Selective Inhibition of Retinal Angiogenesis by Targeting PI3 Kinase

Yolanda Alvarez1*, Olaya Astudillo1, Lasse Jensen2, Alison L. Reynolds1, Nora Waghorne1, Derek P. Brazil3, Yihai Cao2, John J. O’Connor1, Breándán N. Kennedy1*

1 UCD Schools of Biomolecular and Biomedical Sciences, UCD Conway Institute, University College Dublin, Dublin, Ireland, 2 Institute for Microbiology Tumor and Cell Biology, Karolinska Institute, Stockholm, Sweden, 3 Centre for Vision and Vascular Science, School of Medicine, Dentistry and Biomedical Science, Queen’s University Belfast, Belfast, United Kingdom

Abstract
Ocular neovascularisation is a pathological hallmark of some forms of debilitating blindness including diabetic retinopathy, age related macular degeneration and retinopathy of prematurity. Current therapies for delaying unwanted ocular angiogenesis include laser surgery or molecular inhibition of the pro-angiogenic factor VEGF. However, targeting of angiogenic pathways other than, or in combination to VEGF, may lead to more effective and safer inhibitors of intraocular angiogenesis. In a small chemical screen using zebrafish, we identify LY294002 as an effective and selective inhibitor of both developmental and ectopic hyaloid angiogenesis in the eye. LY294002, a PI3 kinase inhibitor, exerts its anti-angiogenic effect in a dose-dependent manner, without perturbing existing vessels. Significantly, LY294002 delivered by intraocular injection, significantly inhibits ocular angiogenesis without systematic side-effects and without diminishing visual function. Thus, targeting of PI3 kinase pathways has the potential to effectively and safely treat neovascularisation in eye disease.

Introduction
Ocular Diseases and Neovascularisation
Functional damage to existing blood vessels and inappropriate development of new blood vessels are hallmarks of prominent forms of blindness. In diabetic retinopathy (DR), which affects ~150 million patients, neovascularisation of vessels in the adult retina occurs [1]. In age-related macular degeneration (AMD), which is estimated to affect ~8 million US patients and ~20 million patients in US plus Europe, neovascularisation of capillaries in the choroid layer adjacent to the retina occurs [2]. Ocular neovascularisation associated with retinopathy of prematurity (ROP) arises when premature infants are placed into high oxygen to alleviate respiratory distress. As a consequence, concomitant regression of the lens-associated hyaloid vasculature and growth of the nascent retinal vasculature ceases. When returned to normoxia, the infant retina becomes hypoxic leading to neovascularisation, persistent hyaloid vasculature and eventual blindness [3]. Thus, a therapeutic goal for DR and AMD is to develop agents that inhibit ocular neovascularisation and/or stabilise existing vessels, without diminishing visual function.

Current Ocular Angiogenesis Treatments
Current interventions for resolving inappropriate growth of new vessels in the eye include laser treatment or molecular therapies targeted to vascular endothelial cell growth factor (VEGF). Although the therapeutic mechanism of action is not fully understood, laser photocoagulation surgery can halt neovascularisation and reduce macular oedema [1,4]. However, success is partial, requires multiple treatments and side effects include cataracts, hemorrhage, retinal detachment and visual field loss.

Variations of VEGF molecular therapy to inhibit ocular neovascularisation include antibodies, siRNAs or small molecule inhibitors [5–10]. Those in clinical use are antibodies targeted to VEGF (e.g. bevacizumab (Avastin®) and ranibizumab (Lucentis®)) which delay the growth of new vessels and delay vision loss. Treatment also requires intraocular injection by retinal specialists, repeat administrations and patient sedation. Concerns regarding the development of siRNAs to VEGF have also arisen as they may act non-specifically [7]. More general concerns regarding the strategy of solely targeting VEGF include, the observed compensatory up-regulation of other pro-angiogenic factors which decrease therapeutic efficacy, and the potential to cause retinal neurodegeneration by inhibiting VEGF’s neuroprotectant function [11,12].

Alternative Anti Angiogenic Targets
Targeting angiogenic molecules other than, or in addition to VEGF, may reveal more effective and safer inhibitors of intraocular neovascularisation. Potential targets include growth factors (e.g. angiopoietin, FGF, HGF, IGF-1, PDGF-B, PIGF), chemokines (e.g. IL8, SDF1, G-CSF), receptors (e.g. CXCR1, FGF-R, PIGF, PDGFR, Tie-receptors), intracellular mediators (e.g. c-kit kinase, PI3 kinase, PKC) and extracellular mediators (e.g. integrins, cadherins) [13].
Several drugs which do not selectively target VEGF have indeed shown anti-angiogenic efficacy in eyes. Pazopanib, PKC412 and TG100572 are small molecule inhibitors that each targets multiple kinases [6,9,14]. Pazopanib (Armala®) which blocks PDGFRs, c-Kit, FGFR and c-fms, and TG100572, which inhibits FGFR, PDGF and VEGF, both suppress choroidal neovascularisation in mouse models [6,9]. TG100572 has improved delivery features as it is effective following local administration as eye drops [6]. PKC412 blocks PKC, VEGF-R, PDGFR and SCF-R isoforms, and in diabetics is reported to reduce macular oedema [14]. However, liver toxicity was observed following oral administration.

