TITLE

EVALUATION OF ONCOGENIC CYSTEINYL LEUKOTRIENE RECEPTOR 2 AS A THERAPEUTIC TARGET FOR UVEAL MELANOMA

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Abstract

Uveal melanoma is a rare, but deadly, form of eye cancer that arises from melanocytes within the uveal tract. Although advances have emerged in treatment of the primary tumour, patients are still faced with vision loss, eye enucleation and lethal metastatic spread of the disease. Approximately 50% of uveal melanoma patients develop metastases, which occur most frequently to the liver. Metastatic patients encounter an extremely poor prognosis; as few as 8% survive beyond 2 years. Understanding of the genetic underpinnings of this fatal disease evolved in recent years with the identification of new oncogenic mutations that drive uveal melanoma pathogenesis. Despite this progress, the lack of successful therapies or a proven *standard-of-care* for uveal melanoma highlights the need for new targeted therapies. This review focuses on the recently identified *CYSLTR2* oncogenic mutation in uveal melanoma. Here, we evaluate the current status of uveal melanoma and investigate how to better understand the role of this *CYSLTR2* mutation in the disease and implications for patients harbouring this mutation.

Epidemiology and aetiology of uveal melanoma

Uveal melanomas, which arise from the choroid (85-90% of cases), ciliary body (5-8% of cases) or iris (3-5% of cases), account for approximately 5.2% of all primary melanomas [1]. Although considered a rare disease, incidence ranges from < 2 per million to > 8 per million across Europe [2], uveal melanoma is the primary intraocular tumour found in adults. The overall incidence of uveal melanoma has remained relatively constant in comparison to other cancer types, but varies by race, sex and country [3]. Males have greater disease incidence than females and uveal melanoma is more common among Caucasians than non-Caucasians [4]. Interestingly, the National Cancer Registry Ireland reports an estimated 62 new cases of neoplasms of the eye and adnexa diagnosed in Ireland between 2015 - 2017 [5], this compares to approximately 1,700 new cases per year in the United States [6] and 430 new cases per year in the United Kingdom [7], suggesting that Ireland has a higher incidence of the disease per capita (1.3 cases per 100,000 per year in Ireland versus 0.52 cases per 100,000 per year in the U.S.).

Uveal melanoma patients are often asymptomatic (30.2% of patients), with disease first diagnosed during routine ophthalmic examination [8]. Patients experience blurred vision, the presence of floaters and/or perceived flashes of light, visual loss and pain in the eye [8].

Risk factors associated with uveal melanoma include an inability to tan, the presence of light coloured eyes (blue or green), fair skin, ocular melanocytosis and the presence of germline mutations in *BAP1* (BRCA – associated protein-1), a tumour suppressor gene found on chromosome 3 [3]. The role of ultraviolet light remains unclear; many uveal melanomas show no evidence of the UV radiation mutational signature commonly found in cutaneous melanoma [9]. However, intermittent ultraviolet exposure through welding arcs and flames is reported as a significant risk factor for uveal melanoma [10].

Importantly, uveal melanoma is clinically and molecularly distinct from cutaneous melanoma, the most common melanoma subtype [11]. Therefore, recent advances in targeted therapies for the treatment of cutaneous melanoma have failed to alter the clinical outcomes of uveal melanoma patients [12]. Disease- and most importantly, mutation-specific therapies for uveal melanoma are critical and likely to provide the most promising therapeutic strategies for uveal melanoma patients.

Prognosis of uveal melanoma

Treatment of primary uveal melanoma

Treatment of the primary disease is surgical, (*e.g.* resection or enucleation) to remove the tumour from the eye, or, more conservative radiation or laser therapy, which aim to preserve the affected eye [13]. Enucleation involves complete removal of the eye and orbital recurrence of the cancer after primary enucleation is rare [14]. Enucleation is common in cases of large (> 8 mm), locally advanced tumours in which vision cannot be retained [4]. However, globe-conserving therapies have become increasing popular after the 2006 Collaborative Ocular Melanoma Study confirmed no differences in survival between patients treated with iodine-125 brachytherapy and enucleation [15].

Brachytherapy for uveal melanoma involves placement of radioactive implants, most commonly emitting iodine-125 (^{125}I) or ruthenium-106 (^{106}R), directly on to the eye for several days [15, 16]. This allows for a concentrated dose of radiotherapy to be delivered directly to the tumour. Laser therapies, such as photodynamic therapy (PDT) and transpupillary thermotherapy (TTT) are also available, however, they are associated with a risk of local tumour recurrence [17, 18].

Treatment of metastatic uveal melanoma

Despite advances in the treatment of the primary ocular tumour, the prognosis of patients that develop metastatic uveal melanoma remains poor and the effect of ocular therapy on metastasis and survival remains uncertain [19]. Approximately 50% of patients develop metastatic disease, with the liver being the most common site (89% of metastatic patients), followed by the lung, bone and soft tissue [20]. The median overall survival from diagnosis of metastatic uveal melanoma ranges from less than 6 months to 13.4 months, with only 8% of patients surviving beyond 2 years [20, 21].

