Presented at abcam, Multidrug Resistance and ABC Transporters Conference, November 2008

ZEBRAFISH: A PREDICTIVE MODEL FOR ASSESSING MODULATORS OF P-GLYCOPROTEIN EFFLUX

Demian Park, Maryann Haldi, Wen Lin Seng, and Patricia McGrath

Phylonix Pharmaceuticals, Inc., Cambridge, Massachusetts, U.S.A.

Abstract

We are developing an *in vivo* assay for assessing drug effects on Pgp efflux in zebrafish brain. Based on retention of rhodamine 123 in the brain region, we determined that Pgp is expressed starting at 5 days post fertilization (dpf) and is fully functioning by 7 dpf. In order to monitor Pgp efflux clearance kinetics, we then injected rhodamine conjugated horseradish peroxidase (rho-HRP) into the midbrain of 7 dpf zebrafish and captured images at varying time points in both untreated (vehicle control) and reference compound treated zebrafish. In these studies, we determined that 3 hours post dye injection was the optimal time point for assessing Pgp efflux in untreated and drug treated animals. To validate our assay, we then assessed effects of 8 characterized compounds. We initially assessed Pgp efflux visually followed by quantitative image based morphometric analysis. We confirmed that 6 positive control drugs: Verapamil, Phenytoin, Loperamide, Cyclosporine, RU486, Quinidine, inhibited Pgp efflux in the zebrafish brain; results for Alanine were inconclusive. Caffeine, a negative control drug, did not exhibit significant inhibitory effect. In these results, 7 of 8 test compounds showed similar results as results in mammalian Pgp model systems. Our results demonstrated that embryonic zebrafish exhibit Pgp efflux transport machinery and effectively transfer xenobiotics from the brain to the blood. Zebrafish have been shown to exhibit comparable drug metabolism as mammals. Since zebrafish brain structure is transparent during early development (day 0-day 22), it is possible to study drug effects on dye retention as well as the molecular machinery underlying brain transporter function without complicated surgery and histology.

Introduction

Blood-Brain Barrier (BBB) and ABC transporters in Zebrafish: Zebrafish in recent years has emerged as an effective vertebrate model system in drug screening, discovery and delivery. In zebrafish, initial neurulation starts in the late stage of gastrulation (9 hpf, hours post fertilization), and by 30 hpf, four separate brain ventricles have formed and are visible throughout development. To assess the similarity of zebrafish and mammalian brains, the BBB in zebrafish has been identified with immunostaining ZO-1, a tight junction specific antibody. We have also assessed transport of BBB impermeable Evans blue dye from the blood vessels to the brain. Our data showed that Evans blue dye did not infiltrate brain tissue in zebrafish older than 2 days. A phylogenetic study has identified 52 ABC transporter genes in zebrafish including all 48 transcribed genes of human ABC transporters including 2 Pgp/MDR1 transporter genes (ABCB1a and ABCB1b), Mdr, ABCB1a and ABCB1b, and four paralogue copies of ABCG2 (ABCG2a, b, c and d). Expression of zABCG2a and zABCG2c mRNA and MRP1 has been observed in zebrafish. Pgp/MDR1 cDNA has been cloned and expression of Pgp/MDR1 by antibodies has been observed in the liver of 5 dpf zebrafish. Our studies also showed that embryonic zebrafish exhibit efflux transport machinery and effectively transfer xenobiotics from the brain to the blood. The transparent zebrafish brain can serve as an excellent model for studying drug retention via ABC transporters

Methods

Drug treatment for Pgp efflux: 7 dpf zebrafish were pre-soaked for one hour with different drug (1, 10, 100 µM) or 0.1% DMSO (carrier control) and kept in 28ºC incubator before injecting rho-HRP into the brain; drug incubation continued during injection and image capture at various time points.

Brain microinjection for Pgp efflux assay: After incubating for one hour at 28ºC, zebrafish were placed in the retainer containing drug solution and 0.32 mM tricaine (ethyl 3-aminobenzoate methanesulfonate) for immobilization; the retainer maintained zebrafish in dorsal position. Microinjection needle containing rho-HRP fluorescent was injected into the midline of optic tecta bordering cerebellum.