Another approach is to selectively target a distinct pro-angiogenic molecule. Indeed recent studies report that the broad-spectrum PI3K inhibitor LY294002 and the αβ3/αβ5 integrin antagonist EMD478761 suppress retinal or choroidal neovascularisation following intraocular injection in rodents [15,16]. A combination of both approaches involves targeting multiple foci of pro-angiogenic pathways with several selective inhibitors. Dorrell et al. report that combination angiothelial therapy with a VEGF aptamer, an integrin antagonist and a proteolytic fragment of tryptophan tRNA synthetase inhibits ocular angiogenesis [17].

Zebralsh Intraocular Vasculature and Chemical Screens

We recently demonstrated that the inner retina of zebralsh is nourished by an intricate vascular network which shares many features with human hyaloid and retinal vasculatures [17]. Initially this intraocular vasculature presents as a hyaloid vasculature more tightly associated with the lens which in adults has migrated to firmly attach to the inner retina. We identified a cohort of genes necessary for normal hyaloid vasculature formation in zebrafish [17]. In addition to amenability towards genetic screens, zebralsh are also suitable for pharmacological screens [18,19]. In relation to intraocular vasculature, Cao et al. recently show that exposure of adult zebralsh to hypoxia combined with treatment with a notch signaling inhibitor is sufficient to induce retinal neovascularisation [20]. In a random screen of small molecules, Kitambi et al. recently identified novel molecules inhibiting hyaloid angiogenesis in zebralsh larvae [21].

Summary

Here, we conduct a targeted screen of known regulators of angiogenesis to identify drugs that inhibit developmental angiogenesis in the zebralsh eye. LY294002, a small molecule inhibitor of PI3 kinase was identified as an effective inhibitor of developmental angiogenesis in the eye. LY294002 inhibits new vessel growth without any apparent effect on existing vessels. The anti-angiogenic effect of LY294002 is dose-dependent and can be phenocopied using an inhibitor to AKT, which itself is known to act downstream of PI3K. Importantly, intraocular administration of LY294002 selectively inhibits angiogenesis in the eye but does not diminish visual function.

Methods

Ethics Statement

All experiments were carried out under ethical approval granted by the UCD animal research ethics committee (http://www.ucd.ie/researchethics/arec.html).

Drug Treatment

*Tg(fli1:EGFP)* zebralsh were maintained according to standard procedures on a 14 h light/10 h dark cycle at 28°C. Embryos were obtained by natural spawning and developmental stages established by time and morphological criteria. At 24 hpf, 5 embryos per well were placed in 400 µl of Embryo Medium/1% DMSO and incubated with drug at 28°C on a 14 h light/10 h dark cycle [22]. Larvae were euthanised, and fixed in 4% PFA at 4°C overnight before analysis.

Quantification of Primary Branch Number

Prior to analysis of the intraocular vasculature, control- and treated-larvae were screened under an Olympus SZX16 microscope for general malformations. The overall patterning of the fin, gut and intersegmental vasculature was examined. Larval lenses from right eyes were dissected, transferred to depression slides and observed by epi-fluorescence microscopy using an Olympus SZX16, or placed in petri-dishes with cover-slip bottoms for confocal microscopy using a Zeiss UV510 META LSM. To orientate, lenses were embedded in 10% methyl-cellulose and manipulated with a 0.5 mm tungsten needle. Number of primary branches radiating from optic disc at the back of lens was determined for each specimen (see white arrows in Fig. 1).

Regenerative Angiogenesis Assay

Adult *Tg(fli1:EGFP)* zebralsh were anaesthetised in 0.02% Tricaine (Sigma) and caudal, dorsal or anal fins amputated with a scalpel below the second branch points. Fish were incubated in tank water or tank water/10 µM LY294002 (Sigma) for 9 days, euthanised in 0.04% Tricaine, decapitated and fixed in 4% PFA (Sigma) for 24 hours at 4°C. Fins were mounted on glass slides in VectaShield (Vector) and visualised on an Olympus SZX12 fluorescent microscope. Images were acquired using a Hamamatsu ORCA-ER camera with OpenLab capturing software and adapted for publication in Photoshop CS2 9.02.