Unfortunately, the prognosis for metastatic patients is bleak. There remains no proven *standard-of-care* available for metastatic uveal melanoma patients [13]. Dacarbazine, a chemotherapeutic used in cutaneous melanoma, has limited therapeutic benefit in uveal melanoma [22]. Fundamental molecular differences in the two melanomas are the obvious reason. Uveal melanomas generally lack the *BRAF* mutations common to cutaneous melanoma and which is an established target for treating disseminated cutaneous disease [23]. Given that >80% of uveal melanomas possess mutations that drive constitutive activation of the MAPK/ERK pathway, drugs targeting this pathway are of major interest [24]. Selumetinib, a small molecule inhibitor of MEK1/2, resulted in improved progression-free survival versus chemotherapy in a phase II clinical trial of uveal melanoma patients [22]. However, no improvement in overall patient survival was reported [22]. Similarly, in a phase III double-blind study, a combination of selumetinib plus dacarbazine did not significantly improve progression free survival in metastatic uveal melanoma patients versus dacarbazine alone [25].

In summary, there is an overwhelming unmet clinical need to develop targeted therapies to improve the prognosis of uveal melanoma patients. Given that the majority of the driver mutations identified to date in uveal melanoma lead to constitutive activation of the MAPK/ERK pathway via aberrant Gαq signalling, the associated G proteins and G protein-coupled receptors represent enticing therapeutic targets for the prevention and/or treatment of the disease.

Genetic alterations in uveal melanoma

Notably, the primary mutations linked with development and progression of uveal melanoma are entirely distinct from those in cutaneous melanoma. Cutaneous melanoma has one of the highest mutational loads amongst cancer types, while uveal melanoma has a low mutational burden [26]. Roberson *et al*. reported a median somatic mutation density of 1.1 per Mb in uveal melanoma, which was markedly lower than in cutaneous melanoma, other melanoma subtypes or other solid tumours [9]. The lack of *bona fide* mutations in uveal melanoma has meant that the scope for targeted therapies is quite limited, with no successful targeted therapies to date.

Uveal melanoma can be subdivided into molecular classes, Class 1 or 2, based on a 15-gene assay developed by Onken *et al*. [27, 28]. In terms of 5-year risk of developing metastases, patients with Class 2 tumours harbour a 72% risk, whereas Class 1 tumours harbour a 21% risk [29].

Several chromosomal abnormalities associated with uveal melanoma can inform a patient's prognosis and their likelihood of metastasis [30]. 8q and 6p gains are frequently observed in uveal melanoma [31], as are losses in 1p, 6q and chromosome 3 [32]. Loss of 1p and chromosome 3, and gain of 8q are associated with worse patient prognosis and often found in Class 2 tumours, whereas gain of 6p is associated with a better patient outcome and commonly associated with Class 1 tumours [27, 33]. In particular, monosomy 3 is an extremely important prognostic test and is frequently associated with metastasis and Class 2 tumours [33].

Additional analysis of 80 uveal melanomas from TCGA (The Cancer Genome Atlas https://cancergenome.nih.gov/) identified four distinct and clinically relevant disease subtypes; two associated with monosomy 3 and poor patient prognosis and two associated with disomy 3 and a more positive patient prognosis [9]. Disomy 3 uveal melanomas

were further divided into transcription-based clusters 1 and 2, while monosomy 3 uveal melanoma were further divided into transcription-based clusters 3 and 4 [9].

Uveal melanomas are predominantly characterised by mutations in *GNAQ* and *GNA11 (*a paralog of *GNAQ*), both of which encode for G-protein alpha subunits and share 90% amino acid sequence homology [34]. Overall, 83% of uveal melanomas contain mutations in either *GNAQ* or *GNA11*, however, these mutations do not correlate with prognosis [35]. *GNAQ* mutations occur almost exclusively at codon 209 and result in glutamine to leucine (p.Gln209Leu), or proline (p.Gln209Pro) amino acid substitutions. In both cases, this mutation occurs within the GTPase domain and results in a constitutively active G-protein [36]. Similarly, mutations in *GNA11* are predominantly found at position Q209 and result in similar downstream consequences [35] In 2016, a recurrent hotspot mutation in *PLCB4*, a downstream target of GNAQ/GNA11 was identified in 2 of 28 samples assayed [37]. PLCβ4 is activated upon binding of a G-protein subunit, resulting in cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce diacylglycerol (DAG) and inositol triphosphate (IP3), and calcium release from the cell. The *PLCB4* hotspot mutation is also a gain-of-function mutation leading to constitutive activation of the MAPK/ERK pathway.