Fluorescence microscopy and image analyses: Following microinjection, zebrafish were maintained in the retainer for T0 image acquisition. After image capture at T0, zebrafish were transferred into numbered microwells of a 24-well plate containing 0.5 ml of drug solution and drug incubation continued at 28ºC. After 3 hours of incubation, zebrafish were returned to the retainer containing drug solution and 0.32 mM tricaine for T3 image capture. Fluorescence signal was measured by morphometric analysis using Image J. Dorsal images of the ROI of the brain in each animal were obtained using the same exposure time and fluorescence gain at 25x magnification. Then, a constant threshold for acceptable fluorescence signal was applied to brain images from each zebrafish. Total fluorescence intensity was derived by multiplying total area (red color) and average fluorescence intensity.

Statistics: All data were presented as mean ± SE (standard error of the mean). ANOVA was used to compare drug treated zebrafish with DMSO controls. Dunnett's t-test (pair-wise comparison) was used to determine which concentration group exhibited significant effect (P <0.05).

Zebrafish Life Cycle

Embryos: 20 hours post fertilization

Larva: 5-days post fertilization

Adult: 3 months old

Results

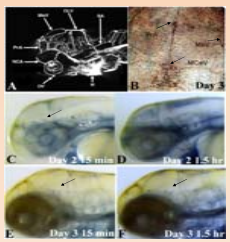


***Figure 1. Confirmation of BBB formation in zebrafish by microangiography, antibody staining and dye injection. (A)** After injecting dextran-rhodamine beads, microangiography was used to image the entire brain vasculature in 3 day zebrafish; the major brain ventricles are completely formed, angiogenic vessels are well formed, and the blood vessel network, which transports nutrients to brain cells, can be visualized in the brain. (**B**) Tight junctions are present during early development. Whole mount ZO-1 antibody immunostaining showed that tight junctions form in the brain (**brown spots**) adjacent to the: mesencephalic vein (**MsV**), middle cerebral vein (**MCeV**), and prosencephalic artery (**PrA**) (arrows). ZO-1 is a tight junction component; mouse monoclonal antibody against rat ZO-1 (Sigma, St Louis, MO) was used in this study. **BA**: basilar artery; **DLV**: dorsal longitudinal anastomotic vessel; **NCA**: nasal ciliary artery; **OV**: optic vein; **H**: heart. **(C-F)** Conventional Evans blue dye injection method was used to confirm the presence of BBB in zebrafish. Evans blue dye was microinjected into the peripheral blood vessels in 2 (**C, D**) and 3 day (**E, F**) zebrafish. Distribution of the dye was assessed in the brain at 15 min (**C, E**) and 1.5 hours (**D, F**) after microinjection. In 2 day zebrafish (**C, D**) Evans blue dye was observed in the brain blood vessels (arrow) at 15 min (**C**), however, permeation of dye to the midbrain and hindbrain region was observed at 1.5 hours (**D**). In contrast, in 3 day zebrafish (**E, F**), after injection, Evans blue dye was retained in the brain blood vessels at both 15 min (**E**) and 1.5 hours (**F**) and was not found in the brain tissue, indicating the presence of BBB in 3 day zebrafish.

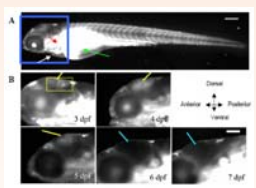


***Figure 2. Identification of optimal stage to assess Pgp efflux functional expression.** Rhodamine 123 was injected into the circulation of: 3, 4, 5, 6, and 7 dpf zebrafish. **(A)** Full body plan of 4 dpf zebrafish, lateral view. Area of interest, **blue box**. The common cardinal vein, the site of circulation injection, **red arrow**. Heart, **white arrow**. Yolk sac, **green arrow**. White scale bar is 100 µm. **(B)** Lateral images of zebrafish head 2 hours post Rhodamine 123 injection; fluorescence signal was detected in the brain of 3, 4, and 5 dpf zebrafish (**yellow lines in A and B**), indicating that the dye successfully crossed the BBB and was retained in the brain region, **yellow box**. However, fluorescence signal was absent in 6 and 7 dpf zebrafish (**blue arrows in B**), indicating the dye was pumped out of the brain, confirming that Pgp efflux system is fully functioning by 7 dpf. White scale bar is 200 µm