Electroretinography

Electroretinograms (ERGs) were performed on *Tg(fli1:EGFP)* zebralsh treated from 2–5 dpf with 10 µM LY294002 (n = 15) or 1% DMSO vehicle (n = 13). ERGs were performed in *vivo* during the afternoon as described by Fleisch et al., with a few modifications [23]. Larvae were paralysed with 0.5 mg/ml mivacurium chloride (Mivacron®) and the recording electrode filled with 0.9% saline. A Kodak projector containing a 300W halogen light source was used for light stimulation. The unattenuated irradiance stimulus was 2.8×10^(-3) µW/cm^2^. Three optical density filters were used to produce flash intensities of −3.0 log, −2.0 log and −1.0 log together with the unattenuated stimulus, −0 log. Flash duration was 100 milliseconds for all intensities, with an interstimulus interval of between 10 seconds and 1 minute, depending on flash intensity. To digitise and visualise data, a NIDAQ 6024E board (National Instruments) and Windows Whole Cell Programme software (WinWCP V3.9.1; courtesy of John Dempster, Strathclyde University, Scotland) were used. Data shown are signal averages of 2–10 responses. The a-wave was measured from baseline to the trough of the a-wave, and the b-wave was measured from the trough of the a-wave to the peak of the b-wave. Raw data from the sample groups were compared using an independent 2-sample t-test with unequal variances.

Visual Behaviour Assay

Visual function was assessed by the optokinetic-response (OKR) assay [24]. Briefly, 5 dpf larvae in Embryo Medium/9% methylcellulose were placed inside a black and white striped drum. The drum was rotated for 30 seconds in clockwise then counterclockwise directions and the number of eye saccades counted.
Retinal Light Microscopy

Larvae were euthanised with a lethal dose of Tricaine and fixed in 0.1 M Sorensen phosphate buffer (pH 7.5)/4% PFA/2.5% glutaraldehyde overnight at RT. Larvae were post-fixed in 0.1 M Sorensen phosphate buffer/1% osmium tetroxide for 1 hour at RT, dehydrated in ascending concentrations of alcohol to 100% and embedded in Epon resin using standard methods [23]. 1 μm sections were stained for 20 seconds with toluidine-blue, and examined by light microscopy using a Leica DMLB bright field illumination microscope and a Leica DFC 480 camera.

Intraocular Drug Treatments

Tg(fli1:EGFP) larvae were anesthetised with 0.02% Tricaine, dechorionated and transferred to 3 cm Petri dishes containing 1 ml of cold-liquid CyGEL™ (Biostatus). Larvae were immobilised in CyGEL at room temperature with the left eye facing upwards for injection (Fig. S1). Immobilised larvae were injected in the left eye at 1 or 2 dpf with 10 μM LY294002 or 1% DMSO diluted in ddH₂O/1% FastRed tracer dye using heat pulled glass capillaries (bore diameter OD/ID: 1/0.58 mm). After intraocular injection, larvae were retrieved by placing the dish on a chilled ice pad to melt the CyGEL and immediately transferred to Embryo Medium at RT for recovery. Larvae were maintained at 28°C until 5 dpf and hyaloid vasculature analysed as described above.

Results

LY294002 Inhibits Developmental Angiogenesis of the Eye

To identify drugs that significantly alter development of new blood vessels in the eye, we performed a small pharmacological screen in zebrafish. The drugs screened (Table S1) are known to control growth factor-, cytokine- or inflammatory-mediated angiogenesis in other tissues, and several are used clinically to treat AMD, cancer or rheumatoid arthritis [26–28]. Tg(fli1:EGFP) larvae which specifically express EGFP in all blood vessels [29] were immersed in drug from 1–5 days post fertilisation (dpf) and the integrity of the hyaloid vasculature determined by fluorescent microscopy of dissected lenses.

None of the growth factors, cytokines or antibodies affect the number of primary branches of hyaloid vasculature (Fig. 1A).

Figure 2. LY294002 results in dose-dependent inhibition of intraocular angiogenesis. 1 dpf Tg(fli1:EGFP) larvae were treated with LY294002 at concentrations ranging from 2.5–20 μM and the number of primary branches of hyaloid vessels determined at 5 dpf. The data graphed is plotted as the average and standard deviation of 3 replicate experiments. LY294002 results in a statistically significant inhibition of hyaloid vasculature development (**p<0.01, t-test).

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However, the small molecule drug LY294002 significantly reduces the number of primary branches of hyaloid vasculature, indicative of anti-angiogenic activity (Fig. 1A). Analysis of wholemount larvae suggests that LY294002 treatment has no significant effect on the intersegmental or trunk vessels (Fig. 1B), which develop earlier than the hyaloid vasculature [17,29]. Confocal analysis

Figure 3. Time-dependent inhibition of intraocular, developmental angiogenesis by LY294002. Tg(fli1:EGFP) larvae were treated with 10 μM LY294002 for the indicated time frames and the number of primary branches of hyaloid vessels determined at 5 dpf. The data is plotted as the average and standard deviation of 3 replicate experiments. 10 μM LY294002 is added for 24 hours (A) or 48 hours (B) starting at 1, 2, 3 or 4 dpf. In C 10 μM LY294002 is added at 1 dpf for 96, 72, 48 or 24 hours. In any experimental variation, the presence of LY294002 from 2–3 dpf is sufficient to result in a statistically significant inhibition of developmental angiogenesis of hyaloid vessels (*p<0.05; **p<0.001, t-test).

