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Title: **Discovery And Development Of The Quininib Series Of Ocular Drugs**

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Abstract
The quininib series, is a novel collection of small molecule drugs with anti-angiogenic, anti-vascular permeability, anti-inflammatory and anti-proliferative activity. Quininib was initially identified as a drug hit during a random chemical library screen for determinants of developmental ocular angiogenesis in zebrafish. To enhance drug efficacy, novel quininib analogues were designed using medicinal chemistry. The resulting quininib drug series has efficacy in *in vitro* and *ex vivo* models of angiogenesis utilizing human cell lines and tissues. *In vivo*, quininib drugs reduce pathological angiogenesis and retinal vascular permeability in rodent models. Quininib acts as a cysteinyl leukotriene (CysLT) receptor antagonist, revealing new roles of these G-protein coupled receptors in developmental angiogenesis of the eye. The quininib series highlighted the potential of CysLT receptors as therapeutic targets for retinal vasculopathies (*e.g.* neovascular age-related macular degeneration, diabetic retinopathy and diabetic macular oedema) and ocular cancers (*e.g.* uveal melanoma).

Ocular Angiogenesis.
Vascularisation of the vertebrate eye is a complex developmental process, controlled by an intricate network of genetic determinants and the coordinated interaction of different cell types including neurons, glia, endothelial, and immune cells (1, 2). The retina, as one of the most metabolically active tissues in the body, depends on a functional vasculature to supply oxygen and nutrients (3). In mammals, two distinct vascular networks nourish the retina: *i)* the choriocapillaris nourishes the photoreceptor layer and *ii)* the retinal vessels, which grow through the ganglion cell layer and nourish the inner layers of the retina (1). The formation of these intraretinal vessels in mammals is synchronised with regression of a transient hyaloid vasculature, which nourishes the developing lens and retina. Genetic or environmental alterations during this complicated process can lead to retinopathy of prematurity (ROP), an important and potentially avoidable cause of perinatal blindness afflicting pre-term infants (4).

Pathological angiogenesis and/or changes in the retinal vessels in the adult are linked to severe ocular pathologies which can ultimately lead to blindness. Neovascular age-related macular degeneration (nAMD) and proliferative diabetic retinopathy (PDR) are leading causes of vision impairment and blindness in working and aged populations, respectively (5). They are both driven by pathological ocular angiogenesis, which presents as pigmentation, exudation, haemorrhage, oedema, or atrophy in the macula upon fundus examination (6). Vision loss caused by pathological ocular angiogenesis places a great economic and social burden on patients. Vision impairment can have a detrimental effect on quality of life, reducing independence due to impaired mobility (7). It is also a major barrier to employment, with evidence that vision impairment is associated with a loss of productivity and income as well as a lower level of total employment (7).

In the last 20 years, understanding of the molecular pathways responsible for retinal angiogenesis and blood vessel physiology has significantly improved (2, 8, 9). Vascular endothelial growth factor (VEGF) was one of the first genes identified as a key player in pathological and developmental ocular angiogenesis (10, 11). Anti-VEGF antibodies then rapidly emerged as promising anti-angiogenic therapies. In 2001,

clinical trials in cancer patients reported that bevacizumab (Avastin®) was safe and efficient at inhibiting tumour angiogenesis, and it was subsequently approved in February 2004 by the FDA to treat colon cancer (12-14). In a parallel clinical trial, pegaptanib (Macugen®), an RNA aptamer neutralizing VEGF, decreased progressive vision loss in nAMD patients and was approved in December 2004 by the FDA to treat nAMD (15). However, ophthalmologists started using intravitreal injections of the much cheaper bevacizumab off-label to treat nAMD with positive results, increasing visual acuity in a greater proportion of patients and by a greater average number of letters than pegaptanib, along with minimal systemic side effects (15-17). In 2006, Genentech launched ranibizumab (Lucentis®) for ophthalmic use, a modified version of bevacizumab designed for better diffusion in the retina but priced significantly higher (18). Subsequent head-to-head clinical trials demonstrated that bevacizumab is noninferior to ranibizumab in the treatment of nAMD (19, 20).

