POSTER 01

TITLE: PHENOTYPE-BASED DISCOVERY OF 2-[(E)-2-(QUINOLIN-2-YL)VINYL]PHENOL AS A NOVEL INHIBITOR OF DEVELOPMENTAL AND PATHOLOGICAL OCULAR ANGIOGENESIS

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Retinal angiogenesis is tightly regulated to meet oxygenation and nutritional requirements. In diseases such as proliferative diabetic retinopathy and neovascular age-related macular degeneration, uncontrolled angiogenesis can lead to blindness. Our goal is to better understand the molecular processes controlling retinal angiogenesis and discover novel drugs that inhibit retinal neovascularisation.

Phenotype-based chemical screens were performed using the ChemBridge Diverset™ library and inhibition of hyaloid vessel angiogenesis in Tg(fli1:EGFP) zebrafish. Hit compounds were tested in mammalian models of angiogenesis. A structural activity relationship (SAR) study was performed and structurally related compounds tested in zebrafish and where efficacious, mammalian models of angiogenesis.

2-[(E)-2-(Quinolin-2-yl)vinyl]phenol, (quininib) robustly inhibits developmental angiogenesis at 4-10 µM in zebrafish and significantly inhibits angiogenic tubule formation in HMEC-1 cells, angiogenic sprouting in aortic ring explants and retinal revascularisation in OIR mice. Quininib are structurally related compounds are well tolerated in zebrafish, human cell lines and murine eyes. Profiling screens of 153 angiogenic and inflammatory targets revealed quininib does not directly target VEGF receptors but antagonises cysteinyl leukotriene receptor 1 (CysLT₁) with an IC₅₀ of ~1.4 µM. Approximately 15 closely related compounds have also shown efficacy in angiogenesis models.

In summary, quininib is a novel anti-angiogenic small molecule CysLT₁ antagonist. Quininib inhibits developmental and pathological angiogenesis in a range of cell and tissue systems, revealing novel physiological roles for CysLT₁. Quininib has potential as a novel therapeutic to treat ocular neovascular pathologies and may complement current anti-VEGF biologicals.
UNFOLDING THE POTENTIAL FOR PROTEASE INHIBITORS IN THE TREATMENT OF OCULAR DISEASES USING A ZEBRAFISH MODEL OF ANGIOGENESIS

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Diabetic macular edema (DME), defined as retinal thickening on or around the centre of the macula, is an ocular manifestation of diabetic retinopathy, one of the major complications of diabetes and a leading cause of blindness in developed countries. KalVista Pharmaceuticals is an ophthalmology company focused primarily on DME with a strong background in developing small molecule serine protease inhibitors. Through collaboration with UCD in the 3D-NET consortium, we aim to identify new development opportunities for our proprietary serine protease inhibitor (SerTryP™) library in treating other ocular disease, such as inflammation, degeneration and neovascularisation. To determine the anti-angiogenic effects of the SerTryP™ library, the Intersegmental Vessel (ISV) assay was performed in Tg(fli1:EGFP) zebrafish from 6 hours post fertilisation (hpf). Each library plate of 80 compounds was pooled into 18 combinations of 8 or 10 compounds and tested at 10 µM in 48-well plates containing 5 zebrafish embryos per well. Pools with positive hits or toxic effects were rescreened individually at 10 µM. Anti-angiogenic effects were analysed by counting the number of ISVs at 48 hpf compared to a vehicle control (1% DMSO). In total 477 drugs were screened in 108 pools. Approximately 11% of pools had a significant anti-angiogenic effect of 9-34% with less than 5% having a toxic effect. Out of 40 drugs rescreened individually, none had toxic effects and 6 showed a significant reduction ranging from 10-25%. To date, the SerTryP™ library screen identified several drug hits with significant but mild anti-angiogenic effect in zebrafish; therefore none so far would be suitable for a potential anti-angiogenic drug candidate. Current focus is on screening the remaining SerTryP™ library compounds for more potent anti-angiogenic drug candidates and investigating their potential in treating other ocular diseases. The next step will involve testing the library in assays of permeability, inflammation and neurodegeneration. (Contact info: jlw@kalvista.com)

