EXPLORE THE ANTICANCER AND ANTIMETASTATIC EFFECT OF SOLUBLE EPOXIDE HYDROLASE INHIBITORS (sEHI) IN THE TREATMENT OF HUMAN UVEAL MELANOMA (UM) IN 2D AND 3D CELL CULTURE MODELS

by

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STATEMENT OF ORIGINALITY

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Hiep Dang

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Abstract

BACKGROUND

Uveal melanoma (UM) is the primary form of eye cancer– a rare but deadly disease with up to 50% of the patients' died from metastasis. There are few proven pharmacological treatments for UM, which are associated with suboptimal clinical outcomes. The pathogenesis of UM is largely unknown, which greatly limited the progress of UM drug discovery.

AIM: This project aims to screen novel drug candidates from a group of soluble epoxide hydrolase inhibitors (sEHIs) for the treatment of UM, which will address the unmet need in this cancer.

METHODS: In this project, I have investigated the impact of a group of sEHIs on cell viability in four different UM cell lines (i.e. C918, 92.1, MP46 and OMM1 originated from primary or metastatic UM) using AlarmaBlue and MTT assays. UC2288 was selected as the lead molecule due to its potent effect in reducing UM cell viability. The subsequent molecular assays evaluated the anti-cancer effect of UC2288 with deriving its IC_{50} values and its impact on cell death using LDH assay in four UM cell lines. And the mitochondrial function and health were assessed using ROS and JC-1 assays with or without UC2288 treatment in UM cell lines. The anti-metastatic effect of UC2288 was explored with wound healing, cell invasion and colony formation assays. UC2288-induced cell death was studied using cell cycle flow cytometry. Additionally, I validated the anti-cancer effect of UC2288 in the 3D UM *in vitro* models - a pilot study.

RESULTS: UC2288 decreased cell viability, induced ROS level and impaired mitochondrial membrane potential in UM cell lines. The IC_{50} values of UC2288 in four UM cell lines were ranged from 1.99 to 3.77 μM. Noteworthy, UC2288 exhibited the most potent cytotoxicity in 92.1 cell line. Further molecular assays proved that UC2288 deceased cell migration and invasion as well as promoted reproductive cell death in UM cell lines. The anti-UM effect of UC2288 was also confirmed in 3D UM cell culture models with the most prominent effect observed in C918 and 92.1.

CONCLUSION: UC2288 has a potent anti-cancer and anti-metastatic effect in UM. The findings in this study suggested that UC2288 is a novel drug candidate for UM, which shall be further investigated for its clinical values.

KEYWORDS: UC2288, Uveal melanoma, soluble epoxide hydrolase inhibitor, 3D cell model, anti-cancer effect.

Chapter 1: Uveal Melanoma

Cancer is among leading causes of death in the world. Hanahan et al. (2010) organised the complexity of such neoplastic diseases for rationalisation using cancers hallmarks. Cancers comprise six biological capabilities, including long-term proliferative signalling, resistance to anti-growth signals, cell death resistance, tissue invasion and metastasis, angiogenesis, and unlimited replication potentials [1, 2]. Human uveal melanoma (UM) is a rare cancer, but it is the most common intraocular malignancy in adults as well as the second most common melanoma. The work reported in this thesis focuses on the drug discovery and development against UM.

1.1 EPIDEMIOLOGY, AETIOLOGY, DIAGNOSIS AND PROGNOSIS OF UM

1.1.1 UM Epidemiology

UM is a rare but deadly cancer that affects the eye, with an incidence rate of 7.6 per million adults in Australia [3]. Although the incidence varies by sex, race and country, the average age-standardised incidence rate (ASRs) of UM in Australia in males and females are 8.4 and 6.9 per million, respectively [3]. At diagnosis, the median age is 62 years and is most commonly found in the Caucasian population [4]. Like any other cancers, the earlier the detection, the better the prognosis and the higher chance of survival. There are currently few treatment options for UM, primarily non-pharmacological approaches such as surgery (e.g. resection, enucleation, exenteration) [5]. More importantly, there are few proven drugs for UM. Evidently, tebentafusp is an approved immunotherapy drug and data in phase 2 clinical trial suggests that it has promising clinical activity while maintain a safety profile in patients with metastatic UM [6]. Despite reported skin related adverse events in 51 patients (40%) due to cytokine release syndrome, the study produced positive endpoint. The rate of disease control was 32% (n=40), and 45% remained in stable status of disease progression at ≥ 8 weeks. Moreover, tumour shrinkage was observed in 44% of the patients, the overall progression-free survival was 2.8 months and that of the median overall survival was 16.8 months. These numbers are significant in uveal melanoma research given the deadliness nature of this disease [6]. Although recent research advancements suggest more treatment options and attempts to improve prognosis, the risk of metastasis and its mortality remain unchanged. Indeed, there are current challenges in developing potential therapies for UM due to the limited knowledge about its cause and pathogenesis [5].