The treatment of blinding ocular neovascular disorders was revolutionised by the introduction of these anti-VEGF biologics to the market. Ranibizumab became a blockbuster drug with label expansions to include macular oedemas, PDR, myopic choroidal neovascularisation, neovascular glaucoma and complications of vein occlusion. These drugs introduced efficacious treatments but also changed how ophthalmologists work. The need for patients to have monthly/bimonthly intravitreal injections by an ophthalmologist created a “tsunami” of intravitreal injections for this speciality and introduced a treatment burden for both medical staff and patients and family members. Like all medications, anti-VEGFs came with adverse effects, with one key disadvantage being the mode of delivery. Monthly injections directly into the vitreous has a small yet inherent risk of infection (endophthalmitis), retinal detachment, and a potential increase in intraocular pressure (15, 21). Bevacizumab is usually aliquoted from the original vial before intravitreal injection, which carries a risk of contamination and thus endophthalmitis (22). Intravitreal injection is not the preferred mode of administration for many patients, some of whom reported pain on injection. Patients are also reliant on carers to attend appointments. Drugs targeting VEGF introduce a higher risk of thromboembolic events (*e.g.* heart attack, stroke) with many of those availing of anti-VEGF therapy already at higher risk due to age.

Unbiased, phenotype-based screens for drugs modulating ocular angiogenesis.
We decided to undertake an unbiased drug screen to identify novel pharmacological modulators of ocular angiogenesis. It was hoped the compounds identified would act on angiogenic pathways independent of VEGF, enhancing our knowledge of the fundamental biology underpinning ocular angiogenesis and potentially offering a distinctive therapeutic target. Several experimental models were considered for the screen including human endothelial cell lines, rodent tissue explants, and model organisms. Although the *in vitro* models offered opportunities for higher efficiency drug screening, it was decided that *in vivo* screening in the more physiologically relevant zebrafish model would be more effective and could uncover small molecule drugs that overcame key pharmacokinetic and pharmacodynamic challenges (23). Zebrafish are a cost-effective *in vivo* model which can be readily scaled up through the use of embryos or larvae for higher-throughput screening. They have orthologues to 82% of human disease-associated genes and the rate of conservation of pharmacological effect of drugs between zebrafish and humans is relatively high (23). Potential limitations of the zebrafish model included the need to exclude screening of biologicals due to presumed poor bioavailability upon administration to the media, and the

likelihood of poor interaction with zebrafish targets for biologicals designed to mammalian proteins (23, 24). Equally, there was a risk that drug hits identified in zebrafish may not translate their biological or therapeutic activity into mammalian models.

The biological assay selected for this screen was quantification of the primary hyaloid vessel (HV) number in 5 days post-fertilisation (dpf) zebrafish larvae (25). This assay determines the ability of interventions to inhibit developmental angiogenesis in the eye, and thus was directly relevant to our objective. A genetic model of pathological angiogenesis (*vhl*) was considered but not selected, as only 25% of the larvae display the phenotype, significantly reducing screening efficiency (26). Other research groups were screening for drugs affecting developmental angiogenesis of intersegmental vessels (ISV) along the zebrafish trunk (27, 28). Ultimately, the HV assay also offered a niche research area to uncover hits that selectively inhibited developing HV but not existing peripheral vasculature. A pilot screen of commercially available compounds found a PI3-kinase inhibitor (LY294002) to inhibit HV angiogenesis without introducing systemic side-effects or diminished visual function (29).

As our aim was to discover novel drugs and novel therapeutic targets, phenotype-based screening was chosen over target-based screening. In phenotype-based drug screening, compounds are investigated for their ability to modulate a chosen trait. In contrast, target-based screening approaches discover chemicals with ability to interact with a single molecular target (30). Phenotypic screens do not require prior identification of a target molecule (target agnostic), which makes them unbiased. They also do not require an understanding of the molecular mechanism of action, only a biological readout or biomarker related to the biological process or disease which can be measured in the screen (30). This provides the opportunity to identify *first-in-class* drugs, targeting all the components involved in a pathway/phenotype. Phenotypic screening accounts for a larger proportion of *first-in-class* small molecule drugs than target-based approaches (31). In our research, serendipity intervened. Mistakenly we were sent the *Tg(fli1:EGFP)* line of fish from a stock centre. Tg[*fli1:EGFP*] transgenic larvae express EGFP in vascular endothelial cells (32) allowing for, simple observation and imaging of HV phenotypes using fluorescent microscopy.

