU) solution. Embryos are raised in 0.003% PTU in E3 medium at 8-24 hours post fertilization (hpf) until time of analysis to inhibit pigmentation.

B. Assessment of Drugs Antithrombotic Effect on Zebrafish Thrombosis

To assess the tested drugs' antithrombotic effect on thrombosis in zebrafish, three FDA-approved antithrombotic drugs (Clopidogrel, 70μg/mL; Tirofiban, 60μg/mL; Aspirin, 5μg/mL) were selected. Twenty larvae at 2dpf were randomly selected and raised in six-well plate with 5 mL E3 medium each well. Zebrafish were cotreated with 1μmol/L ponatinib and a candidate drug for the following 24h. Zebrafish treated with 0.1% DMSO was served as a vehicle control. After treatment, the zebrafish larvae were stained with O-dianisidine to quantify the caudal vein red blood cells (RBCs) which reversely correlated with the degree of thrombosis. Caudal vein RBCs counts were performed using the LAS hardware configurator image analysis software (Leica). The effect of a test drug was calculated based on the formula below:

$$\text{Efficacy (\%)} = [\text{IOD(drug)}-\text{IOD(model)}] / [\text{IOD(vehicle)} - \text{IOD(model)}]\times 100\% \quad (1)$$

(IOD means Integrated Optical Density)

C. Confocal Microscope

Zebrafish larvae were immobilized in 3% methyl cellulose and images were acquired in the identical lighting intensity. Image the larvae using an inverted, fluorescent, laser-scanning confocal microscope (Zeiss LSM 800).

D. Statistical Analysis

The results are expressed as mean±SEM, the statistical differences between groups were analysed by one-way ANOVA followed by Dunnett's test. All tests were carried out at 5% level of significance (two-tailed, $p < 0.05$). All experiments were repeated at least three times.

III. Results

In zebrafish embryo, the blood circulation began at 24hpf, and the caudal vein blood flow velocities could be measured by simply tracking movements of red blood cells (RBCs) in the embryo's body, which are easily identifiable due to transparent skin [6]. At 3dpf, bright CD41 GFP+ cells could be detected in our transgenic CD41-eGFP (yst101) line, as shown in Fig.1.

Three target-specific drugs: Aspirin, Tirofiban and Clopidogrel were used to assess the ponatinib induced caudal vein thrombosis in zebrafish. In a pilot study, we found that NOAELs (No Observed Adverse Effect Level) were 5μg/mL for aspirin, 60μg/mL for tirofiban and 70μg/mL for clopidogrel respectively. After co-treatment with ponatinib, clopidogrel was found little anti-thrombotic effect on the caudal vein in CD41-eGFP (yst101) transgenic zebrafish, whereas tirofiban and aspirin significantly decreased the thrombosis (Fig.2). The preventive efficacy of thrombosis was indicated in Table 1.