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Figure 4. The Akt inhibitor SH6 phenocopies the PI3K inhibitor LY294002. Comparison of hyaloid vasculature patterning in DMSO-treated control larvae (A), 10 μM LY294002 treated larvae (B) and larvae treated with the Akt inhibitor SH6 at 10 μM (C). All larvae are from the Tg(fli1:EGFP) line and were treated from 1–5 dpf. Like LY294002, SH6 affects angiogenesis of the hyaloid vessels. Abnormal patterning of the hyaloid vasculature is observed with SH6 (C) resembling LY294002-treated larvae (B), though no significant reduction in primary vessel number is observed with SH6 (D). The data shown in panel D is plotted as the average and standard deviation of 3 replicate experiments (**p<0.001, t-test).

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clearly demonstrates that treatment with 10 μM LY294002 reduces the branch number and patterning of the hyaloid vasculature (Fig. 1C). This result suggests that the PI3K inhibitor, LY294002, inhibits the formation of new vessels but does not reduce the number of existing vessels systemically.

Dose- and Time-Dependent Inhibition of Intraocular Angiogenesis by LY294002

Treating larvae from 1–5 dpf, with a range of LY294002 concentrations, reveals a dose-dependent inhibition of hyaloid vessel angiogenesis with an IC_{50} of ~12 μM (Fig. 2). At concentrations from 7.5–12.5 μM LY294002, statistically significant inhibition of hyaloid angiogenesis is observed in the absence of gross morphological defects. Larvae incubated in 10 μM LY294002 occasionally display mild oedema indicative of systemic side effects. At concentrations of 17.5 μM LY294002 and above, systemic toxic effects including oedema, bent body axis and craniofacial abnormalities are observed. Thus, 10 μM LY294002 was utilised for all additional experiments.

To determine if LY294002 inhibits hyaloid angiogenesis during a specific developmental time-frame, 1–4 dpf Tg(fli1:EGFP) larvae were treated for 24, 48, 72 or 96 hours, and hyaloid vasculature analysed at 5 dpf (Fig. 3). If LY294002 is added to 1 or 4 dpf larvae for 24 hours, development of the hyaloid branches is unaffected (Fig. 3 A & C). However, the presence of LY294002

![Image](image_url)

Figure 5. LY294002 attenuates ectopic developmental angiogenesis in obd mutants. Wildtype Tg(fli1:EGFP) larvae (wt) or out of bounds mutants in the Tg(fli1:EGFP) background (obd) were treated with 10 μM LY294002 from 1–5 dpf before dissecting lenses and analysing hyaloid vasculature development. LY294002 inhibits normal developmental angiogenesis in wildtype Tg(fli1:EGFP) larvae as seen by the reduced vessel patterning in lateral views of the lens (A, D). LY294002 also inhibits the extraneous hyaloid branch patterning (B) and branch number (C) observed in obd mutants as observed by reduction of branch patterning to near normal levels (E and F). Arrows depict the orientation of the lens, pointing in the direction from the optic disk to lens. G) Quantification of the reduction in the extraneous primary hyaloid branches number in LY294002-treated obd mutants. The data is plotted as the average and standard deviation of larvae (obd−/− n = 7, obd−/+ treated: n = 8) from 2 independent experiments (**p<0.001, t-test). OD: Optic Disc.

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from 2–3 dpf is sufficient to result in a statistically significant inhibition of hyaloid angiogenesis. These results are consistent with LY294002 blocking developmental angiogenesis of new hyaloid vessels but exerting no effect on existing intraocular vessels.

The PI3K-Akt Pathway Controls Hyaloid Angiogenesis

To corroborate the anti-angiogenic activity of LY294002 on hyaloid vasculature being mediated via inhibition of PI3K signalling, we selectively inhibited the activity of Akt/PKB, a downstream target of PI3K [30]. Analysis of hyaloid vessels at 5 dpf demonstrates that SH6, a PKB/Akt inhibitor, can phenocopy LY294002. Both drugs at 10 μM, result in a striking difference in the patterning of hyaloid vessels (Fig. 7A–C). Interestingly, LY294002, but not SH6, also significantly reduces the number of primary hyaloid vessels (Fig. 7D).

LY294002 Attenuates Ectopic and Regenerative Angiogenesis

The ability of LY294002 to inhibit extraneous angiogenesis was determined in out of bounds (obd) mutants. As a consequence of a recessive mutation in plexin D, obd /-/- larvae present with ectopic developmental angiogenesis [17,31]. Treatment of obd /-/- larvae with 10 μM LY294002 inhibits ectopic hyaloid angiogenesis and can reduce branch patterning near wild type levels (Fig. 5).