Recurrent mutations in splicing factor SF3B1 occur at codon 625 in approximately 18.6% of tumours and are associated with low-grade uveal melanomas with good prognosis [38]. Similarly, mutations in *EIF1AX* are associated with better patient outcomes [39]. *SF3B1* and *EIFAX* mutations appear to occur most frequently in uveal melanomas with disomy 3, which rarely metastasize and are often grouped into the Class 1 category [39].

BAP1 (BRCA associated protein-1) mutations are found in approximately 84% of metastasizing uveal melanoma tumours [40]. *BAP1* maps to chromosome 3p21.1 and is implicated as a tumour suppressor gene [26]. Both somatic and germline mutations in *BAP1* occur in uveal melanoma patients [40]. *SF3B1* and *BAP1* mutations are almost mutually exclusive, as also suggested for *BAP1* and *EIF1AX [41]*, suggesting that they represent alternative pathways in tumour progression [38].

Recently, Moore *et al*. analysed DNA data from 136 uveal melanoma patients and identified seven significantly mutated codons in six genes [41]. Amongst those identified were *GNAQ, GNA11, PLCB4, SF3B1,* and *EIF1AX*; all previously linked to uveal melanoma. Interestingly, they also identified a c.386T>A mutation in cysteinyl leukotriene receptor 2 (*CYSLTR2*) which encodes a p.Leu129Gln substitution not previously described in the literature [41].

This activating, recurrent hotspot mutation in *CYSLTR2* was identified in 4 of 136 uveal melanoma patient samples analysed from different cohorts [41]. Interestingly, this mutation was found only in patients lacking *GNAQ, GNA11* or *PLCB4* mutations, all of which are established driver mutations in uveal melanoma. The presence of mutually exclusive somatic mutations in *GNAQ, GNA11, CYSLTR2* and *PLCB4* was further confirmed in a comprehensive analysis of patient samples in the Rare Tumor Project of The Cancer Genome Atlas (TCGA) by Robertson *et al* [9]. As mutually exclusive mutations often operate in the same pathway, this data suggests that the newly identified *CYSLTR2* mutation is associated with the same pathway as previously identified driver mutations, highlighting the importance of this *CYSLTR2*/Gαq/11/*PLCB4* pathway and of Gαq signalling in uveal melanoma oncogenesis.

Mutations in *GNAQ* and *GNA11* are not predictive of prognosis or the likelihood of metastases. However, patients lacking *GNAQ* or *GNA11* mutations have worse disease-free and overall survival than those with these mutations. This suggests that patients harbouring alternative mutations such as *CYSLTR2* or *PLCB4* may have a worse prognosis than those carrying *GNAQ/GNA11* mutations [35].

Activating mutations in *GNAQ* or *GNA11* are found in >80% of all uveal melanomas, irrespective of tumour class, and are also frequent in blue nevi, melanocytic nevi found in the dermal layer of the skin. Mutations in either *CYSLTR2* or *PLCB4* likely account for an additional 8-10% of activating mutations. Robertson *et al.* reported that neither *CYSLTR2* nor *PLCB4* mutations preferentially localized to a specific subset of uveal melanoma, consistent with mutations in these genes functioning like *GNAQ* and *GNA11* mutations to drive tumorigenesis without initiating metastasis [9]. One theory suggests that the mutation associated with the *CYSLTR2*/Gαq/11/PLCB4 pathway occur early in tumour progression and are important initiating events but are not sufficient for malignant transformation. In contrast, genomic *BAP1* pathway mutations occur later in the progression of uveal melanoma and likely correspond with tumour metastasis [40]. Thus, simultaneous targeting of both Gαq coupled receptor signalling and *BAP1*

signalling pathway mutations might have synergistic therapeutic effects in the treatment of uveal melanoma. Targeting of the *BAP1* pathway has proven effective in different cancer types. Indeed, olaparib, an oral PARP inhibitor, has anti-tumour activity in metastatic breast cancer patients with germline *BRCA* mutations [42].

Cysteinyl leukotriene signalling

The novel oncogenic mutations in *CYSLTR2* warrant further investigation of the associated signalling pathway in the pathogenesis and treatment of ocular melanoma. The cysteinyl leukotrienes (CysLTs), LTC_4, LTD_4 and LTE_4, are a group of inflammatory, lipid, signalling molecules that mediate both acute and chronic inflammation. Indeed, cysteinyl leukotriene receptor antagonists are routinely prescribed in the treatment of asthma and allergic rhinitis [43, 44]. These eicosanoids are synthesized from arachidonic acid (AA) in the cell membrane upon cell activation. The 5-lipoxygenase enzyme (5-LOX) interacts with a 5-lipoxygenase activating protein (FLAP) which enhances the activity of 5-LOX to convert AA mobilised to the cytosol to the unstable leukotriene LTA_4 [45]. LTA_4 is further hydrolysed to LTB_4 or LTC_4 via LTC_4 synthase. Intracellularly synthesized LTC_4 is exported from the cell via multidrug resistance-associated proteins and rapidly metabolised to the remaining cysteinyl leukotrienes, LTD_4 or LTE_4 [46]. Synthesis of the CysLTs occurs predominantly in immune cells such as neutrophils, eosinophils, monocytes, macrophages and mast cells [47].