1.1.2 UM Aetiology

UM originates from melanocytes in the uveal tract including choroid, ciliary body and iris [5]. UM is most commonly present in the choroid, which accounts for 85-90% of all UM cases, where 3-5% cases occur in the iris and 5%-8% in the ciliary body. Although the pathogenetic cause of UM remains unknown, various predisposing factors have been reported in literature, such as light-coloured eyes, fair skin, an inability to tan, ocular melanocytotic and BAP1 mutations. It has been considered that people with light skin colour or blue- or grey-coloured eyes have a higher risk of developing UM. This may be because less melanin is presented in the choroid and retinal pigment epithelium, resulting in less protection from ultraviolet light [7]. A study found that germline BAP1 mutations in UM patients are associated with the risk of subsequent tumours like cutaneous melanoma [8].

The UM tumours present at each anatomical location has distinctive clinical features. Although iris melanoma is much less common, it is diagnosed 10-20 years earlier than those found in other anatomical locations [9]. Iris melanoma is mainly discovered by incident findings due to iris colour changes and pupil distortion [10]; whereas the ciliary body and choroidal melanoma exhibit symptoms such as blurred vision, floaters, visible tumour and pain, even asymptomatic [11]. Consequently, ciliary body melanoma is diagnosed at a later stage as tumours are hidden behind the iris and, at the time of discovery, they are already relatively large and extend circumferentially, expanding across the entire ciliary body [12].

1.1.3 UM Diagnosis and Prognosis

At the time of diagnosis, the most common symptom of UM is blurred vision, which accounts for 37.8% of the cases, but many patients are asymptomatic (30.2%) [11]. Clinical examinations for iris melanoma are performed using slit-lamp biomicroscopy or fine-needle aspiration biopsy to validate the diagnosis [13]. In contrast, diagnosing small choroidal melanoma poses a challenge due to its location. Previously, enucleation was the only option for eyes that were diagnosed with choroidal melanoma, however with the evolution of modern diagnostic techniques such as indirect ophthalmoscopy or ultrasonography improved the efficacy of diagnostic [14, 15]. Similarly, diagnosing ciliary body melanoma is also strenuous given that it is a rare type of ocular tumour, and often categorise as “very small ciliary melanoma” - fast local invasion yet lack of symptoms in small-sized tumours [16]. Fortunately, recent breakthrough that allow small ciliary body melanomas to be detected including those

less than 4mm is ultrasound microscopy (UBM) [17]. Despite the improved local disease control and better prognosis than assumed, up to 50% of patients may still develop metastases into the liver, lung, skin/soft tissues, which has an estimated 66% for 10-year relative survival rate [18].

1.2 CURRENT TREATMENT

There are limited drug therapies for UM. Currently, surgery has been used to remove or destroy a tumour directly, which results in blindness [19]. On the contrary, radiation can treat the tumour while preserving some vision; however, it is deemed ineffective if the disease has spread. Although conservative therapeutic options for managing primary tumours such as surgical excision and/or irradiation therapies, offer excellent local tumour control, to manage metastatic UM represents a significant unmet need [19]. It is acknowledged that the current treatment options are often "borrowed from the melanoma of the skin but is not a good fit", as these two diseases have different pathobiology [20]. The prognosis, survival rate and quality of life in primary UM cases have significantly improved over the recent years, but there remains no efficient therapeutic algorithm for controlling the speed of metastasis in this deadly disease.