The anti-angiogenic efficacy and safety of LY294002 in the context of adult tissues was determined using a fin regeneration assay [32]. The extent of angiogenesis in regenerating fins was determined in adult fish following caudal, dorsal or anal fin clipping. Compared to controls, LY294002 significantly inhibits regenerative angiogenesis (Fig. 6 and Fig S2). LY294002 also inhibits regeneration length, which is reported to be angiogenesis-dependent [32] (Fig. 6 and Fig S2). Otherwise, no toxic effects were observed, and LY294002- and control-treated adults were indistinguishable in relation to behaviour, ventilation rate and swimming patterns.

LY294002 Inhibits Retinal Angiogenesis without Affecting Retinal Function

The electroretinogram (ERG) is a diagnostic method for quantifying outer retinal function. ERGs from larvae treated with LY2940002 or vehicle from 2–5 dpf show equivalent responses at 4 flash intensities (Fig. 7A–C). At maximum flash intensity, the a-wave measure of photoreceptor hyperpolarisation has amplitudes of −28±5.8 μV or −31±3.5 μV, and implicit times of 61±7.6 milliseconds (ms) or 68±7.7 ms in control and drug-treated larvae, respectively. Similarly, the b-wave which reflects signal transmission to bipolar cells has amplitudes of 199±20.5 μV or 208±28 μV and implicit times of 157±10.5 ms or 169±10.1 ms in control and drug-treated larvae, respectively (Fig. 7A–C). In agreement, the retinal histology of larvae treated with LY294002 from 2–5 dpf is indistinguishable from controls. Both samples consist of laminated retinas with prominent optic nerves and transparent lenses (Fig. 7D).

Retinal histology in larvae treated earlier from 1–5 dpf does feature apoptotic nuclei and tissue vacuolation (Fig. 7D). This is consistent with a loss of visual function in larvae treated with LY294002 for just 24 hours beginning at 1 dpf and suggests a role for PI3K in early retinal development, independent of angiogenesis. In summary, treatment with LY294002 can block hyaloid angiogenesis with no detrimental effect on retinal function.

Intraocular LY294002 Selectively Inhibits Angiogenesis and not Visual Behaviour

Finally, we determined if localised delivery of LY294002 can inhibit intraocular angiogenesis, and do so without systemic side-effects, or effects on retinal function. Compared to larvae injected with vehicle alone, larvae injected with 10 μM LY294002 do display significant inhibition of hyaloid vessel angiogenesis (Fig. 8A, C). Unlike the mild oedema observed in larvae treated systemically with LY294002, intraocular injection of LY294002 has no obvious systemic side-effects. Visual function in larvae injected intraocularly with LY294002 was analysed by the optokinetic response (OKR) [24]. In this context of adult tissues was determined using a fin regeneration assay [32]. The extent of angiogenesis in regenerating fins was determined in adult fish following caudal, dorsal or anal fin clipping. Compared to controls, LY294002 significantly inhibits regenerative angiogenesis (Fig. 6 and Fig S2). LY294002 also inhibits regeneration length, which is reported to be angiogenesis-dependent [32] (Fig. 6 and Fig S2). Otherwise, no toxic effects were observed, and LY294002- and control-treated adults were indistinguishable in relation to behaviour, ventilation rate and swimming patterns.

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assay, fish with normal visual function track rotating black and white stripes via reproducible saccades. No difference is observed in the OKR response of fish injected intraocularly with vehicle alone or with LY294002 (Fig. 8B). The results indicate that intraocular administration of LY294002 can selectively block angiogenesis in the eye without detrimental effects on visual function.

Discussion

Summary

We report here evidence demonstrating that the small molecule inhibitor LY294002 can be an effective and safe inhibitor of ocular angiogenesis in vivo. LY294002 inhibits normal and extraneous developmental angiogenesis in the zebrafish eye, in dose- and time-dependent manners. Systemic administration of LY294002 results in oedema and locomotor activity defects are indicated (data not shown). However, intraocular administration of LY294002 can selectively inhibit intraocular angiogenesis without affecting retinal morphology or visual function.

LY294002- An Ocular Anti-Angiogenic

Does LY294002 or its cellular target have potential for clinical use as an ocular anti-angiogenic? Our data demonstrates that LY294002 is an effective inhibitor of hyaloid vasculature angiogenesis in the zebrafish eye. In addition, recent studies report that intraocular administration of LY294002 reduces unwanted retinal and choroidal neovascularisation in murine models [15,16]. The mouse studies did not address potential effects of LY294002 on retinal function, but our study shows that short-term treatment with LY294002 can have no effect on ocular morphology or visual physiology. LY294002 is most likely exerting its anti-angiogenic activity by inhibiting the PI3K signalling pathway, which is known to mediate angiogenesis [39]. Indeed, LY294002 is a classic, broad-spectrum inhibitor of phosphoinositide 3-kinases (PI3K) [33]. The PI3K enzyme family act as second messengers during signal transduction events associated with diverse cellular activities, including migration, proliferation and differentiation [39]. Recent evidence suggests that LY294002 also inhibits casein kinase, GSK3β and mTOR (a PI3K-related kinase) [34]. LY294002, however, was previously excluded from
clinal development due to toxicity issues. These may arise from the broad-spectrum of PI3Ks inhibited, off-target effects or systemic administration.