ENHANCEMENT OF ANTI-ANGIOGENIC EFFICACY USING DRUG COMBINATIONS TARGETING MULTIPLE SIGNALLING PATHWAYS IN VIVO

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Purpose: Ocular neovascularisation is a pathological hallmark common to a number of debilitating ocular diseases including diabetic retinopathy and age-related macular degeneration. Current therapies include the use of anti-VEGF monoclonal antibodies. Unfortunately, the high financial cost, lack of efficacy and need for frequent intraocular injections associated with anti-VEGF therapies mean that alternative therapies are required. A number of signalling pathways including the MAPK/ERK, Sonic Hedgehog (Shh), Wnt, and the PI3K/AKT/mTOR pathways are known to
be upregulated in ocular neovascularisation. Using drug combinations that target multiple signalling pathways may allow for enhanced efficacy, reduced toxic effects due to the requirement for decreased drug concentrations and decreased likelihood of drug resistance developing.

Methods: The intersegmental vessel assay was performed in Tg(fli1:EGFP) zebrafish to determine the anti-angiogenic efficacy of compounds based on their ability to inhibit intersegmental vessel growth in comparison to a vehicle control (n=3, N=30). Similarly, the hyaloid vessel assay was conducted to elucidate the efficacy of drug combinations based on number of primary branches inhibited (n=3, N=30).

Results: The efficacy of various drug treatments; both individually and in combination, for the treatment of angiogenesis were evaluated. The small molecule inhibitors were ranked in order of efficacy and the highest ranking drugs were chosen to be tested in combination (Sorafenib, Cyclopamine, Palomid 529 and TAK 733). Dual inhibition of the MAPK/ERK and Shh pathways (5 μM Sorafenib + 5 μM Cyclopamine) had the most significant effect on the inhibition of angiogenesis in vivo. The additive effect may be due to feedback-loop inhibition and/or positive cross talk between both pathways. Targeting the MAPK and PI3K pathways in combination (5 μM Sorafenib + 5 μM Palomid 529) also showed significant antiangiogenic activity.

Conclusion: Combinations of the highest ranking drug compounds had significant anti-angiogenic effects in both the zebrafish developmental angiogenesis assays. Such combinations of small molecule inhibitors may have the potential to replace current anti-VEGF therapies for the treatment of diabetic retinopathy and age-related macular degeneration.

POSTER 04
SYNTHESIS AND BIOLOGICAL EVALUATION OF STABLE HETEROAROMATIC LIPOXIN A4 ANALOGUES

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Lipoxins are trihydroxytetraene-containing eicosanoids that are biosynthesised from arachidonic acid by lipoxygenase enzymes and possess potent and selective anti-inflammatory activity. There are two native lipoxins; LXA4 and LXB4. These were first isolated from human leukocytes by Serhan and Samuelsson in 1984.1 They activate the ALX receptor on polymorphonuclear leukocytes (PMNs) and monocytes preventing the migration of neutrophils to sites of inflammation thus acting as stop signals. Due to this anti-inflammatory activity, lipoxins are of interest as potential drug candidates for the treatment of a range of inflammatory diseases. Studies indicate that LXA4 may have a fundamental role in protecting ocular function and promoting corneal wound healing thus making it an attractive and new therapeutic alternative to currently available anti-inflammatory drugs for the management of ocular inflammatory diseases.2 LXA4 is rapidly metabolised in vivo and in an effort to prevent this, recent research has been carried out towards the development of more stable analogues.3 Substantial work has been carried out by our research group in this area, with the successful synthesis of stable benzene and pyridine analogues.4,5 These analogues were found to significantly
increase phagocytosis of apoptotic PMN’s compared to native LXA4 and to suppress key cytokines involved in inflammatory diseases.

Our research group is carrying out an extensive SAR lipoxin programme in an effort to design and develop further analogues with increased potency and stability. This poster will outline the synthesis of some novel heteroaromatic LXA4 analogues which features a Suzuki-Miyaura cross-coupling and an asymmetric hydrogenation as the key reactions. The preliminary biological evaluation of these analogues will also be presented.


POSTER 05
CAN DRUG COMBINATIONS TARGETING PI3K/AKT/MTOR PATHWAY INHIBIT ANGIOGENESIS, INFLAMMATION OR PERMEABILITY IN HUMAN RPE AND ENDOTHELIAL CELL LINES?