Tebentafusp is the only drug approved in clinical setting for UM so far. It is an immunotherapeutic drug that is only effective for UM patients with specific human leukocyte antigens [21]. Enormous attempts have been made to in UM discovery and development. Clinical trials on targeted therapies like multi-kinase inhibitors (eg. sorafenib and cabozantinib) in UM patients have shown no beneficial effect in patient survival or were terminated without conclusions [22]. Moreover, receptor tyrosine kinases (RTKs) have been assessed for its potential of being therapeutic targets. For instance, several small molecule drugs and antibodies targeting RTKs and their downstream signalling pathways, have been shown to be promising when tested in-vitro. Moreover, current drug treatments for primary and metastatic UM include cytotoxic drugs such as selumetinib (MEK inhibitor) or crizotinib (C-Met inhibitor). Selumetinib is currently in Phase II trial however when combined with dacarbazine, this drug combination in Phase 3. Despite exhibiting tolerable safety profile, it did not yield significant enhancement in patient survival when compared to the placebo plus dacarbazine [21]. Although the viability of UM cells with GNAQ and GNA11 mutations have been shown to decrease upon the treatment of crizotinib (a c-Met inhibitor), it is ineffective in mouse

xenograft possibly due to the signalling crosstalk [23]. Other small molecules drug such as proteasomal inhibitors, inhibitors of histone deacetylases alone or in combination with other agents, genetically disrupt the pathogenesis microRNAs (miRNAs). In general, candidate drugs tested so far have not shown promising effects in treating UM [4, 24, 25]. Therefore, there remains an urgent need to identify new and effective drugs to treat the primary and metastatic UM.

Other than cytotoxic drugs, there are several clinical trials regarding natural compounds used in UM such as curcumin, flavonoids and retinoids. According to the literature, natural compounds have potentially promising efficacies in vitro and/or in vivo in UM models. There is, however, insufficient evidence to support the clinical applications in UM trials. Given the heterogeneity of UM, perhaps cytotoxic drugs might be more effective than natural compounds [26]

1.3 CONCLUSION

The non-pharmacology approaches are only effective for patients with early diagnosis. Given that there are few drug therapies available for UM and most of the pharmalogical agents studied in UM showed unsatisfactory outcomes,there is an unmet need in UM drug discovery. It is highly anticipated that new pharmacology agents targeting cancer mechanism and/or metabolism could be further explored in UM.

Chapter 2: Is targeting Mitochondria a viable approach in the treatment of human Uveal Melanoma?

2.1 Introduction

Given that mitochondria are so-called "the powerhouse of the cells", they are the most studied sub-compartments in eukaryotic cells. They have a distinctive structure composed of four unique sub-structures with distinct corresponding functions: the inner mitochondrial membrane (IMM), the mitochondrial matrix, the intermembrane space (IMS), and the outer mitochondrial membrane (OMM) (Figure 1) [27]. The shape of mitochondrial is essential for its metabolic activity. Mitochondria play a critical role in cell growth and death, such as initiating necrotic cell death and abnormal reactive oxygen species (ROS) production, ATP depletion and Ca^{2+} homeostasis failure [28]. These vital biochemical functions serve as a point of convergence for intracellular and extracellular cues, which trigger cell death signals [29]. These processes are closely associated with neurodegenerative diseases, diabetes and cancers. For instance, as part of sustaining cell survival and lethal functions, mitochondria produce ATPs (adenosine triphosphates) that are regulated by mitochondrial outer membrane permeation (MOMP) [30]. MOMP is mediated by proapoptotic proteins such as Bax (Bcl-2 associated X protein), Bcl-2 (B-cell lymphoma 2), and Bak (Bcl-2 antagonist). Thus, such activities of mitochondria may be deemed as therapeutic targets as to their fatal or remedial effects on cell survival and normal function [31]. Therefore, mitochondria have been considered as targets in the development of agents for highly prevalent diseases especially cancers.