TABLE I. Drugs Used to Characterize Ponatinib-Induced Thrombosis in Zebrafish

Drugs	Preventive efficacy (%)
Clopidogrel	-2.94
Tirofiban	87.84
Aspirin	83.01

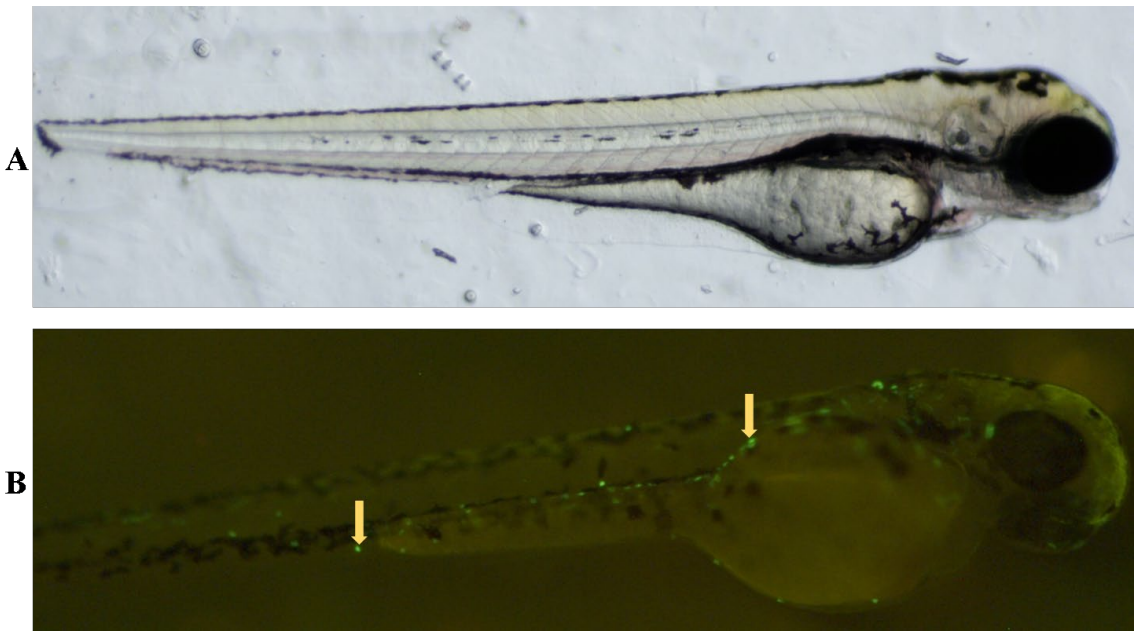

Figure 1. Photomicrographs showing CD41-eGFP (yst101) transgenic embryos are presented. (A) 3pdf wild-type zebrafish. (B) 3pdf CD41-eGFP (yst101) transgenic zebrafish, bright CD41 GFP+ cells are detected in the region between dorsal aorta and caudal vein (arrows,B).