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Age-related macular degeneration (AMD), diabetic retinopathy (DR) and diabetic macula edema (DME) are characterised by inflammation, retinal angiogenesis and increased retinal vascular permeability (RVP). There is an urgent need to develop better therapies to treat these diseases. Previously, we showed that combinations of PI3K/Akt/mTOR (PAM) pathway inhibitors have additive or synergistic anti-angiogenic activity in vivo (Sasore and Kennedy 2014). Here, we assess the anti-angiogenic, anti-inflammatory or anti-RVP of these drugs in human RPE and endothelial cells.

PAM pathway target genes (pik3ca, pik3r1, akt1, MTOR, RPS6KB1 and EIF4EBP1) are expressed in human ARPE-19. The most active drug combinations additively inhibit phosphorylation of p70S6K protein in human RPE cells. Preliminary data indicate that individual PAM pathway drugs reduce sVCAM-1 expression, with drug combinations having stronger effects. These drugs also reverse barrier disruption induced in ARPE-19 cells as determined by localization of ZO-1. In HMEC-1 cells, these combinations also inhibit in vitro tubule formation.
In summary, our study identifies combinations of PI3K/Akt/mTOR pathway inhibitors that are effective inhibitors of angiogenesis and cellular permeability in vitro. Further studies will evaluate the effect of these drugs on inflammation and permeability using multiplex ELISA and trans-epithelial/endothelial electrical resistance (TEER), respectively. In parallel, the activity of these drug combinations will also be assessed in mouse model of developmental angiogenesis. Therefore, further investigations of the PI3K pathway and drug combinations hold promise as inhibitors of retinal angiogenesis, inflammation and retinal vascular permeability.

POSTER 06
STABLE LIPOXIN ANALOGUES FOR BIOLOGICAL EVALUATION?

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Lipoxins are a family of bioactive compounds which are synthesised in the body from arachidonic acid for the resolution of inflammation. Lipoxin A4 (LXA4) and Lipoxin B4 (LXB4) were first isolated from human leukocytes in 1984 and have displayed therapeutic potential in the treatment of chronic inflammatory diseases, including ocular uveitis and diabetic retinopathy.1 However, their therapeutic potential is limited by their rapid metabolism in vivo.2

Recent work in this research group involved the design of LXA4 analogues that would possess a greater resistance to metabolism while retaining the lipoxin anti-inflammatory activity. Analogues containing benzene (1) and pyridine (2) have been synthesised and were found to display improved biostability and increased potency compared to the native LXA4.3,4

Herein we present the asymmetric synthesis of further novel heteroaromatic analogues of LXA4. The synthetic strategy includes a cross-dehydrogenative coupling protocol, a palladium - catalysed Heck reaction and asymmetric transfer hydrogenation as some of the key steps. These analogues are currently undergoing biological evaluation for anti-inflammatory activity and preliminary results will be presented.
PHARMACOLOGICAL RESTORATION OF VISUAL FUNCTION IN A BLIND ZEBRAFISH MUTANT FOLLOWING HISTONE DEACETYLASE INHIBITOR (HDACi) TREATMENT

Conor Daly, Lisa Shine, Theresa Heffernan, Eugene Dillon, David Duffy, Gerard Cagney, Breandán N. Kennedy.

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Background: Controversially, pharmacological inhibition of Histone Deacetylase (HDAC) proteins is in clinical trial for the treatment of inherited retinal degenerations. Previous studies report that patients suffering from the inherited retinal degeneration Retinitis Pigmentosa (RP) may show improved visual field and acuity following treatment with the HDAC inhibitor valproic acid (VPA). Utilising zebrafish models of retinal degeneration we rescued retinal morphology and visual function of a blind zebrafish mutant (dye mutant) by treatment with HDACi.

Methods: Visual function was assessed by Optokinetic Response (OKR) and Visual Motor Response (VMR) assays. Cone photoreceptor outer segment (OS) morphology, ciliary marginal zone (CMZ) apoptosis and cone photoreceptor outer segment (OS) length were assessed by light microscopy. Larvae were drug treated with HDACi (1 µM TSA, 10 µM MC1568 and 10 µM MS275) with or without 100-500 nM ANA-12 from 3-5 dpf at 28.5 °C. An unbiased shotgun proteomic analysis of TSA-treated dye eyes was carried out by LC-MS/MS and the resulting dataset analysed by Ingenuity Pathway Analysis (IPA) software.