Thus, the CysLTs are a group of structurally similar but functionally different lipid mediators that exert their biological effects via binding to the GPCRs (G-protein-coupled receptors), $CysLT_1$ and $CysLT_2$[(1)] [48]. $CysLT_1$ and $CysLT_2$ are located on the plasma membrane [49, 50], however, both receptors possess the ability to localize to the nuclear membrane [51, 52]. LTC4 and LTD4 binds $CysLT_2$ with low, but equal affinity, LTD4 and LTC4 bind $CysLT_1$ with high and low affinity, respectively [49]. Neither receptor subtype exhibits substantial affinity for LTE^4 [48]. However, additional CysLT receptors, GPR17 and GPR99 have been reported. GPR17 is a G protein-coupled orphan receptor with homology to both the P2Y and CysLT receptors. GRP17 is reported as a ligand-independent negative regulator of $CysLT_1$ [53]. GPR99, also described as cysteinyl leukotriene receptor E (CysLTE) or $CysLT_3$ is proposed as a potential LTE^4 selective cysteinyl leukotriene receptor [46].

Cysteinyl leukotriene signalling in cancer

Cysteinyl leukotriene signalling is implicated in inflammation, bronchoconstriction, increased vascular permeability, mucus production and white blood cell recruitment [54-56]. A recent review evaluated links between CysLT receptors and many hallmarks of cancer including angiogenesis, sustained proliferative signalling, migration and invasion [57]. Interestingly, overexpression of $CysLT_1$ presents in colorectal cancer, prostate cancer, renal cell carcinoma, transitional cell carcinoma and testicular cancer [58-61]. Tsai *et al.* conducted a large, population-based study to investigate the effect of leukotriene receptor antagonists on the risk of cancer development in newly diagnosed asthmatic patients. Leukotriene receptor antagonists decreased the risk of 14 different cancers analysed in a dose dependent manner, suggesting that CysLT receptor antagonism provides a cancer-protective effect [62].

Moore *et al*. identified the recurrent hotspot mutation in *CYSLTR2* as a driver oncogene [41]. The oncogenic properties of the $CysLT_2$ were later supported by Möller *et al.* who identified the same Leu129Gln hotspot mutation in blue nevi [63]. Interestingly, in other cancer types $CysLT_2$ exerts anti-cancer properties. CRC patients with high nuclear $CysLT_1$ expression have a poor prognosis, while patients with high nuclear $CysLT_2$ expression have a better overall prognosis, suggesting that $CysLT_2$ is protective in CRC [64]. Magnusson *et al.* reported a similar phenomenon in breast cancer patients, whereby patients with large tumours exhibiting high $CysLT_1$ and low $CysLT_2$ expression levels had a significantly reduced survival [65]. Indeed, it is suggested that regulation of $CysLT_2$, leading to increased expression of the receptor, may have anti-tumour properties in CRC [66, 67].

Two *CYSLTR2* mutations, p.Arg136His and p.Arg136Cys, were identified in colorectal cancer [41]. However, with exception to the Leu129Gln hotspot mutation in uveal melanoma and blue nevi, *CYSLTR2* is not significantly mutated in any other cancer types, nor have other hotspot mutations been identified, suggesting this is a unique driver mutation in uveal melanoma and blue nevi. However, *CYSLTR2* is overexpressed in certain acute myeloid leukaemia subtypes [68].

This raises an interesting question about the role played by the different cysteinyl leukotriene receptors in various cancer subtypes. Increased expression of endogenous $CysLT_2$ has a protective effect linked to negative regulation of

$CysLT_1$ [69, 70]. Lack of $CysLT_2$ receptors may facilitate the formation of $CysLT_1$ homodimers, leading to heightened LTD_4 signalling which may promote a pro-tumorigenic phenotype [48]. Constitutive activation of $CysLT_2$ in uveal melanoma acts as an oncogene, suggesting opposing effects to those documented in colorectal and breast cancer. It will be interesting to determine if the oncogenic *CYSLTR2* mutation influences $CysLT_1$ signalling, expression or localization.

Cysteinyl leukotriene receptor 2 as a uveal melanoma oncogene

The $CysLT_2$ mutation associated with uveal melanoma and more recently, blue nevi, occurs at Leu129, which is situated in transmembrane helix 3, a functional hub of the receptor. This mutation leads to constitutive activation of the receptor and endogenous signalling, leaving it unresponsive to leukotriene stimulation *in vitro* [41]. Moore *et al.* characterised the oncogenic potential of this mutation by stably expressing the mutant Leu129Gln $CysLT_2$ in melan-a cells [41]. Mutant Leu129Gln, but not wild-type $CysLT_2$, conferred TPA(12-O-Tetradecanoylphorbol-13-acetat)-independent growth *in vitro* [41]. In agreement, siRNA mediated knockdown of exogenous *CYSLTR2* reduced the growth of melan-a cells grown in the presence or absence of TPA but had no effect on those expressing the wild-type receptor [41]. This exciting preliminary *in vitro* data suggests that inhibition of $CysLT_2$ in patients harbouring this oncogenic mutation may have therapeutic potential in the treatment of uveal melanoma.