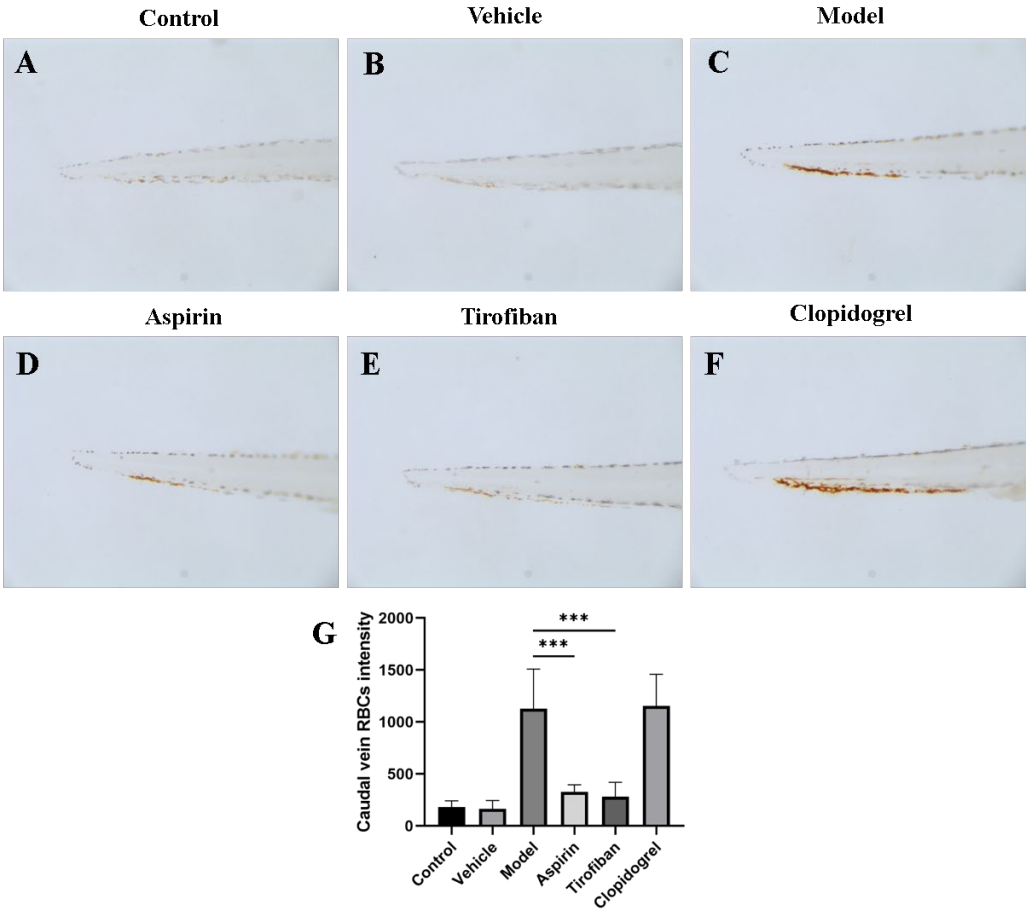

Figure 2. Caudal vein RBCs and quantitative analysis of preventive efficacy in thrombotic zebrafish co-treated with target specific drugs for 24h. The RBCs' intensity at 2dpf treated with 1μmol/L ponatinib for 24h (C) significantly increased compared with control zebrafish (A, B). Embryo co-treated with Aspirin (D) and Tirofiban (E) decreased the caudal vein RBCs' intensity. Clopidogrel (F) had no preventive efficacy in thrombotic. Compared with model: ***$p < 0.001$. **$p < 0.01$. RBC, red blood cell.

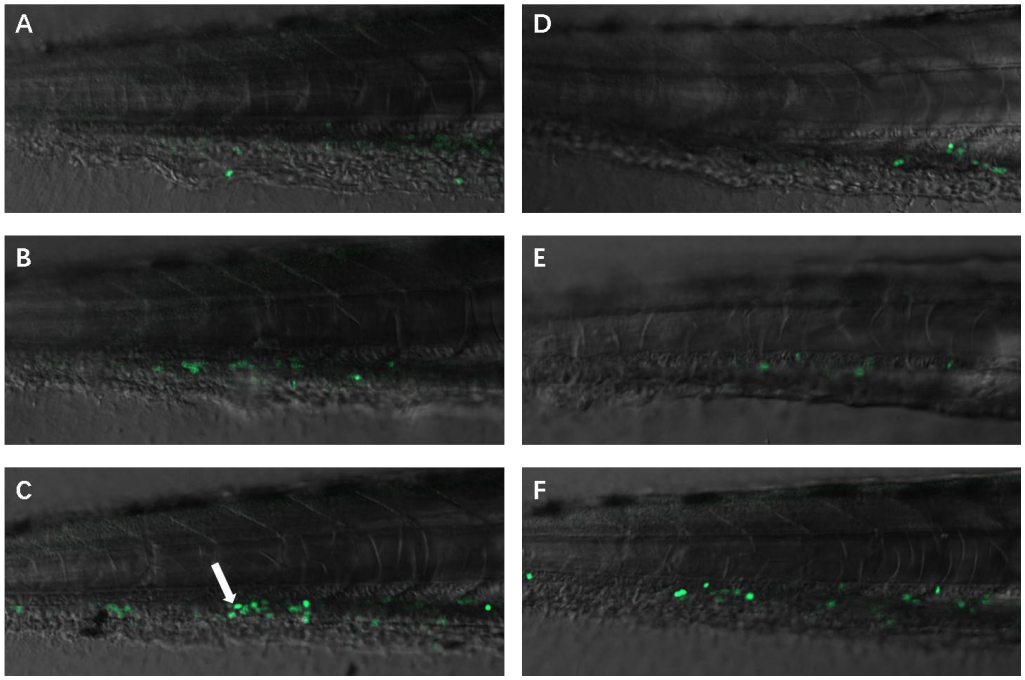

Figure 3. Thrombocytes adhered to the site of injury in caudal vein after treatment. Following endothelial injury mediated by Ponatinib, the CD41-eGFP (yst101) line zebrafish could visualize thrombocytes through green fluorescence. Platelets adhered and aggregated at the site of endothelial injury (C), in contrast to uninjured vessels (A, B). Embryo cotreated with aspirin (D) and tirofiban (E) significantly decreased the thrombocytes adhesion, and clopidogrel (F) had no effect on thrombocytes adhesion.