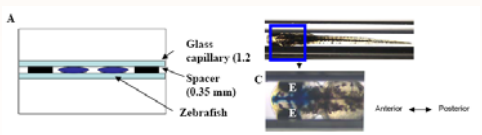


***Figure 3. Specially designed slide retainer facilitates dorsal dye injection into zebrafish brain.** (A) A cartoon illustration of the slide retainer design; 3 retainer rows can be built on each slide and 5 zebrafish can be maintained on one slide. (B) Image of 4 dpf zebrafish inside the retainer, dorsal side up. Blue box indicates the area that was magnified in (C); an image of 4 dpf zebrafish injected with trypan blue in the brain. E indicates eyes.

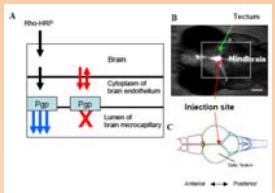


***Figure 4. Rho-HRP clearance from the brain.** (A) A schematic diagram of rho-HRP movement. In presence of Pgp efflux (blue arrows), rho-HRP in the brain moves into the cytoplasm of brain endothelium (black arrows) where it is pumped out into the lumen of brain microcapillaries. In presence of Pgp inhibitor (red x), rho-HRP accumulates in the brain and the cytoplasm of brain endothelium (red arrows). (B) Dorsal view of fluorescence brain image of 7 dpf zebrafish injected with rho-HRP at 20x magnification. The region of interest (ROI) is indicated by white box. White scale bar is 100 µm. (C) A cartoon of zebrafish brain in dorsal position (Wullimann and Reichert 1996). Injection site is indicated by red arrows.

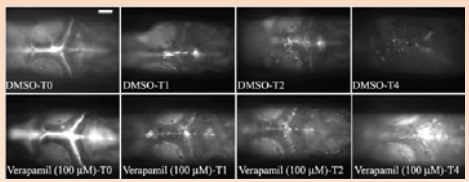
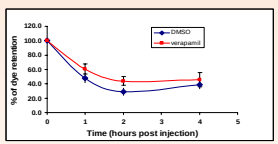


***Figure 5. Kinetics of rho-HRP clearance.** 7 dpf zebrafish were soaked in either: 0.1% DMSO or Verapamil for one hour before injecting rho-HRP into the brain. Dorsal view ROI images were captured at 25X magnification at: 15 minutes post injection (designated as T0) and at 1, 2, and 4 hpi designated as T1, T2, and T4, respectively. Fluorescence signal decreased at T1, T2 in both conditions. By T4, minimal fluorescence signal was detected in 0.1% DMSO control animal. In contrast, fluorescence signal remained strong in Verapamil treated animals, indicating reduced Pgp mediated Rho-HRP clearance. Anterior to the left; white scale bar is 100 µm.



Time point	0.1% DMSO	Verapamil (100 µM)
T0	100%	100%
T1	48 ± 6%	61 ± 7%
T2	29 ± 5%	44 ± 6%
T4	39 ± 6%	46 ± 10%

***Figure 6. Kinetic curves of rho-HRP clearance.** Morphometric analysis was performed on brain images, as shown in Figure 7. Fluorescence intensity at each time point was expressed as % intensity at T0 and plotted against time to obtain kinetic curves. Each data point represents mean ± SE (n = 10).