Melan-a cells applied by Moore *et al.* are a melanocyte, non-tumorigenic cell line derived from the embryonic skin of 18-day-old C57BL mice and require phorbol-esters such as TPA for growth [71]. While melan-a cells are commonly used in melanoma research [35, 36], it will be important to also investigate the effects of this oncogenic mutation in human derived uveal melanoma cells. When mutant Leu129Gln was stably expressed in Mel290 cells, a human uveal melanoma cell line lacking *GNAQ* or *GNA11* mutations, the expression of melanocyte-lineage specific genes was significantly upregulated by RT-qPCR analysis compared to empty vector and wild-type control [41]. It will be interesting to examine whether expression of the oncogenic Leu129Gln mutation alters the cellular phenotype or additional hallmarks of cancer in uveal melanoma *in vitro* and *in vivo*. The effect of knockdown, or indeed knockout of the receptor in uveal melanoma cells also remains to be established. Similar experiments could also be conducted and validated using the Mel285 uveal melanoma cancer cell line, which is also reported as wild-type for both *GNAQ* and *GNA11* [72].

To strengthen the $CysLT_2$/Gαq/11/PLCβ4 pathway hypothesis, steps should be taken to examine the downstream signalling effects associated with the constitutively active Leu129Gln *CYSLTR2* mutation. Given that the best understood signalling pathway in uveal melanoma is the MAPK/ERK pathway, which is known to be activated by *GNAQ* and *GNA11* mutations [34], it is likely the *CYSLTR2* mutation upregulates this pathway. *GNAQ* and *GNA11* mutated uveal melanoma cell lines cause increased expression of phosphorylated MEK and phosphorylated ERK, which can be abolished via knockdown of the respective gene [36, 73]. In *GNAQ* and *GNA11* mutated cell lines, MAPK pathway activation occurs as a result of PKC activation [73]. As such, levels of p-MARCKS, a substrate of PKC, are detectable in uveal melanoma cells harbouring these mutations and can also be suppressed following knockdown [73]. Similar results would be expected from cell lines expressing the *CYSLTR2* mutation.

Given the well documented role of CysLT receptors in angiogenesis and inflammation, additional IHC and expression analysis could examine the vascular and inflammatory status of the Leu129Gln expressing cells. Cysteinyl leukotriene receptor antagonists can promote anti-angiogenic activity via a VEGF-independent pathway [74, 75]. It will be interesting to examine the levels of VEGF and other associated angiogenic markers in the oncogene background. Given the cross-regulation that occurs between the CysLT receptor subtypes, investigation into the effect of the Leu129Gln mutation on the expression of $CysLT_1$ is warranted.

Moore *et al.* also reported tumorigenic properties of the Leu129Gln *CYSLTR2* mutation *in vivo*. Leu129Gln expressing cells engrafted subcutaneously into immunocompromised mice significantly accelerated tumour formation versus the empty vector control [41]. These findings demand further investigation using additional model organisms and more advanced preclinical tumour models to evaluate the role of cysteinyl leukotriene signalling in uveal melanoma *in vivo* and to determine the relevance of *CYSLTR2* mutations to the patient disease.

Patient derived xenograft (PDX) models of uveal melanoma

Patient-derived xenograft (PDX) models have become a powerful tool in cancer research. PDX models are generated when cancerous cells or tissue taken directly from a patient's tumour are implanted into an immunocompromised mouse. Accumulating evidence suggests that PDX models have major advantages over the traditional cell line derived xenograft models as they show less divergence from the original patient tumour and more closely resemble the patient sample in terms of histology, gene expression, therapeutic response and metastatic behaviour [76-78].

Heterotopic uveal melanoma PDX models were previously generated, with a 28% engraftment success rate [79, 80]. Tumours taken from primary ocular tumours or metastases were implanted into the interscapular fat pad of SCID (severe combined immunodeficiency) female mice [80]. While useful for pharmacological studies, subcutaneous PDX models come with limitations. Firstly, they present a low engraftment rate and a slow tumour growth. Moreover, as expected, the vast majority of human solid tumours that grow subcutaneously in mice do not metastasize.

Orthotopic PDX (PDOX) or orthoxenografts are generated when the tumour is implanted into the organ of its origin. PDOX models better recall molecular features, histology, metastasis and drug response patterns, making them more suitable for translational research [81]. Recently, PDOX models using uveal melanoma liver metastases were developed with 10 of 12 hepatic metastasis specimens successfully xenografted into immunocompromised mice [82]. Similarly, orthotopic transplantation of uveal melanoma tumours directly into the eye will be extremely important to truly model the correct tumour environment. Exciting preliminary data shows the successful development of orthotopic uveal melanoma xenografts implanted directly into the eye *(Figures 1 & 2)*. To our knowledge, this is the first report of successful orthotopic transplantation into in the eye . These PDOX models will undoubtedly prove invaluable tools in the field of uveal melanoma research and for the identification of therapeutic strategies.