Ponatinib induced thrombosis caudal vein vascular endothelial injury, and platelet adhesion and aggregation could be clearly seen by confocal laser microscope in CD41-eGFP (yst101) transgenic line with fluorescently tagged thrombocytes (Fig.3C). As FDA-approved antithrombotic drugs, aspirin and tirofiban could inhibit platelet adhesion and aggregation significantly in this model, except clopidogrel. Both caudal vein thrombosis (Fig.2F. and Fig.3F.) and blood flow velocity recovery test indicated that clopidogrel had no effects on inhibiting platelets adhesion and aggregation in this model. But in a pilot study, clopidogrel had good antithrombotic effect on wild type zebrafish thrombosis model induced by Ponatinib (data not shown).

In summary, ponatinib could induce thrombosis in CD41-eGFP (yst101) zebrafish, which slow down the caudal vein blood flow. Platelets adhesion and aggregation at the site of endothelial injury. Antithrombotic drugs aspirin and tirofiban could compensate this condition significantly. Clopidogrel had no effects on inhibiting platelets adhesion and aggregation in this model.

IV. Discussion

Platelets are a component of blood whose function is to react to bleeding from blood vessel injury by clumping, thereby initiating a blood clot. During embryonic and larval development, both circulating and nonmobile GFP+ cells are visible.

Ponatinib uniquely induces a prothrombotic state due increased platelet activation and reduced vessel wall anti-thrombosis [7]. Antiplatelet drugs have different mechanism of action. Aspirin's antithrombotic effect is mediated by inhibition of blood platelets. The drug blocks a platelet enzyme, cyclo-oxygenase, by acetylating the enzyme's active site. Inhibition of the enzyme blocks production of an important prothrombotic agent known as thromboxane A2. Thromboxane A2 causes activation and aggregation of platelets, which is an early step in thrombosis. Tirofiban is a reversible antagonist of fibrinogen binding to the GP IIb/IIIa receptor, the major platelet surface receptor involved in platelet aggregation. When administered intravenously, tirofiban inhibits ex vivo platelet aggregation in a dose- and concentration-dependent manner.

Clopidogrel, as an antagonist against the platelet P2Y12 adenosine diphosphate (ADP) receptor, reduces platelet aggregation in many species [4], [8], [9]. The administration of clopidogrel results in a decreased responsiveness of the vascular smooth muscle to 5-HT [5], and 5-HT release from activated platelets is significantly decreased treated with clopidogrel. For these reasons, clopidogrel might be a drug that can decrease platelet hyperactivity, secretion of 5-HT, and subsequent platelet aggregate formation during inflammatory conditions and the prodromal stages of laminitis. While clopidogrel therapy resulted in inhibition of ADP-induced platelet aggregation, a significant difference was not demonstrated for ponatinib-induced aggregation. Clopidogrel required hepatic metabolism by several CYP enzymes in order to generate the active metabolite [10], and have no antiplatelet effect in this study.

The results suggest that the CD41-eGFP (yst101) transgenic zebrafish line used in the study might have affection to certain hepatic metabolism enzyme, either synthesis or activity, which potentially induced by the insertion of transgene in this case. The specific reasons merited further study.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Yi Wen and Liu Hu conducted the research; Yanjun Jiang developed the CD41-Egfp (yst101) transgenic fish; Ye Rong photographed by confocal microscopy; Ruixue Wu analyzed the data; all authors had approved the final version.