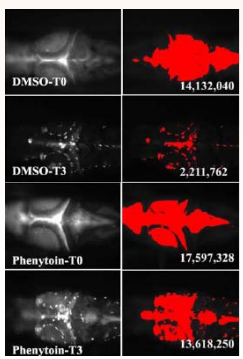


***Figure 7. Morphometric analysis of acquired fluorescence images.** Fluorescence images of ROI region from individual zebrafish were captured at 25X. T0 and T3 images of: a) DMSO control and b) 100 µM Phenytoin treated zebrafish brains were acquired, as shown in the Left panels. The same threshold was applied to images which were then converted to red color (right panels). Total fluorescence intensity (area X average fluorescence intensity) in the red colored area was measured using Image J software. Dye retention was 15.8% ((2,211,762/14,132,040) x 100%) for DMSO treated animals and 77.4% ((13,618,250/17,597,328) x 100%) for Phenytoin treated animals. Anterior to the left.

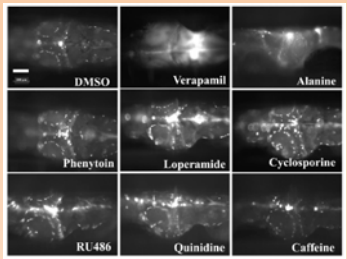


***Figure 8. Drug effects on Pgp efflux. Clearance of rho-HRP from the brain was used to assess Pgp efflux.** Varying drug concentrations were pre-incubated with 7 dpf zebrafish for one hour before injecting rho-HRP into the brain. Representative T3 images of ROI in brain, dorsal view, are shown. At T3, Verapamil, Phenytoin, Loperamide, Cyclosporine, RU486, Quinidine, 6 positive control compounds, all exhibited stronger fluorescence intensity than the DMSO control, indicating that theses drugs inhibited Pgp efflux. DMSO and Caffeine treated zebrafish exhibited limited fluorescence intensity at T3, indicating that Caffeine, a negative control compound did not inhibit Pgp efflux. However, Alanine, a negative control compound exhibited stronger fluorescence intensity than DMSO control, indicating that Alanine inhibited Pgp efflux. Anterior to left. White scale bar is 100 µm.

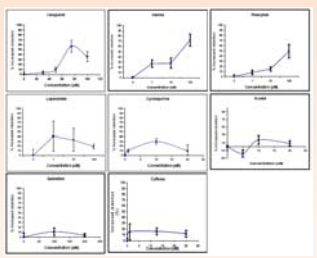


***Figure 9. Dose response curves of drug effects on Rho-HRP retention.** X axis represents drug concentration. Y axis represents % increased Rho-HRP retention. Each point represents mean ± SE (5 ≤ N ≤ 12).

Compounds	Pharmaceutical Function in Mammals	Pgp Efflux in Zebrafish (In Vivo)	Pgp Efflux in Cells/ Tissues (In Vitro)	Pgp Efflux in Mammals (In Vivo)	Correct Prediction
Verapamil	Calcium channel blocker	Inhibition	Inhibition[1]	Inhibition[2]	yes
Alanine	Amino acid	Inhibition	NA	NA	Inconclusive
Phenytoin	Antiepileptic agent	Inhibition	Inhibition[3]	Inhibition[4]	yes
Loperamide	Immunosuppressant	Inhibition	Inhibition[5]	NA	yes
Cyclosporine	Immunosuppressant	Inhibition	Inhibition[6]	Inhibition[7]	yes
Mefepristone (RU486)	Progesterone antagonist	Inhibition	Inhibition[8]	NA	yes
Quinidine	Antiarrhythmic agent	Inhibition	Inhibition[9]	Inhibition[10]	yes
Caffeine	Neural stimulator	None	None	NA	yes

Figure 10. Comparison of Pgp efflux inhibitors in zebrafish, cells/tissues, and mammals

Conclusions

Drugs known to inhibit Pgp efflux invitro and invivo systems show similar effects in zebrafish.

Zebrafish assays are rapid, quantitative, and reproducible. Only small amounts of drug are needed.

Zebrafish *in vivo* PGP efflux assay provide useful information that supplements conventional mammalian assays.

PHYLONIX