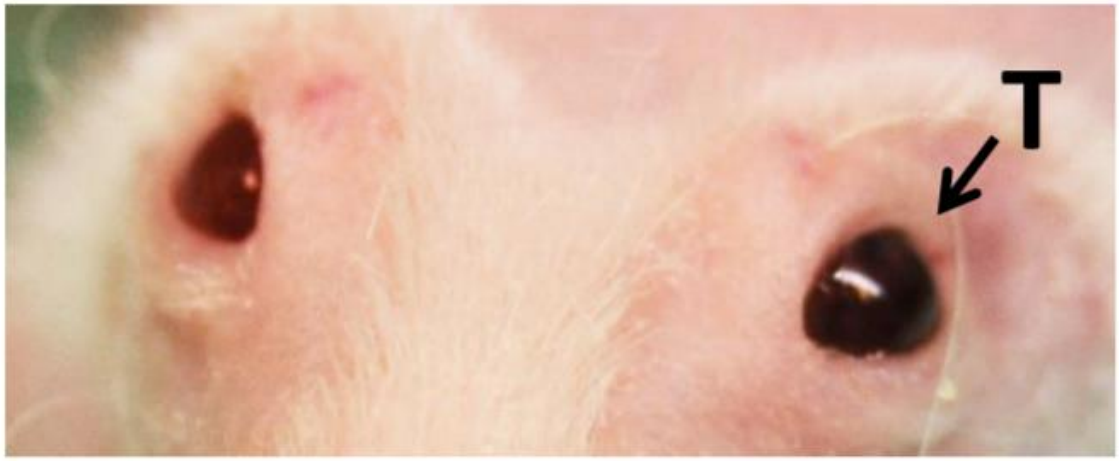

Figure 1 SCID mouse with orthotopically engrafted uveal melanoma tumour (T). This PDOX model was generated from human tumour tissue obtained from enucleation. A small tumour fragment was mechanically disaggregated, mixed with Matrigel and injected into eye.