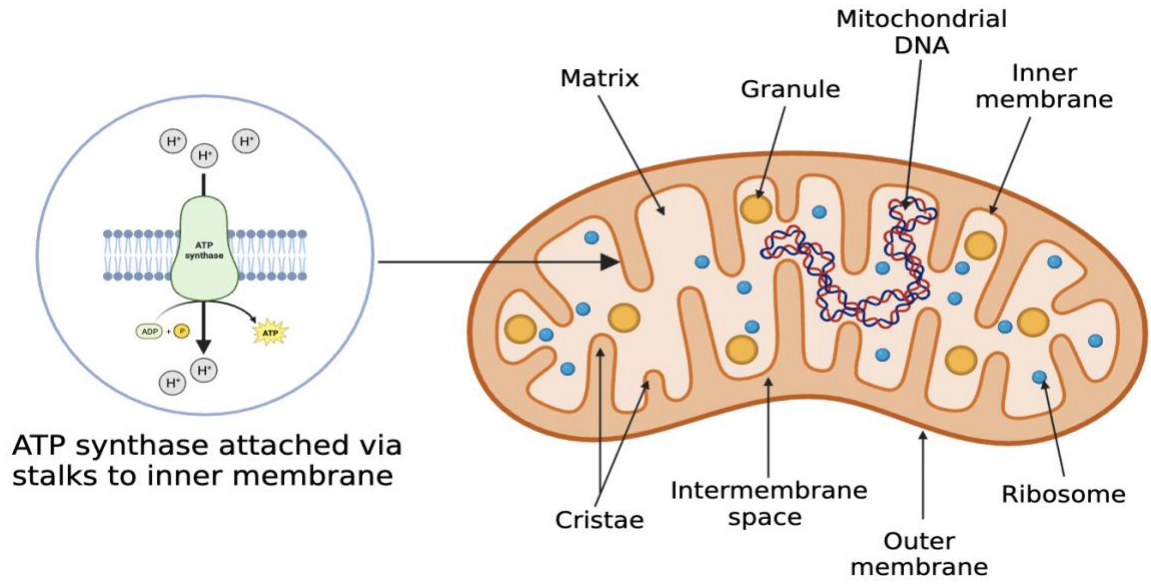


Figure 1. The general structure of mitochondrial membrane.

The mitochondrial matrix is the spaces enclosed by inner membrane. It is the organelle responsible for numerous cellular oxidations and ATP production. It is formed with two membranes: Inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM). The highly permeable OMM acts as a barrier to the diffusion process and mediates signal transduction in and out of the mitochondria. IMM is selectively permeable, where electron transport chain and ATP production occur. [32]

2.1.1 Dysregulated mitochondrial dynamics and metabolism in chronic diseases

Mitochondria are the critical organelles for life-sustaining chemical reactions, which provide energy and elementary units for cell viability and proliferation. The dysregulated function of mitochondria in metabolism is associated with a number of prevalent diseases including diabetes, obesity, cancers and eye diseases [33].

Mitochondria coordinate various cellular functions, and the dynamic network is orchestrated by extracellular nutrient levels and intracellular energy needs. Mitochondrial dysfunction is mainly characterised by an inefficient electron transport chain (ETC) and a decrease in the synthesis of high-energy molecules such as ATPs, contributing to the development of chronic diseases [33]. ETC consists of several protein complexes that involve in redox reactions, which results in the production of ATP in the system of oxidative phosphorylation (OXPHOS), as an electrochemical gradient is formed (Fig. 2). OXPHOS is composed of two parts: ETC and

chemiosmosis. ETC passes electrons through a series of redox reactions and releases energy to form a protein gradient [34]. ATPs are generated and consequently generate ROS, which contributes to both homeostatic signalling and oxidative stress during pathologies [35]. ETC is the core component of mitochondria, where the OXPHOS process coupled with ATP generation also results in ROS production [35]. While the ETC generates ROS needed for homeostasis, ETC dysfunction reduces mitochondrial energy production and increases ROS generation. This process induces the onset and development of a series of biological changes, like obesity and ageing, as well as pathologies in all organ systems [35]. ROS overproduction is the underlying cause of a number of chronic inflammatory diseases through activating pathways such as NF- κB [36].

At the cellular level of ETC, electrons going through chain of proteins leads to a release in energy production (Fig 2). The complex I consists of NADH dehydrogenase, flavin mononucleotide and eight Fe-S clusters and plays important roles in apoptosis of programmed cell death [37]. Complex II (succinate dehydrogenase) is the second entry point of the ETC [37]. Following that, coenzyme Q (ubiquinone (CoQ) consists of a hydrophobic tail and quinone), mediates the transfer of electrons to complex III [34]. Complex III (cytochrome C reductase) transfers electrons across intermembrane space to cytochrome c. The last ETC protein is Complex IV (cytochrome c oxidase) oxidises cytochrome c and also transfers electrons to oxygen [34]. Mitochondrial ETC is important for bioenergetics, redox control as well as biosynthesis in cancer cell growth and metastases. Thus, it has been considered as a promising anti-cancer therapeutic target.