Refined Targeting of PI3K Pathways

Our data suggests that LY294002 can have selective anti-angiogenic effects in the eye following intraocular administration. No perturbations of retinal function or morphology are observed. Future studies will need to determine if the lack of toxicity is due to local administration to the eye, species difference or short-term treatment.

However, instead of using a broad-spectrum PI3K inhibitor like LY294002, it should also be possible to selectively interfere with the subset of PI3K signals associated with pathological angiogenesis in the eye. The development of isoform-selective PI3K inhibitors has already led to their approval for clinical trials in cancer [35,36]. The PI3K family is divided into several classes based on protein structure and substrate targets. The signal transduction mechanism is best understood for Class I PI3Ks [39]. These are heterodimers, comprising of one of four p110 catalytic subunits (α, β, δ or γ) plus a regulatory subunit. Growth factor or G protein-coupled receptor activation recruits the regulatory subunit of PI3K resulting in activation of the catalytic subunit. Subsequent conversion of PI(4,5)P2 to PI(3,4,5)P3 promotes the recruitment of proteins exemplified by AKT to the plasma membrane. AKT is phosphorylated by phosphoinositide-dependent protein kinases and targets proteins involved in angiogenesis, apoptosis, cell growth and glucose uptake [37,38].

Analysis of p110δ and γ reveals specific roles for these isoforms in the immune system. Indeed, IC87114, an inhibitor selective for p110δ, was subsequently found to inhibit a form of leukaemia and is now in clinical trial [39]. Similarly, genetic analyses demonstrate that the p110δ isoform is selectively required for angiogenesis [40].

To our knowledge no p110δ-selective inhibitor has gone on to clinical development. However, several potential p110δ selective inhibitors have been reported [35,39]. Our data with LY294002 strongly support testing the efficacy and safety of p110δ-selective PI3K inhibitors in models of ocular neovascularisation.

An alternative approach to isoform-selective PI3K inhibitors is the use of a modified version of LY294002. Until recently, it was generally considered that broad-spectrum PI3K inhibitors would not have clinical use due to toxicity arising from inhibition of physiological signalling. However, SF1126 is a modified version of LY294002 linked to a peptide sequence that targets it to the vasculature [41]. Future studies are warranted to test the efficacy and safety of this prodrug in models of ocular angiogenesis.

Screens for Ocular Anti-Angiogenics in Zebrafish

Here we provide further proof-of-principle that chemical screens in zebrafish can uncover novel anti-angiogenic drugs for the eye. The screens are relatively simple with 5 larvae per well incubated from 1–5 days post-fertilisation with an experimental drug. Assessment of ocular angiogenesis is facilitated by the use of the Tg(fli1:EGFP) line and a fluorescent dissecting microscope. The limiting step is the labour-intensive dissection of lenses but this may be overcome with improvements in imaging in vivo. Kitambi et al. recently identified several drugs that perturb intraocular angiogenesis in vivo [21] and our study demonstrates how such drugs can be further characterised to address specificity and safety.

Figure 8. Intraocular injection of LY294002 inhibits angiogenesis but not visual function. A) The hyaloid vasculature of 5 dpf larvae intraocularly injected with 10 μM LY294002 at 2 dpf shows abnormal patterning and reduced numbers of primary branches compared with controls injected with 1% DMSO (left images are lateral views, and right images are dorsal views of lenses. OD: optic disc; scale bars: 50 μm) B) Graph of the percentage of 5 dpf larvae with optokinetic responses (OKR) following intraocular injection of LY294002 at 1 or 2 dpf. The Fisher test shows no statistically significant difference in visual function when LY294002 is injected intraocularly at 1 or 2 dpf (p values shown on table) C) Graph showing percentage of 5 dpf larvae with normal hyaloid vasculature after injection of LY294002 at 1 or 2 dpf. Intraocular LY294002 at 1 or 2 dpf, significantly inhibits hyaloid angiogenesis. Data graphed from 3 independent experiments; LY294002 at 1 dpf, n = 17; 1% DMSO at 1 dpf, n = 9; LY294002 at 2 dpf, n = 26; 1% DMSO at 2 dpf, n = 13 (**p<0.001, Chi-Square test). doi:10.1371/journal.pone.0007867.g008

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appear most suited to stable small molecule chemicals as antibodies or recombinant proteins had no effect, most likely due to poor bioavailability in the eye or degradation.

Future Perspectives

In summary, we provide evidence that a broad-spectrum PI3K inhibitor can attenuate ocular angiogenesis without perturbing visual function. Thus, there can be cautious optimism about the potential clinical use of PI3K inhibitors to treat unwanted ocular neovascularisation. This could involve administration of an isomform-specific inhibitor or a vascular-targeted prodrug, either as monotherapies or part of combination angiostatic therapies.