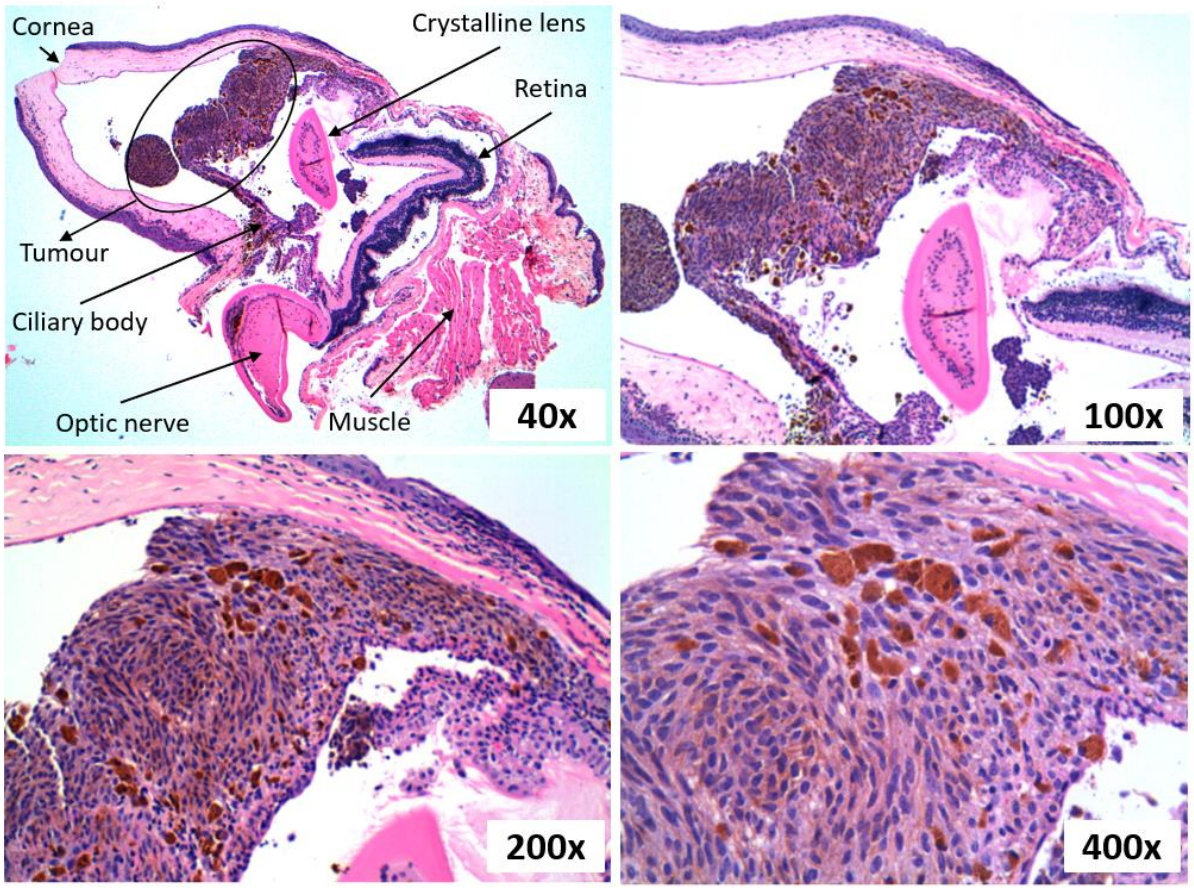

Figure 2 Histology of PDOX model of uveal melanoma showing evidence of tumour growth in the ciliary body. Tumour cells are spindle shaped, heavily pigmented in some areas with uniform nuclei and are arranged in a spiral pattern.

Undoubtedly, large numbers of PDOX models are needed to accurately reflect the mutational diversity found in uveal melanoma and to reflect the different sites in which uveal melanomas are found (choroid, ciliary body and iris). It has been reported that tumours harbouring *GNA11* mutations grow significantly better than *GNAQ* mutated tumours and that metastatic tumours engrafted more successfully than those taken from the eye when implanted subcutaneously [79]. It will be of interest to see the effect of *CYSLTR2* and *PLCB4* mutations on PDX development. Given the rarity of these mutations in uveal melanoma patients it may take some time to generate PDX models with the desired mutations. However, the generation of PDOX models derived from patients harbouring the *CYSLTR2* mutation would allow for more in depth analysis of this mutation and its role in disease progression, metastasis and drug responsiveness. Once a successful PDOX model harbouring the *CYSLTR2* mutation is established, the tumour can be expanded to generate a tumour bearing colony of mice in molecular pathology and therapeutic efficacy can be analysed. Given the rarity of *CYSLTR2* mutations, this approach will offer a quicker and more comprehensive method of analysing the consequences of this mutation. It will be exciting to examine the effect of CysLT receptor antagonists in cell lines and *in vivo* models expressing the mutant *CYSLTR2*. However, given the constitutively active nature of the mutant receptor, it is likely that regular antagonists of the receptor will be ineffective.

Inverse agonists to target $CysLTR_2$

$CysLT_1$ antagonists, montelukast, zafirlukast and pranlukast are prescribed for the treatment of asthma and allergic rhinitis. BAY u9773 is a non-selective cysteinyl leukotriene receptor antagonist at both $CysLT_1$ and $CysLT_2$ receptors [83], while HAMI 3379 is described as a potent and selective $CysLT_2$ antagonist [84].

Aberrant expression and activity of GPCRs in cancer is well established and they have become a compelling therapeutic target in the disease. In order to effectively target the Leu129Gln mutation in *CYSLTR2* an inverse agonist that selectively targets this receptor will be required. Inverse agonists preferentially bind to and stabilize a constitutively active receptor, maintaining the receptor in an inactive state and thus have intrinsic negative activity [85]. This differs to a neutral antagonist which can block the actions of agonists and inverse agonists. Neutral antagonists exhibit equal preference for both the active and inactive state and have no intrinsic activity [85].

GPCRs represent one of the most common drug targets and yet there are few examples of anti-tumour agents that directly target these receptors [86]. Even fewer examples of inverse agonists as anti-cancer agents are available. However, ALX-065, a biparatopic nanobody that acts as inverse agonist, blocks spontaneous activation of the CXC4 receptor and inhibits cell migration [87, 88], suggesting that inverse agonists may have the potential to act as successful chemotherapeutic agents.

Given that inverse agonists targeting $CysLT_1$ are currently in clinical use [89], it is certainly possible that an inverse agonist acting at $CysLT_2$ is available. Indeed, many compounds that were previously classified as antagonists, actually possess inverse agonist activity [90], suggesting that some anti-cancer GPCR antagonists may in fact mediate their effects through inverse agonism. BAY u9973 does not act as an inverse agonist at $CysLT_1$ [89], however, it exhibits weak potency at the human $CysLT_1$ and the exact activity of this drug at $CysLT_2$ remains to be studied. In addition, it will be important to test HAMI 3379 to determine if this selective antagonist possesses similar inverse agonist capabilities which could be used to target the constitutively active $CysLT_2$ receptor.

The relevance of a *CYSLTR2* mutation to the patient disease

The *CYSLTR2* mutation can be considered a rare mutation in a rare form of cancer. Moore *et al.* identified this mutation in 4 of 136 patients (~3% of study subjects) [41]. Three of the identified samples came from a cohort of 80 samples taken from the TCGA, while one additional sample came from a cohort of 22 samples from the University of Duisburg-Essen (UNI-UDE). In the United States, approximately 1,700 patients are diagnosed with this cancer each year [6], suggesting that a potential 51 newly diagnosed patients have $CysLT_2$ mutations.