A key consideration before carrying out a drug screen is the compound library selection. As our intention was to discover novel drugs, a screen of a randomised chemical library was performed, rather than a library of market approved drugs or one focused on a particular biological process. The criteria in which we based our library selection of a diverse, unbiased compound set included: *i)* desirable physiochemical properties (*e.g.* adhering to Lipinski's rule of 5 (33)) *ii)* presence of drug-like and lead-like scaffolds with differing complexity; and *iii)* a range of structurally diverse compounds and low clustering density to maximise the explored chemical space and *iv)* molecular weight under 400 g/mol to assist with bioavailability (34). Some clustering is essential for additional quantitative structural activity studies. Examining the activity of several cluster members can uncover key pharmacophores and elucidate mechanism of action. Uniqueness is another important factor; the more unique a compound is the greater its patentability. However, it can be risky to screen purely novel compounds and as such most libraries will include a mixture of common and unique scaffolds (35). Logistically, the supplier should be cost effective, deliver in a timely manner, provide the library in an easy-to-use format and have a constant stock

of the compounds for quick repurchase/restocking should hits require additional analysis (36).

After consideration of the above, we ultimately selected the Chembridge Diverset™ Express pick MF6 subset library for the HV screen. This consists of 50,000 compounds selected from an original pool using 3D conformer analysis and 2D structural similarity analysis. Filtering methods applied by the supplier to generate this subset include selecting compounds with druglike and lead-like physiochemical properties such as MW≤500, clogP≤5, TPSA≤100, rotatable bonds ≤8, hydrogen bond acceptors ≤10 and hydrogen bond donors ≤5. Chemicals with undesirable groups and non-druglike properties are removed. Compounds were also selected to provide a wide structural diversity and pharmacophore coverage within the subset, while still containing clusters of compounds with similarity. The library was available in pre-plated sets, with all compounds available individually from the manufacturer for further analysis.

Discovery of quininib within the HV Screen.

In our HV screen, 10 μM of each library compound was screened in *Tg(fli1:EGFP)* larvae. The HV attach to the lens at 2.5 dpf and develop an organised hemispherical basket pattern around the lens by 5 dpf (25, 37). Five larvae were treated per well of a 48-well plate with drugs administered to the media at 2 dpf and the lenses dissected from euthanised larvae at 5 dpf for HV analysis (29). Arguably, this manual approach could be considered less efficient than an automated analysis. However, dissection and quantification of HVs is relatively fast, taking between 5 and 10 minutes for each drug. Automated systems are expensive, require extra time to prepare the samples and at the time were not effective at imaging through the refractive lens. A primary hit was defined as producing >50% reduction in primary hyaloid vessels in at least 3 out of 5 larvae. Of 1760 chemicals screened, ten primary hits were identified (0.5% hit success) consistent with similar screens (37, 38). Replicate experiments on these primary hits validated 4 as secondary hits (0.23%), which were re-ranked based on their ability to inhibit HV formation in a dose response experiment. In our screen, 2-*[*(*E*)-2-(Quinolin-2-yl)vinyl]phenol (quininib) was ranked the highest in efficacy and potency with up to 85% inhibition, extensively preventing the growth of entire hyaloid vasculature basket pattern (39). A challenge with randomised chemical library screens is that when a hit is identified, the target and molecular mechanism of action must be elucidated (30). A concern was that all significant hits would act in the VEGF pathway, therefore not fulfilling our aims of finding a novel therapeutic target to overcome contemporary issues with VEGF targeting or providing novel insights into developmental angiogenesis of the eye.

Development of the quininib series.

Target profiling was used to investigate the pathway through which quininib was exerting its anti-angiogenic effects. Both the SelectScreen® Kinase Profiling Service (Invitrogen) of 22 kinases and the Premier Screen of 140 protein kinase targets (Dundee) were screened. Neither showed a target with inhibition greater than the threshold of 50% (39). VEGFR1-3 were among the targets analysed and ruled out, suggesting a novel anti-angiogenic mechanism of action. From a literature search, quininib and analogues were identified as putative cysteinyl leukotriene 1 receptor ($CysLT_1$) antagonists and endothelin-converting enzyme 2 (ECE2) inhibitors (40, 41). ECE1 was ruled as a target based on only 20% inhibition in target profiling (39).