Results: The dye mutant has reduced visual behaviour and several defects in retinal morphology compared to sibling larvae. HDACi treatment of dye results in improved OKR and VMR, rescue of gross morphological defects, an 80% decrease in the number of dead cells in the CMZ and an increase in cone photoreceptor OS length. Proteomic analysis identified significantly differentially expressed proteins in response to treatment, and pathway analysis identified Bdnf as a major contributing mechanism to rescue. ANA-12 treatment blocks Bdnf/TrkB signaling and HDACi mediated rescue in dye.

Conclusions: HDAC inhibition is effective in restoring visual function and rescuing morphological defects in a zebrafish model of retinal degeneration.

References:

**POSTER 08**

**ENHANCED DRUG DELIVERY TO RODENT RETINAS FOLLOWING INNER BLOOD-RETINA BARRIER MODULATION.**

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**PURPOSE**

To enhance retinal delivery of hsp90 inhibitor 17-DMAG and to assess therapeutic efficacy in two rodent models of RP, study mechanism of action of 17-DMAG and quantify drug uptake by mass spectrometry.

**METHODS**

Doxycycline-inducible iBRB modulation; ERG analysis; LC/MS/MS; cell culture; RT-PCR; immunoblotting; histology.

**RESULTS**

Modulation of iBRB enhances delivery of 17-DMAG to retina as evidenced by LC/MS/MS quantitation. Retinal degeneration retarded in eyes in which drug delivery was enhanced compared to controls. Ongoing research indicates that the mode of action involves components of the unfolded protein response.

**CONCLUSION**

Transient inducible modulation of the iBRB is a promising method for enhancing delivery of therapeutics to retina. Hsp90 inhibitors are just one class of many compounds which could be of therapeutic benefit in ocular disorders.

**POSTER 09**

**FUNCTIONAL INVESTIGATIONS OF CONE-ROD DYSTROPHY ASSOCIATED WITH RAB28.**

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Cilia are sensory organelles which are present on the surfaces of most cell types. In photoreceptors, the cilium is modified to form the outer segment, where light detecting opsins are localised. Many genes with ciliary functions are associated with a number of retinal dystrophies, including cone-rod dystrophy, Leber congenital amaurosis and retinitis pigmentosa. RAB28 is a member of the RAS superfamily of small GTPases which has recently been linked to a form of autosomal recessive cone-rod dystrophy (CRD); an inherited disorder whereby the photoreceptors of the retina begin to degenerate and die, resulting in permanent blindness. Localisation of rat RAB28 to the base of the connecting cilium (CC) of photoreceptors suggests a possible role for RAB28 in cilia.

We are taking a dual-model approach in dissecting the molecular function of RAB28, combining the genetic tractability of C. elegans and the vertebrate biology of zebrafish. We are also making use of the novel genome editing technology CRISPR to generate a zebrafish knockout model of Rab28 associated CRD. This will allow us to
understand the role of RAB28 in cilia generally and in photoreceptors specifically, as well as the pathomechanism of its associated disorder.

In C. elegans, a Prab-28::gfp promoter fusion is expressed in ciliated sensory neurons and GFP-tagged RAB-28 localises to the cilium, undergoing a ciliary transport process known as intraflagellar transport (IFT). This transport is disrupted by mutations in the IFT machinery. Despite this association, behavioural and microscopy based assays used to assess C. elegans ciliary mutants have so far shown that rab-28 -/- worms have no obvious ciliary defects. Neither has a genetic interaction so far been found between rab-28 and two other ciliary GTPase genes, arl-3 and arl-13. We are also currently raising zebrafish injected with CRISPR constructs targeting rab28.

POSTER 10

NORGESTREL: A PROGESTERONE ANALOGUE; ACTS AS A NEUROPROTECTANT IN TWO MOUSE MODELS OF RETINITIS PIGMENTOSA.