The UNI-UDE sample came from the enucleated eye of a 77-year-old male treatment naïve for the disease. This tumour was positive for monosomy 3 and possessed a *BAP1* mutation. Sample V4 A9ED from TCGA was a stage IIIa tumour from a Caucasian male, diagnosed at 42 years old. Sample YZ A982 was a stage IIIb tumour from a Caucasian female, diagnosed at 79 years old. Sample VD AA80 was a stage IIb tumour from a now deceased male of unknown ethnicity, diagnosed at 77 years old. Given the limited number of patient samples available it is difficult to extrapolate meaningful inferences from the data in terms of tumour and patient characteristics. In the future, with additional patient samples it will be possible to determine whether *CYSLTR2* mutations influence patient survival or the development of metastases.

Blue nevi are common melanocytic tumours that occur in the dermal layer of the skin [63]. Blue nevi generally lack *BRAF* and *NRAS* mutations commonly found in neoplasms of epithelial melanocytes [36]. Instead, blue nevi display a similar genetic profile to that found in uveal melanomas, and frequently possess recurrent activating mutations in *GNAQ* and *GNA11* [36, 91]. *BAP1* mutations are reported in metastatic blue nevi, further strengthening the role of *BAP1* in metastatic potential and poor patient outcomes in certain cancer subtypes [92, 93]. Based on this knowledge and the additional findings of $CysLT_2$ and PLCβ4 mutations in uveal melanoma, Möller *et al.* sought to analyse a cohort of blue nevi lacking *GNAQ* or *GNA11* mutations to determine if driver mutations in *CYSLTR2* and *PLCB4* are also present. 3% of tumours analysed harboured a mutation in *CYSLTR2*, which is identical to the frequency of the mutation reported in uveal melanoma [41, 63]. Moreover, the mutation in *CYSLTR2* was the same c.386T>A, L129Q, mutually exclusive, hotspot mutation identified in uveal melanoma samples by Moore *et al.* [63]. The three *CYSLTR2* mutations reported by Möller *et al*. were found in morphologically benign common blue nevi [63].

These findings highlight the strikingly similar genetic similarities between the two melanocytic tumour types affecting different organ systems, and that similar treatment strategies may be effective against both types of neoplasms.

Given the rare frequency of *CYSLTR2* mutations in uveal melanoma and blue nevi, it is important to continue to study large numbers of tumours to further understand the role of cysteinyl leukotriene receptor 2 in disease and to validate its utility as a therapeutic target. Similarly, the prognosis and survival of those patients identified with *CYSLTR2* mutations should be closely monitored. Furthermore, over-expression and CRISPR/Cas9 mediated knock-out or knock-in strategies targeting the cysteinyl leukotriene receptor 2 will help to further validate its role as a uveal melanoma oncogene and to test the therapeutic potential of targeting the receptor.

Conclusion

There is an overwhelming unmet clinical need to develop new therapeutic strategies for the treatment of uveal melanoma. To date, no targeted therapy has proven successful in the treatment of this disease. The cysteinyl leukotrienes play an established role in inflammation and angiogenesis and have an established role in other cancer subtypes. Moreover, the cysteinyl leukotrienes have been successfully targeted in other diseases and antagonists have demonstrated anti-tumour properties *in vitro* and *in vivo*. The *CYSLTR2* hotspot mutation identified in uveal melanoma acts as an activating, oncogenic driver mutation and may have therapeutic potential in the subset of patients harbouring this mutation. Further *in vitro* and *in vivo* analysis is warranted to fully appreciate the implications of this mutation in terms of altered signalling, likelihood of metastasis and patient prognosis. Similarly, due to the low incidence of the disease, it is not feasible to conduct numerous clinical trials, especially those that are mutation specific. The development of orthotopic PDX models harbouring specific *CYSLTR2* mutations are likely the best way to model the patient disease and to determine the effectiveness of drug strategies targeting this mutation.

FOOTNOTES

(1) Correct nomenclature of the cysteinyl leukotriene receptors ($CysLT_1$ and $CysLT_2$) as per the IUPHAR/BPS Guide to PHARMACOLOGY [94].

AUTHOR CONTRIBUTIONS

KS was the primary author of the review. PSH and AMB contributed intellectual input. JMP, AV and AP were responsible for PDOX model development and drafted a section for the review. BNK contributed significant intellectual input, revised and edited the review. All authors reviewed the final manuscript.

CONFLICT OF INTEREST STATEMENT

KS is an employee of Genomics Medicine Ireland. AV is the chief scientific officer and co-founder of Xenopat S.L. AP is the chief executive officer and co-founder of Xenopat S.L.

The other authors declare no competing financial interests that could be construed as a potential conflict of interest.

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