$CysLT_1$ and $CysLT_2$ are G-protein coupled receptors (GPCRs) which activate similar downstream signalling events including inositol phosphate accumulation (42). They are stimulated by endogenous ligands leukotriene (LT)C_4, LTD_4, LTE_4 and LTF_4, with LTD_4 having the greatest affinity for $CysLT_1$ and both LTC_4 and LTD_4 having equal affinity for $CysLT_2$ (42, 43). Out of 153 targets profiled only $CysLT_1$ was significantly inhibited (107%) by quininib (39). Quininib antagonised LTD_4 binding to $CysLT_1$ more potently than to $CysLT_2$ (39). Downstream inhibitors of the CysLT pathway phenocopied quininib in HV assays, supporting this as a pathway through which quininib exerted anti-angiogenic effects. The role of CysLTs in inflammation is well characterised, and $CysLT_1$ antagonists such as montelukast are used clinically for the treatment of asthma (44), but this was the first indication of their therapeutic potential for ocular angiogenesis.

To identify more efficacious quininib variations, 24 structurally distinct analogues were synthesised and investigated for anti-angiogenic activity using the zebrafish intersegmental vessel assay (45). 12 analogues showed significant inhibition of developmental angiogenesis, with one formulation Q22 (2-quinolin-2-yl-ylethynylphenol) having activity equivalent to quininib and two formulations, Q18 ((*Z*)-2-(2-(quinolin-2-yl)vinyl) phenol HCl)) and Q8 ((*E*)-2-(2-quinolin-2-yl-vinyl)-benzene-1,4-diol), having greater activity than quininib. Q22 contains an alkyne linkage between the quinoline and phenyl ring, Q18 is the Z-entantiomer of quininib and Q8 has an additional hydroxy group compared to quininib. Q8 was the most potent and effective analogue in reducing ISV developmental angiogenesis in zebrafish, and in HMEC-1 endothelial cell migration and tubule formation inhibition (45). The increased potency of Q8 compared to quininib could be attributed to the additional phenyl ring hydroxy group in the R_4 position, as it may facilitate enhanced $CysLT_1$ interaction due to increased hydrogen bonding or π-π interactions through the aryl group (41). Cell-based receptor assays confirmed significant inhibition of $CysLT_1$ receptor by Q8, but not of $CysLT_2$ or VEGFR1-3 at equivalent concentrations. Q8 was also investigated in combination with the anti-VEGF bevacizumab (45). Compared with either drug treatment alone, the combination showed a significant additive reduction in tubule formation, indicating that Q8 acts via a VEGF-independent pathway.

Quininib was tested in *in vitro*, *ex vivo* and *in vivo* mammalian angiogenesis models. Prior to animal models, the safety and efficacy of quininib in mammalian cells was confirmed in dermally derived human microvascular endothelial cells (HMEC-1) (39). Consistent with an anti-angiogenic profile, 5–20 μM quininib reduced HMEC-1 viability by 20–30% following 96-hour treatment. Importantly, 10 μM quininib significantly inhibited tubule formation in HMEC-1 cells, a surrogate measure of angiogenesis (46). Subsequent *ex vivo* experiments in rodents determined that 10 μM quininib reduced (non-ocular) sprouting angiogenesis in mouse aortic rings by 36%, in comparison to a 43% reduction with 10 μM montelukast, a clinically available $CysLT_1$ antagonist (39). In human tissue, 10 μM quininib significantly reduced the secretion of angiogenic and inflammatory factors (*e.g.* IL-6, IL-8, VEGF, ENA-78, GRO-α, TNF, IL-1β and MCP-1) from *ex vivo* human colorectal cancer explants (47). In mouse, quininib administered intraperitoneally is safe and well tolerated up to 50 mg/kg every 3 days (47). In relation to murine ocular safety, a maximum tolerated dose of 200 μM quininib administered intravitreally was identified. Mice exhibited normal retinal morphology in 200 μM quininib-injected eyes: evidenced by the presence of all cell types, normal lamination, and a lack of pyknotic nuclei. In the *in vivo* ocular context, it was initially planned to

administer quininib topically as eye drops. Technically, however, this proved too challenging in our oxygen induced retinopathy (OIR) model as mouse pups had not opened their eyes by postnatal day 12 (48). Thus, quininib was injected intravitreally. 0.5 μM quininib injected once into the vitreous of P12 OIR mouse pups inhibited retinal revascularization by P17 such that there was a 1.5-fold increase in avascular area compared to vehicle controls (39). Montelukast had a negligible effect on revascularisation at this concentration. Thus, quininib prevented the regrowth of the normal intraretinal vessels in mouse, a process comparable to developmental angiogenesis.