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Purpose: Following a drug screen of over 900 FDA approved compounds, our group identified the synthetic progestin, Norgestrel, as a neuroprotectant in models of retinitis pigmentosa (RP) [1]. Here, we investigated the mechanism(s) through which Norgestrel may exert its protective effects. Progesterone acts in the CNS through a number of receptors [2-4]. Thus, we sought to identify which receptor Norgestrel may be acting through. Also, given the association of reactive oxygen species (ROS) with progesterone [5], and their implication in RP [6], we investigated the effects of Norgestrel on ROS.

Methods: rd10 mice were maintained on a norgestrel diet from P10, for time-points indicated. Balb/c mice administered Norgestrel were subjected to damaging white light for 2hours. Photoreceptor cell death and architecture were investigated by means of TUNEL assays and immunofluorescence staining. Receptor expression and gene silencing were studied in 661W mouse photoreceptor-derived cells by means of rtPCR and receptor-specific targeted siRNA. Cells were stressed by serum starvation. To study retinal ROS, Balb/c mice received i.p. injections of the superoxide probe dihydroethidium (DHE).

Results: Norgestrel markedly reduced photoreceptor cell death and rescued cell morphology in the rd10 and light damage models of RP. Using 661W cells, the expression of a number of progesterone receptors was identified. PGRMC1 showed highest expression, and when genetically silenced the protective effects of Norgestrel on stressed cells were abrogated. Finally, ROS induced in the retina by light damage was inhibited by Norgestrel pre-treatment, as visualised by a decrease in DHE staining in the photoreceptor cells.
A NEW EXPERIMENTAL IN VIVO FRAMEWORK TO STUDY RETINAL CHANGES ASSOCIATED TO THE DEVELOPMENT OF CEREBRAL MALARIA.

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Cerebral malaria (CM) is one of the most serious complications of Plasmodium falciparum infection, predominantly in children < five years of age. The pathogenesis is predominantly believed to be a multi-factorial process that involve, among others, an exacerbated host inflammatory response and the sequestration of parasitized red blood cells in the microvasculature. Nevertheless, the study of the clinical time-course of CM during infection in humans has relied to the use of clinical case series and case-control studies, post-mortem surveys, in vitro studies, or animal models. On the other hand, retinal vasculature has been demonstrated intimately associated with the pathogenesis of CM.

Changes in cerebral microvasculature such as blood brain barrier disruption, up-regulation of adhesion molecule expression and sequestration of parasitized erythrocytes have been also reported in retinal microvasculature, offering an opportunity to study CM using non-invasive methods. In this work, we present an experimental murine malaria model in which a heterogeneous course of P. berghei lethal malaria is produced, allowing to compare the clinical progress of CM mice vs. the progression non cerebral malaria (NCM) using the same parasite-host genetic backgrounds. As several cerebral microvasculature markers of CM have been correlated with CM mice, this model provides a novel experimental framework to study retinal changes associated to the development of CM.

DRUG POOLING AS A NEW METHOD TO IMPROVE PHENOTYPE-BASED DISCOVERY OF OCULAR DRUGS USING A ZEBRAFISH MODEL OF ANGIOGENESIS.

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Purpose
Proliferative forms of macular degeneration and diabetic retinopathy are associated with ocular neovascularisation. Current treatments, involving multiple injections of anti-VEGF in the eye, are uncomfortable, costly and risk eye damage. There is a need to develop drugs more effective and more easily administered. Previously, we individually screened compounds from chemical libraries using the zebrafish hyaloid vasculature (HV) assay. To increase throughput we developed a drug pooling strategy.
Methods
We determined the maximum tolerated dose of DMSO in the zebrafish HV assay and spiked for proof-of-principle assay pools with 10 μM sunitinib. For each 80-well library plate, vertical and horizontal drug pools containing 8 or 10 compounds each at 100 μM in 10% DMSO were generated and these were tested at 10 μM in 48-well plates containing 5 zebrafish embryos per well.

Anti-angiogenic effects were quantified by counting the number of primary hyaloid vessels in dissected lenses at 5 dpf.

Results
100% of pools spiked with 10 μM sunitinib significantly reduced HV formation. 360 Chembridge Diverset® library drug pools were screened, comprising 1600 drugs. 22 pools had a >40% reduction. Of the 190 drugs re-screened individually, 1 reduced primary HV by 50% at 10 μM and by 75% at 20 μM without significant changes in survival or morphology. Overall 40% less larvae and approx. 50% less time were needed to complete the library screening compared to individual testing of drugs.