Supporting Information

Figure S1 Intraocular injection of LY294002 in zebrafish larvae. A) ~5 dechorionated zebrafish larvae (48 hpf in the figure) are immobilized in CyGEL at RT and orientated with left eye facing upwards. Dechorionation is extremely important as non-dechorionated larvae collapse in contact with CyGEL. B) Intraocular injection (0.1–0.2 μl/eye) is performed with a heat pulled glass capillary. C) After intraocular injection the Petri dish is placed on a chilled ice pad to melt CyGEL and larvae are transferred gently but immediately to a plate with RT embryo medium for recovery. Found at: doi:10.1371/journal.pone.0007867.s001 (4.36 MB TIF)

Figure S2 Regenerative angiogenesis in zebrafish dorsal or anal fins is inhibited by LY294002. The dorsal (A, C and E) or anal fins (B, D and F) of adult Tg(fli1:EGFP) zebrafish were amputated and left to regenerate in either tank water or tank water supplemented with 10 μM LY294002. Bright field micrographs (A and B) or fluorescent micrographs (C and D) of representative fins at 9 days post amputation are shown. The maximal, average blood vessel length in the regenerated tissue was quantified at the third fin ray from either side of each image and the results are depicted in (E and F). LY294002 significantly inhibits regenerative angiogenesis in both dorsal and anal fins (**p<0.001; t-test). Yellow lines in C and D indicate the amputation planes. n=8 fin rays in 4 fish. Scale bars = 500 μm. Found at: doi:10.1371/journal.pone.0007867.s002 (4.74 MB TIF)

Table S1 Pro- and Antiangiogenic drugs screened for effects on angiogenesis of the zebrafish hyaloid vasculature. Found at: doi:10.1371/journal.pone.0007867.s003 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: BNK. Performed the experiments: YA OAF LDEJ ALR NW. Analyzed the data: YA OAF LDEJ ALR NW DPB YC JJO BNK. Wrote the paper: YA BNK.