Quininib microparticles for prolonged ocular release.

For development of quininib drugs into therapeutic agents, it was necessary to show pre-clinically that they could be effectively delivered to the eye with a similar or better dosing regimen to anti-VEGF agents on the market. The ocular bioavailability of quininib when systemically or topically administered was not known, therefore the approach taken was to develop a microparticle formulation of quininib for intravitreal injection (49). Hyaluronan or hyaluronic acid (HA) is a naturally occurring glycosaminoglycan, consisting of repeating disaccharide units of D-glucuronic acid and d-N-acetylglucosamine subunits linked via alternating beta-1,3 and beta-1,4 glycosidic linkages (50). It is present endogenously in the body, including in the vitreous humour and retina (51, 52), and thus is non-immunogenic when administered. HA has received considerable interest in facilitating the delivery of a wide range of therapeutics (53, 54), including for approved ocular drugs such as Provisc® used as a surgical aid during cataract extraction. . On this basis, we synthesised and characterised a quininib-HA microparticle formulation in a two-step process (49). Scanning electron microscopy (SEM) revealed hollow, needle-shaped microparticles, which may be advantageous as elongated particles were documented to adhere to endothelial cells better than spherical particles (55). The encapsulation efficiency of quininib in the microparticles was 90% (49). Characterisation of the pharmacokinetic release profile showed that 20% of quininib was released from hydrated solutions over 16 weeks (49).

The quininib-HA microparticles significantly reduced HV vessel formation compared to empty-HA formulation (49). There was no significant difference in HV inhibition between neat quininib and quininib released from HA after 10 weeks (49). This data warranted further testing of the HA-quininib formulation in a pre-clinical model; a bespoke rat model of retinal vascular permeability (RVP), a pathological hallmark of nAMD and PDR. We developed a customised leukotriene-induced model of RVP in Brown Norway rats triggered by intravitreal injection of LTD_4 and LTC_4 (49). The larger rat eye (compared to mice) enabled injection of a greater volume of the quininib-HA microneedles. RVP was significantly reduced in rats pre-treated with quininib-HA microneedles compared to those pre-treated with neat quininib. This provided *in vivo* evidence of efficacy of the sustained release formulation after one month and of the ability of quininib to attenuate RVP in addition to angiogenesis.

An unforeseen role for CysLT receptors in Uveal Melanoma

Unexpectedly in 2016, the quininib drug series became relevant to ocular cancer. Uveal melanoma (UM) is a rare form of ocular cancer that arises from melanocytes within the uveal tract (56, 57). Despite advances in treatment of the primary tumour, approximately half of UM patients develop liver metastasis, most commonly through