Conclusion
The drug pooling strategy is a cost-effective and time-saving approach to find unbiased new inhibitors of developmental angiogenesis in the eye and can be easily applied to other chemical screens.

POSTER 13
SUSTAINED RELEASE FROM BIODEGRADABLE MICROPARTICLES OF A BIOACTIVE CYSTEINYL LEUKOTRIENE RECEPTOR ANTAGONIST FOR THE TREATMENT OF OCULAR NEOVASCULARISATION.

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Purpose:
Vision loss in age-related macular degeneration and diabetic retinopathy is due to the excessive growth of leaky abnormal blood vessels to the back of the eye (ocular neovascularisation). There is an unmet need in these diseases to improve current delivery modalities to the eye, which is highlighted by the requirement of current gold-standard anti-VEGF drugs to be administered bi-/monthly by intraocular injection. Our objective is to enhance the delivery of bespoke novel anti-angiogenic drugs using biodegradable sustained-release devices.

Methods:
A novel VEGF-independent, cysteinyl leukotriene receptor antagonist (Quininib [QB]) previously identified as anti-angiogenic in zebrafish, cells and mice was formulated into biodegradable PLGA and alginate microparticles. Drug-loaded microparticles were characterised in terms of shape, size and loading efficiency. Drug release from microparticle subtypes was determined by in vitro release studies using HPLC. The efficacy of released drug was evaluated in vitro with tubule formation assays and in vivo using larval zebrafish angiogenesis assays.

Results:
QB was successfully formulated into PLGA and alginate microspheres of ~1-2μm. Notably, in vitro release studies demonstrate high concentrations of QB drug released from PLGA and alginate microparticles for over three months.
Importantly, QB released from microparticles retained anti-angiogenic efficacy in vitro and in vivo. Future directions will test the safety, pharmacokinetics and efficacy of these Quininib-loaded microparticle formulations in rodents.

Conclusion:
We successfully encapsulated a small molecule cysteinyl leukotriene receptor antagonist into a slow release preparation. The ultimate goal is to determine if these encapsulated anti-angiogenic drugs offer an improved sustained and effective treatment for ocular neovascularisation.

POSTER 14
IDENTIFICATION OF NOVEL CONE PHOTORECEPTOR-ENRICHED FACTORS THAT ARE CONSERVED IN ZEBRAFISH, MOUSE AND HUMAN.

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Purpose
The inability of the retina to detect/transmit light-triggered signals is largely responsible for incurable blinding conditions such as age-related macular degeneration, and cone-rod dystrophy, due to dysfunction or death of photoreceptor cells. Cone photoreceptors mediate colour vision and high visual acuity under brighter light conditions. Notably, our understanding regarding i) factors regulating cone photoreceptor differentiation from retinal progenitor cells, and ii) factors maintaining cone photoreceptor survival remains limited. This research aimed to identify novel factors expressed in cone photoreceptors in zebrafish and mouse, and in the macula of humans, using sequencing technologies.

Methods
Microarray analysis of flow-sorted cone photoreceptors from the transgenic zebrafish line Tg(gnat2:EGFP) identified cone-enriched factors. RNAseq data of photoreceptors of the cone dominant Nrl -/- mouse and rod dominant Nrl-GFP mouse were compared to identify evolutionarily conserved cone-enriched factors. Human retinal RNAseq data was analysed to confirm evolutionary conservation, and retinal enrichment. Genes were ranked based on their cone enrichment. PCR was performed in wild type (Tü) zebrafish eyes to confirm expression of highest-ranking cone-enriched factors during development. Gene knockdown of one of these high-ranking genes clul1 was performed using morpholino technology. The visual behaviour of clul1 morphants was assessed using the Optokinetic Response, and retinal integrity was examined using light microscopy.