References

1. Frank RN (2004) Diabetic retinopathy. N Engl J Med 350(1): 48–58.
2. Jager RD, Miller WF, Miller JW (2000) Age-related macular degeneration. N Engl J Med 352(24): 2606–17.
3. Pierce EA, Foley ED, Smith LE (1996) Regulation of vascular endothelial growth factor by oxygen in a model of retinopathy of prematurity. Arch Ophthalmol 114(11): 1299–304.
4. Hernandez C, Simo R (2007) Strategies for blocking angiogenesis in diabetic retinopathy: from basic science to clinical practice. Expert Opin Investig Drugs 16(8): 1289–96.
5. Avery RL, Peramici DJ, Rabena MD, Castellari AA, Nasir MA, et al. (2006) Intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. Ophthalmology 113(3): 363–372 e3.
6. Doukas J, Mahesh S, Umeda N, Kachi S, Akiyama H, et al. (2008) Topical administration of a multi-targeted kinase inhibitor suppresses choroidal neovascularization and retinal edema. J Cell Physiol 216(1): 29–37.
7. Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, et al. (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. J Immunol 180(7): 591–7.
8. Rosenfeld PJ, Heier JS, Hantsbarger G, Shams N (2006) Tolerability and efficacy of multiple escalating doses of ranibizumab (Lucentis) for neovascular age-related macular degeneration. Ophthalmology 113(3): 29–37 e5.
9. Takahashi K, Sashin Y, Sashin Y, King AG, Levin R, et al. (2009) Suppression and regression of choroidal neovascularization by the multitargeted kinase inhibitor pazopanib. Arch Ophthalmol 127(4): 494–9.
10. Group ES (2003) Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization due to age-related macular degeneration: phase II study results. Ophthalmology 110(9): 979–96.
11. Dorrell MI, Aguilar E, Scheppele K, Barnet FH, Friedlender M (2007) Combination angiostatic therapy completely inhibits ocular tumor angiogenesis. Proc Natl Acad Sci U S A 104(3): 967–72.
12. Saint-Geneix M, Mahuraj AS, Wahle TE, Tucker BA, Sekiya Y, et al. (2008) Endogenous VEGF is required for visual function: evidence for a survival role on muller cells and photoreceptors. PLoS One 3(11): e3554.
13. Ferrari N, Kerbel RS (2005) Angiogenesis as a therapeutic target. Nature 430(7007): 967–74.
14. Campochiaro PA (2004) Reduction of diabetic macular edema by oral administration of the kinase inhibitor PKC412. Invest Ophthalmol Vis Sci 45(13): 3920–22.
15. Yang XM, Wang YS, Zhang J, Li Y, Xu JF, et al. (2009) Role of PI3K/Akt and MEK/ERK in mediating hypoxia-induced expression of HIF-1alpha and VEGF in laser-induced rat choroidal neovascularization. Invest Ophthalmol Vis Sci 50(4): 1783–91.
16. Yu WZ, Zhou H, Li XX, Yan Z, Dong JQ (2008) Effects of the phosphatidylinositol 3 kinase inhibitor in a mouse model of retinal neovascularization. Ophthalmic Res 40(1): 19–25.
17. Alvarez Y, Cederlund ML, Cotrell DC, Bill BR, Ecker SC, et al. (2007) Genetic determinants of hyaloid and retinal vasculature in zebrafish. BMC Dev Biol 7: 114.
18. Peterson RT, Link BA, Dowling JE, Schreiber SL (2000) Small molecule developmental screens reveal the logic and timing of vertebrate development. Proc Natl Acad Sci U S A 97(24): 12965–9.
19. Zou L, Peterson RT (2005) In vivo drug discovery in the zebrafish. Nat Rev Drug Discov 4(1): 35–44.
20. Cao R, Jensen LD, Soll I, Hauptmann G, Cao Y (2008) Hypoxia-induced retinal angiogenesis in zebrafish as a model to study retinopathy. PLoS One 3(7): e2748.
21. Kimathi SS, McColloch KJ, Peterson RT, Malicki JJ (2009) Small molecule screen for compounds that affect vascular development in the zebrafish retina. Mech Dev 126(5-6): 464–77.
22. Westerfield M (2000) The zebrafish book. A guide for the laboratory use of zebrafish Danio rerio. 4th ed. Eugene: Univ. of Oregon Press.
23. Fleisch VJ, T, Neuhaus S (2008) Electroretinogram (ERG) Measurements in Larval Zebrafish, C.S.H.P. doi:10.1101/pdb.prot4973, Editor.
24. Brockerhoff SE (2006) Measuring the optokinetic response of zebrafish larvae. Nat Protoc 1(5): 2448–51.
25. Hatav MA (1987) Principles and Techniques of electron Microscopy. London: Macmillan press.
26. Ellis LM, Hinkel DJ (2000) VEGF-targeted therapy: mechanisms of anti-tumour activity, Nat Rev Cancer 3(9): 579–91.
27. Cao Y (2008) Molecular mechanisms and therapeutic development of angiogenesis inhibitors. Adv Cancer Res 100: 111–31.
28. You SA, Kwok SK, Kim WU (2008) Proinflammatory role of vascular endothelial growth factor in the pathogenesis of rheumatoid arthritis: prospects for therapeutic intervention. Mediators Inflammation 2008: 120978 p.
29. Lawson ND, Weinstein BM (2002) In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 240(2): 307–18.
30. Engelman JA, Luo J, Cantley LC (2006) The evolution of phosphatidylinositol 3-kinase signaling. Nat Rev Cancer 5(5): 413–27.
31. Torres-Vazquez J, Gilder AD, Fraser SD, Berk JD, Van NP, et al. (2004) Semaphorin-plexin signaling guides patterning of the developing vasculature. Dev Cell 7(1): 117–23.
32. Bayliss PE, Bellavance KL, Whitehead GG, Abrams JM, Aegerter S, et al. (2006) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. J Immunol 176(2): 591–7.
33. Vlahos CJ, Matter WF, Hui KY, Brown RF (1994) A specific inhibitor of Semaphorin-plexin signaling guides patterning of the developing vasculature. Dev Cell 7(1): 117–23.
34. Gharbi SI, Zvelebil MJ, Shuttleworth SJ, Hancox T, Saghir N, et al. (2007) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. J Immunol 176(2): 591–7.
35. Ihle NT, Powis G (2009) Take your PIK: phosphatidylinositol 3-kinase inhibitors move into the clinic. Nat Rev Cancer 9(3): 92–3.
36. Jia S, Roberts TM, Zhao JJ (2009) Should individual PI3 kinase isoforms be targeted in cancer? Curr Opin Cell Biol 21(2): 199–208.

37. Brazil DP, Park J, Hemmings BA (2002) PKB binding proteins. Getting in on the Akt. Cell 111(3): 293–303.

38. Manning BD, Cantley LC (2007) AKT/PKB signaling: navigating downstream. Cell 129(7): 1261–74.

39. Billottet C, Banerjee L, Vanhaesebroeck B, Khwaja A (2009) Inhibition of class I phosphoinositide 3-kinase activity impairs proliferation and triggers apoptosis in acute promyelocytic leukemia without affecting atra-induced differentiation. Cancer Res 69(3): 1027–36.

40. Graupera M, Guillermet-Guibert J, Foukas LC, Phng LK, Cain RJ, et al. (2008) Angiogenesis selectively requires the p110alpha isoform of PI3K to control endothelial cell migration. Nature 453(7193): 662–6.

41. Garlich JR, De P, Dey N, Su JD, Peng X, et al. (2008) A vascular targeted pan phosphoinositide 3-kinase inhibitor prodrug, SF1126, with antitumor and antiangiogenic activity. Cancer Res 68(1): 206–15.