hematogenous spread. Once UM has disseminated, patients are faced with an extremely poor prognosis. There are currently no effective standard-of-care therapies available for metastatic UM, with overall survival ranging from 4 to 19 months (58). Importantly, UM is entirely distinct to cutaneous melanoma, therefore advances in the treatment of cutaneous melanoma do not translate to UM. Mutations in *GNAQ* or *GNA11* are identified in >80% of all UM (59). In 2016, Moore *et al*. reported a previously unidentified recurrent mutation in *CYSLTR2* in primary UM patients (60). This mutation leads to constitutive activation of the receptor, resulting in *CYSLTR2* acting as an oncogene in a small subset (~4%) of UM (60). UM develops in one of the most capillary-rich tissues and spreads predominantly through the bloodstream. Highly vascularised UM is more aggressive and associated with a worse prognosis (61, 62). Therefore, it is unsurprising that angiogenesis is considered a highly important process in UM pathogenesis (63, 64). To date, anti-angiogenic therapy in UM has focused predominantly on VEGF, however the experimental and clinical results are conflicting (65). Bevacizumab is reported to both reduce (66) and accelerate (67) the growth of primary UM in *in vivo* mouse models. In primary UM patients, Bevacizumab does not halt tumour progression (68). Alternative anti-angiogenic drugs and pathways were therefore of interest. Our knowledge of the anti-angiogenic properties of the quininib series, coupled with the finding that *CYSLTR2* is a UM oncogene, led us to hypothesize that CysLT receptors are a relevant therapeutic target in UM (69). Using the published TCGA dataset (70) and *de novo* immunohistochemical approaches in primary UM patient samples, we discovered high expression of $CysLT_1$ associated with reduced overall survival and reduced survival from metastatic disease (71). In primary and metastatic human UM cell lines (harbouring *GNAQ* mutations, not the *CYSLTR2* oncogene), we observed $CysLT_1$ antagonists (quininib and 1,4-dihydroxy quininib), but not a $CysLT_2$ specific antagonist (HAMI 3379), to significantly alter the survival, long-term proliferation, metabolism, and secretion of inflammatory and angiogenic factors *in vitro* (71). Interestingly, 24-hour treatment with 20 μM quininib series drugs increases the secreted levels of angiogenic markers in primary and metastatic UM cells (71). In primary UM cells, 20 μM quininib significantly increased secretion of VEGF-C and bFGF, while 20 μM 1,4-dihydroxy quininib significantly increased secretion of Flt-1. In metastatic UM cells 20 μM quininib significantly increased secretion of Flt-1 and VEGF-A. Flt-1 binds VEGF-A with tenfold higher affinity than VEGFR2 (72) and negatively modulates angiogenesis as a decoy receptor trapping VEGF (73).This hints towards anti-angiogenic properties of quininib in metastatic UM cells. The parallel upregulation of VEGF-C and bFGF following treatment with quininib in primary UM cells may be compensatory result of a form of “pseudohypoxia” as previously described in UM and other tumour types (67). Our *in vitro* data was supported in zebrafish models whereby quininib drugs significantly inhibit the growth of both primary and metastatic zebrafish xenograft models (71).

What have we learned from the quininb series discovery?

The role of CysLTs in inflammation has been expanded to include an unknown role for $CysLT_1$ in developmental and pathological ocular angiogenesis. In agreement, recent retrospective studies report oral montelukast is associated with significantly reduced odds of nAMD and PDR (Pham, B. et al. Association for Research in Vision & Ophthalmology. *Invest Ophthalmol Vis Sci,* 2021; 8; 1152, Karaca, I. et al. Association for Research in Vision & Ophthalmology. *Invest Ophthalmol Vis Sci,* 2021; 8; 275). These are encouraging observations for the therapeutic potential of quininib, which has greater ocular anti-angiogenic activity than montelukast *in vivo* (39).

Quininib links $CysLT_1$ with developmental angiogenesis (39). In contrast, *Cysltr1 and Cystlr2* knockout mice do not show significantly impaired angiogenesis (74). This may reflect compensatory receptor upregulation or additional quininib targets. The quininib series also highlighted a previously unforeseen therapeutic opportunity in UM. Antagonists to $CysLT_1$, but not $CysLT_2$, alter cell survival and long-term proliferation, in UM models (71) supporting antagonism of $CysLT_1$ as a therapeutic strategy in UM.

Similar *in vivo,* phenotype-based screens could be undertaken to gain knowledge in other aspects of ocular biology. There are zebrafish models for ocular disease including cataracts, AMD, IRD, glaucoma, diabetic retinopathy and ciliopathies (75). Chemical screens with transgenic models can investigate rod outer segment (ROS) renewal or modulators of dominant retinitis pigmentosa (76). CRISPR/Cas9 technology facilitates robust disease modelling and target validation. The quininib series was identified in a "*one compound, one well*" approach. Advanced approaches such as orthogonal pooling where drugs within wells are combined into pools can expedite screening and reduce animal numbers (77). Integration of computational techniques can significantly reduce experimental costs by filtering the numbers of compounds required for testing. Future ocular drug screens can also benefit from enhanced phenotyping technologies enabling high-throughput manipulation, orientation and imaging of zebrafish larvae (78). These advances in model design, screening protocols and technologies can all be implemented in future drug screens to maximise hit output and drug candidate development while minimising costs and animal usage.

What is the future for the Quininib drug series?