Results
28 novel, evolutionarily conserved, cone-enriched genes were identified in zebrafish and mice, using microarray, and RNAseq analysis, respectively. High-ranking genes were then confirmed to be present during development in wild type zebrafish. Knockdown of the gene clul1 resulted in a statistically significant effect on visual behaviour, and did not result in any significant morphological differences to the retina of zebrafish larvae.
Conclusion

Novel, evolutionarily conserved cone photoreceptor-enriched factors were identified. These genes were confirmed to be expressed in the eye of developing zebrafish using RTPCR. Knockdown of one of these genes, clul1 using morpholino technology resulted in a statistically significant effect on visual function, without any morphological changes to the retina.

POSTER 15

VITAMIN D RECEPTOR AGONISTS SIGNIFICANTLY ATTENUATE OCULAR DEVELOPMENTAL ANGIogenesis.

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Purpose

Angiogenesis is a pivotal process in development and disease. Pathological ocular angiogenesis can ultimately cause blindness in diseases such as neovascular age related macular degeneration (nAMD). Current gold-standard nAMD therapeutics are associated with resistance, repeated intra-ocular injections, high costs and adverse reactions. Therefore, a clinical need remains to develop improved therapies with greater efficacy and safety profiles.

Methods & Results

A phenotype-based screen of the ICCB Known Bioactives Library was performed to identify a novel safe small molecule inhibitor of ocular angiogenesis. Compounds were screened for inhibition of ocular hyaloid vessel (HV) and trunk inter-segmental vessel (ISV) development in Tg(fli1:EGFP) zebrafish larvae. The biological active form of vitamin D “Calcitriol” represents a lead hit. Calcitriol and several vitamin D receptor agonists (VDRA) significantly inhibited ocular developmental angiogenesis by up to 50%. Vitamin D receptor (VDR) expression was analysed by RT-PCR and found to be present in the eye and trunk of larvae. Several miRNA have shown differential expression in larvae in response to Calcitriol treatment. Here, we show miRNA 21 to be upregulated in the eye of Calcitriol treated larvae.

Safety studies used light microscopy to evaluate retinal morphology and the optokinetic response (OKR) assay to assess visual function in response to treatment. VDRAs had an adverse effect on larval OKR despite normal retinal lamination/morphology. Human retinal pigment epithelial cell (ARPE-19) viability by MTT, human retinal endothelial cells (ACBRI 181) tubule formation and mouse aortic ring sprouting angiogenesis in response to treatment was quantified. ARPE-19 cells show dose-dependent cytotoxicity to VDRAs, tubule formation in ACBRI 181 cells was unaltered by VDRAs and preliminary data shows VDRAs to reduce aortic ring sprouting.

Conclusion

Here, we demonstrate that VDRAs significantly and specifically inhibit ocular angiogenesis during zebrafish development. Future experiments will evaluate the molecular mechanisms of VDRAs anti-angiogenic activity and their efficacy in pre-clinical mouse models.
POSTER 16
DEFECTIVE PHOTORECEPTORS UNDERLIE INHERITED BLINDNESS IN THE RAIFTEIRÍ MUTANT

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Purpose: To characterise the basis of the inherited visual function defect in the Raifteirí mutant.

Methods: Optokinetic response (OKR) screens were performed on N-ethyl-N-nitrosourea (ENU)-mutagenised zebrafish to identify families with recessive defects in visual function. Retinal morphology was characterised by microscopy and immunohistochemistry using retinal cell-specific markers. Apoptosis levels were determined by TUNEL staining. Circadian behaviours were ascertained using Zebralab (Viewpoint). Electroretinograms were recorded. Linkage analysis was performed using a standard zebrafish mapping panel.

Results: A novel mutant designated Raifteirí (raf) was identified by OKR screens. Raf mutants show no visual response and are paler with slightly smaller eyes. Locomotor activity levels are ~50% of normal siblings however raf mutants exhibit circadian behaviour and a shadow response. No difference in apoptosis is observed between mutants and siblings. Light microscopy and DAPI staining show raf mutants to have normal retinal lamination. Staining of the inner retina suggests that cell differentiation is equivalent in siblings and mutants. In contrast, staining of the outer retina with photoreceptor-specific markers shows an absence of rod photoreceptors in all but peripheral retina. In addition cone photoreceptors appear stunted and there is a reduction in outer segment staining in raf mutants. Electron microscopy data shows the presence of a small number of stunted outer segments in the central retina of raf mutants. Raf mutants show no electrophysiologial response at lower light intensities and a tiny, latent response at higher flash intensities. The raf gene has been mapped to a marker on the proximal part of zebrafish linkage group 23.