Acknowledgment

This work was supported by a grant from Zhejiang Yangshengtang Co., Ltd.

References

[1] I. L. Zon and T. R. Peterson, "In vivo drug discovery in the zebrafish," *Nature Reviews Drug Discovery*, vol. 4, no. 1, pp. 35-44, 2005.

[2] W. S. Huang, C. A. Metcalf, R. Sundaramoorthi, *et al.*, "Discovery of 3-[2-(imidazo[1,2-b]pyridazin-3-yl)ethynyl]-4-methyl-N-{4-[(4-methylpiperazin-1-yl)methyl]-3-(trifluoromethyl) phenyl}benzamide (AP24534), a potent, orally active pan-inhibitor of breakpoint cluster region-abelson (BCR-ABL) kinase including," *J Med Chem*, vol. 53, no. 12, pp. 4701-4719, 2010.

[3] FDA. (2013). *Drug Safety and Availability [EB/OL]*. [Online]. Available: http://www.fda.gov/drugs/drugsafety/ucm373040.html

[4] D. F. Hogan, *et al.*, "Antiplatelet effects and pharmacodynamics of clopidogrel in cats," *Javma-Journal of the American Veterinary Medical Association*, vol. 225, no. 9, pp. 1406-1411, 2004.

[5] B. L. Coomber, *et al.*, "Clopidogrel induced suppression of bovine platelet activation in vitro and a preliminary study of its effect on the development of Mannheimia haemolytica induced pneumonia," *Veterinary Journal*, vol. 171, no. 1, pp. 126-134, 2006.

[6] T. J. Shin., *et al.*, "High-resolution cardiovascular function confirms functional orthology of myocardial contractility pathways in zebrafish," *Physiological Genomics*, vol. 42, no. 2, pp. 300-309, 2010.

[7] A. H. Schmaier, *et al.*, "Ponatinib and cardiovascular complications," *Blood*, vol. 128, no. 3055, 2016.

[8] U. S. Tantry, *et al.*, "First analysis of the relation between CYP2C19 genotype and pharmacodynamics in patients treated with ticagrelor versus clopidogrel," *Circulation-cardiovascular Genetics*, 2010.

[9] B. M. Brainard, *et al.*, "Pharmacodynamic and pharmacokinetic evaluation of clopidogrel and the carboxylic acid metabolite SR 26334 in healthy dogs," *American Journal of Veterinary Research*, vol. 71, no. 7, pp. 822-830, 2010.

[10] J. L. Mega, *et al.*, "Cytochrome P-450 polymorphisms and response to clopidogrel," *The New England Journal of Medicine*, vol. 360, no. 4, pp. 354-362, 2009.

Yanjun Jiang is Pharmacology Researcher of Department of Basic Research, Zhejiang Yangshengtang Institute of Natural Medication Co., Ltd., Hangzhou, Zhejiang, China. Her research interests are developmental biology, regenerative medicine, and disease modeling.

Ye Rong is Pharmacology Researcher of Department of Basic Research, Zhejiang Yangshengtang Institute of Natural Medication Co., Ltd., Hangzhou, Zhejiang, China. Her research interests are developmental biology, regenerative medicine, and disease modeling.

Ruixue Wu is Assistant Pharmacology Researcher of Department of Basic Research, Zhejiang Yangshengtang Institute of Natural Medication Co., Ltd., Hangzhou, Zhejiang, China. Her research interests are developmental biology, regenerative medicine, and disease modeling.

Yi Wen is Senior Pharmacology Researcher of Department of Basic Research, Zhejiang Yangshengtang Institute of Natural Medication Co., Ltd., Hangzhou, Zhejiang, China. Her research interests are developmental biology, regenerative medicine, and disease modeling.

Liu Hu is Head of Department of Basic Research, Zhejiang Yangshengtang Institute of Natural Medication Co., Ltd., Hangzhou, Zhejiang, China. Her research interests are natural products and healthy living