In Europe, the number of people with early and late-stage AMD is predicted to increase to 21.5 and 4.8 million, respectively, by 2040 (79). 60 million people in Europe suffer from diabetes, 40% of which will develop DR. Given that the number of diabetics is expected to increase by a further 10 million in the next 14 years, as many as 28 million Europeans could suffer from DR visual impairment by 2035 (80). The current standard of care to treat ocular neovascularisations (e.g. DR and AMD) involves patients receiving periodic injections of anti-VEGF biologicals into the vitreous 7-12 times per annum (19, 81-83). What has become clear is that blockage of the VEGF pathway is not always an effective treatment. In nAMD, the SEVEN UP trial reported ranibizumab to improve visual acuity in one third of patients, halt disease progression in another third, but ineffective in the final third of patients (84). This is even greater for PDR patients, with approximately 50% non-responders (85, 86). In addition, some patients develop resistance to treatment after initially responding. One explanation is that attenuated VEGF signalling results in compensatory upregulation of non-VEGF angiogenic factors allowing disease progression. The difficulties associated with regular intravitreal delivery also remain. Newer anti-VEGF drugs have emerged, with emphasis put on extending the interval between injections (Aflibercept (87)), maintaining a higher ocular concentration of anti-VEGFs (Brolucizumab (88)), developing drugs with more than one target *e.g.,* Fovista, a platelet-derived growth factor (PDGF) antagonist, administered in combination with the anti-VEGF agent ranibizumab which failed at phase 3 trials (89), sustained release formulations and a refillable device (90). In many cases, clinical trials have struggled to show superiority and meet endpoints of increased efficacy compared to standard of care. Quininib has the potential to overcome some of these limitations, to be used either in combination with current anti-VEGF agents to increase efficacy and or as a single agent in those

who do not respond to current anti-VEGF agents. It may be possible to administer quininib drugs orally or topically, however, further studies are needed to demonstrate acceptable ocular pharmacokinetic properties. The quininib drugs show an additive effect *in vitro* when used in combination with bevacizumab (Avastin®) (45). Further studies are needed to validate this additive effect in an *in vivo* ocular model. There remains an urgent, clinical need for treatments for UM. Our preclinical data warrant further investigation of the disease relevance of CysLT receptors in metastatic UM and provide a rationale for analysing the therapeutic potential of $CysLT_1$ antagonism, potentially as a combination therapy. Ongoing research focuses on recapitulating our results using quininib and 1,4-dihydroxy quininib in patient-derived xenograft models and in *ex vivo* explant cultures generated from UM patient tumours. There is also potential to use quininib drugs beyond ocular indications. In 2005 it was predicted that approximately 500,000,000 people would benefit from anti-angiogenic therapies over the following 20-30 years (91). Cancer makes up a large proportion of those in need of antiangiogenic drugs and there is ongoing research on the use of quininib in colorectal cancer (47).

In summary, we discovered a series of novel anti-angiogenic drugs which target the CysLT pathway, which was previously unknown to act in ocular angiogenesis. Ocular anti-angiogenic action has been observed in zebrafish, mouse, and rat models of pathological angiogenesis which is associated with many serious ocular disorders causing much of global blindness, along with ocular cancer uveal melanoma. The development of the quininib series also exemplifies the utility of both random drugs screens and the use of zebrafish in drug discovery. There remain key questions to be answered before the clinical utility of the quininib series can be fully known, but this series has the potential to overcome shortcomings in current ocular anti-angiogenic agents by targeting a novel, independent pathway with a small molecule agent.

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Figures.