Conclusions: We have identified and partially characterised a novel zebrafish model of inherited blindness. Electroretinographic and histological analyses suggest that specific defects in raf outer retina underlie loss of visual function. A number of interesting candidate genes lie on linkage group 23, which are currently being sequenced.
POSTER 17
HYALURONIC ACID MICRONEEDLES ALLOW FOR SUSTAINED RELEASE OF ANTI-ANGIOGENIC QUININIB

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Purpose
Pathologic neovascularisation is a hallmark of proliferative Diabetic Retinopathy and Age-related Macular Degeneration. Current pharmacologic interventions targeting VEGF are effective in only a subset of patients and require multiple intraocular injections associated with iatrogenic infection. Thus, our goal is to develop novel, VEGF-independent, anti-angiogenic drugs with sustained ocular-release. Previous work demonstrates quininib to successfully inhibit formation of blood vessels in in vitro, ex vivo and in vivo models of angiogenesis. Here, sustained release of quininib from a novel hyaluronic acid microneedle formulation is determined in vitro, and the safety and efficacy evaluated in vivo.

Methods
Quininib-hyaluronic acid microneedles were formulated via a salt precipitation method from a quininib-HA solution and cross-linked with 4-arm-PEG-amine prior to freeze-drying. Microneedle conformation was characterised by scanning electron microscopy. The zeta potential (charge) of the microneedles was determined by electrophoretic light scattering. The concentration of quininib released in vitro from the microneedles was quantified by HPLC. Anti-angiogenic activity was assessed in a zebrafish hyaloid developmental angiogenesis assay, and a rodent model of oxygen induced retinopathy.

Results
The Quininib-HA formulation generated hollow needle-shaped particles with a charge of -35.5 mV. The majority of particles were >1.0 µm in size with 30% of particles ranging between 0.5-1.0 µm. The incorporation of quininib into these microneedles was 98%. In vitro, 20% quininib was released over 4 months; in the presence of increasing concentrations of hyaluronidase a maximum of 60% quininib was released over 4 months. Quininib released from these microparticles inhibited hyaloid vessel development in zebrafish and a maximum tolerated dose study indicates these microneedles are safe in rodent eyes.

Conclusion
Quininib-HA microneedles are safe in vivo and Quininib released from these microneedles effectively inhibits angiogenesis in vivo. Current work is assessing activity of Quininib-HA microneedles in a month long study of ocular permeability in vivo.
The majority of patients with severe age-related macular degeneration (AMD) or diabetic retinopathy (DR) exhibit pathological neovascularization of the choroid or retina respectively, associated with edema and reduced vision. Currently, anti-VEGF treatment is given to eliminate pathological vessels and stop edema, but many are resistant and thus alternative targets are needed. It is hypothesized that hypoxia is an important factor responsible for sustained angiogenesis in these diseases, but the mechanisms involved in neovascularization in the eye, in particular in response to hypoxia, remain poorly investigated. As hypoxia as an isolated pathological stimuli cannot be studied in rodent models, we have used adult zebrafish to study mechanisms of hypoxia-induced choroidal- or retinal neovascularization.

Here we for the first time present a thorough anatomical and functional characterization of the zebrafish choroid vasculature. The zebrafish choroid contains a dense, multi-layered vasculature, which have a weaker blood-brain barrier than the retinal vessels. Interestingly, choroidal vessels develop mostly by vasculogenesis and intussusception, while retinal vascularization relies on sprouting angiogenesis. Hypoxia-induced neovascularization similarly led to predominantly intussusive or angiogenic growth of the choroidal or retinal vasculatures respectively and a severe disruption of blood-brain barrier integrity and function in both tissues. RNA sequencing of endothelial cells from the choroid or retina demonstrated that choroidal and retinal vasculatures have entirely different transcriptomes, and that they exhibited up-regulation of different and non-overlapping, angiogenic genes following exposure to hypoxia. In particular we found differential engagement of VEGF-VEGFR signaling pathways in the retinal and choroidal vessels, which suggest that these vasculatures utilize different ligands and receptors. These findings may have far-reaching implications for the use of anti-VEGF drugs for treatment of retinopathies in the future.