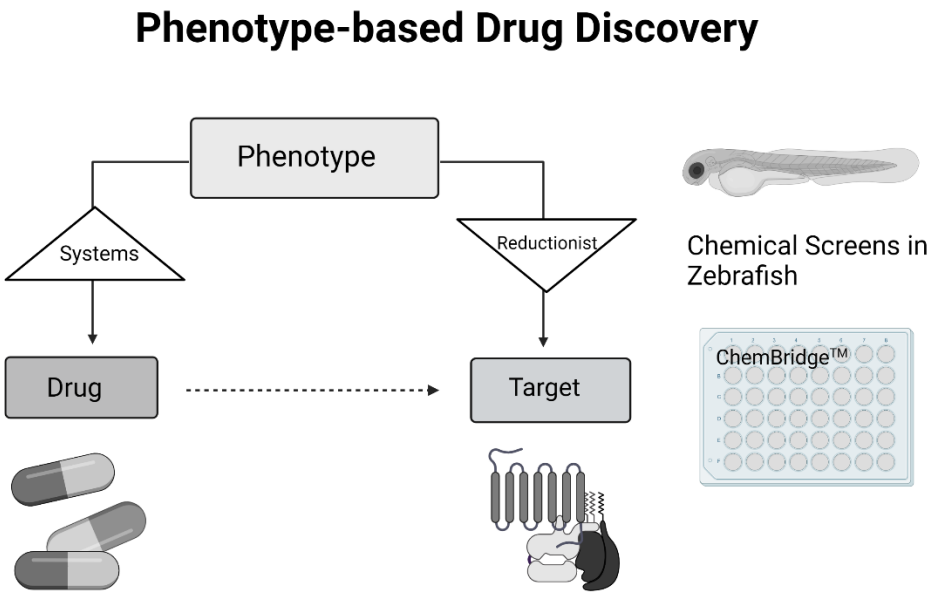

Figure 1.
Schematic detailing the strategy involved in the discovery of the quininib series of ocular drugs. Phenotype-based drug screening differs from target-based approaches as it is not biased towards a known target, instead focusing on a difference in phenotype which could occur due to an unknown target or a combination of targets. In the discovery of quininib, a ChemBridge™ random chemical library was used to screen for compounds affecting angiogenesis in zebrafish. Created with BioRender.com.

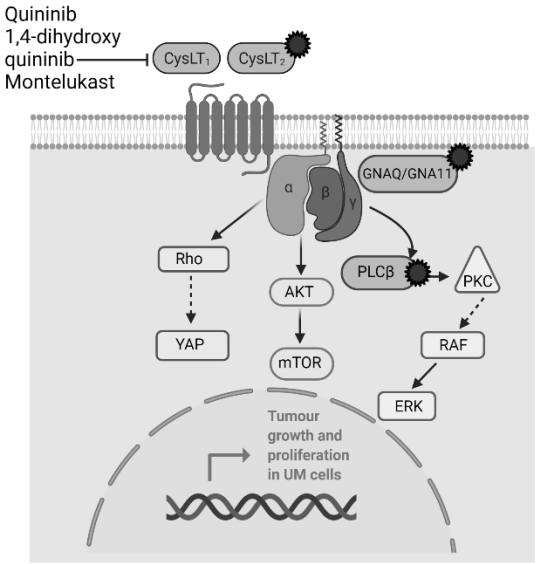

Figure 2.
$CysLT_1$ antagonists act upstream of the most common mutations in UM. $G_{\alpha q}$ pathway mutations in *GNAQ*, *GNA11*, *PLCB4*, and *CYSLTR2* (as indicated by*) are mutually exclusive

in UM and trigger the activation of $G_{\alpha q}$ signalling and related downstream pathways which promote tumour growth and proliferation. Created with BioRender.com.

Table 1: Results from *in vivo* Testing of the Quininib Drug Series (see main for references)

Compound	**IUPAC Name**	**Hyaloid Vessel Assay**	**Ocular Preclinical Disease Model**
Quininib (Q1)	2-[(*E*)-2-Quinolin-2-yl)vinyl]phenol	50% inhibition at 10 μM	1.5-fold increase in avascular area - Mice model of oxygen induced retinopathy
			26.2% reduction in tumour size - UM zebrafish xenograft (OMM2.5 cell line)
Q8	(E)-2-(2-Quinolin-2-yl-vinyl)- -benzene-1,4-diol HCl salt	33% inhibition at 5 μM	21.7% reduction in tumour size - UM zebrafish xenograft (OMM2.5 cell line)
Q18	(Z)-2-(2-(Quinolin-2-yl)vinyl) phenol HCl salt	33% inhibition at 5 μM	-
Q22	2-Quinolin-2-yl-ylethynyl-phenol HCl salt	53% inhibition at 10 μM	-
Quininib-HA formulation	Not applicable	~40% inhibition compared to empty-HA	Significantly attenuated leaked from retinal vessels - Rat model of retinal vascular permeability

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