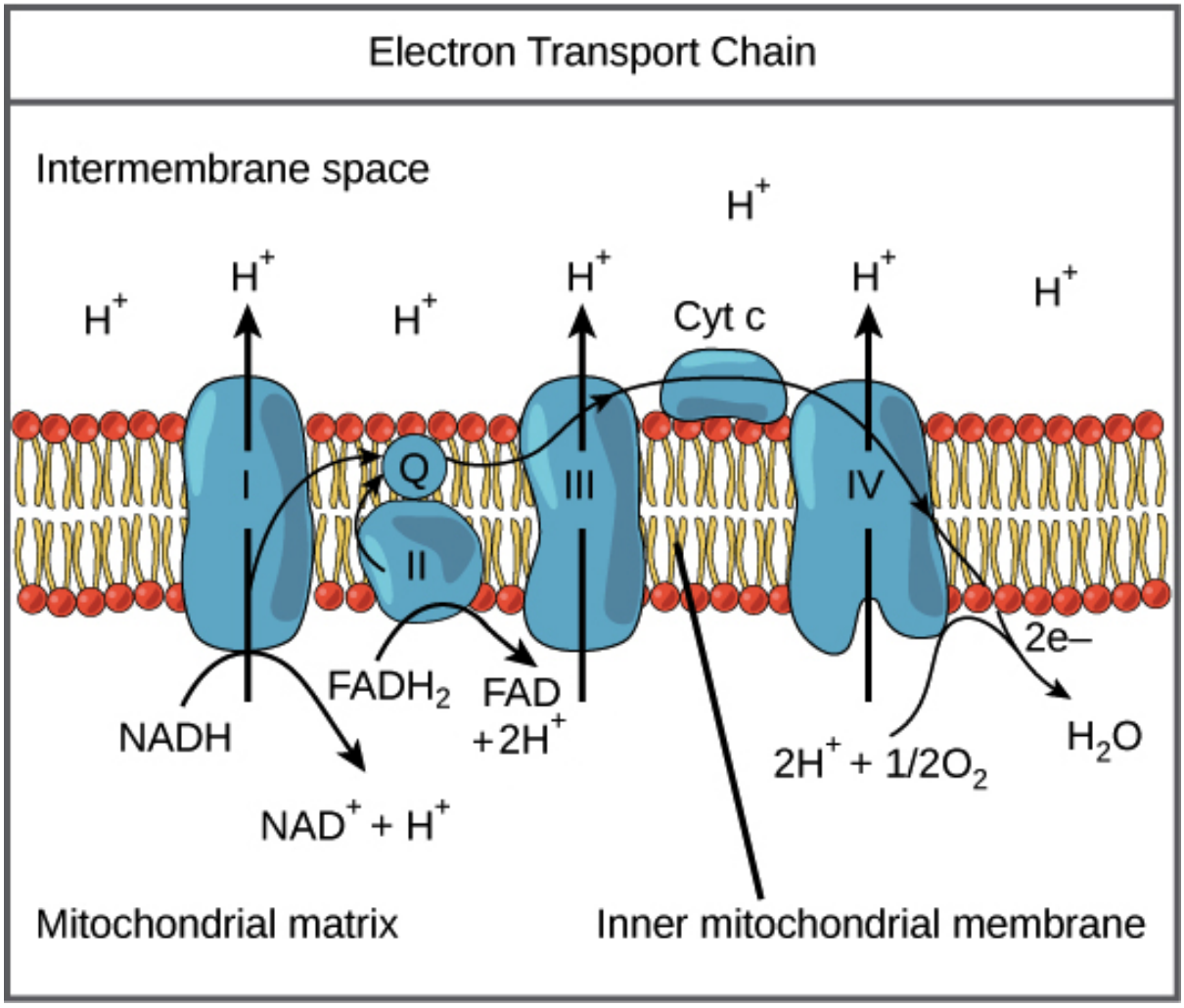


Figure 2. The illustration of mitochondrial electron transport chain [Khan Academy]

Moreover, upon stimulation, mitochondria undergo consistent interchanges between fusion and fission processes, (Fig. 3). Fusion involves the process of complementation from mixing multiple partially damaged mitochondria to mitigate stress; while fission is involved in the generation of new mitochondria by removing damaged mitochondria, and facilitates apoptosis [38]. Both of these processes involve GTPases and the dysregulated GTPases cause disruption in cellular metabolism and mitochondrial dynamics [33]. Mitochondrial fusion contributes to enhance metabolic competence and facilitates repairing damaged mitochondria. Furthermore, mitochondrial fission uncoupled respiration, reduced ATP synthesis and cause mitochondrial degradation.

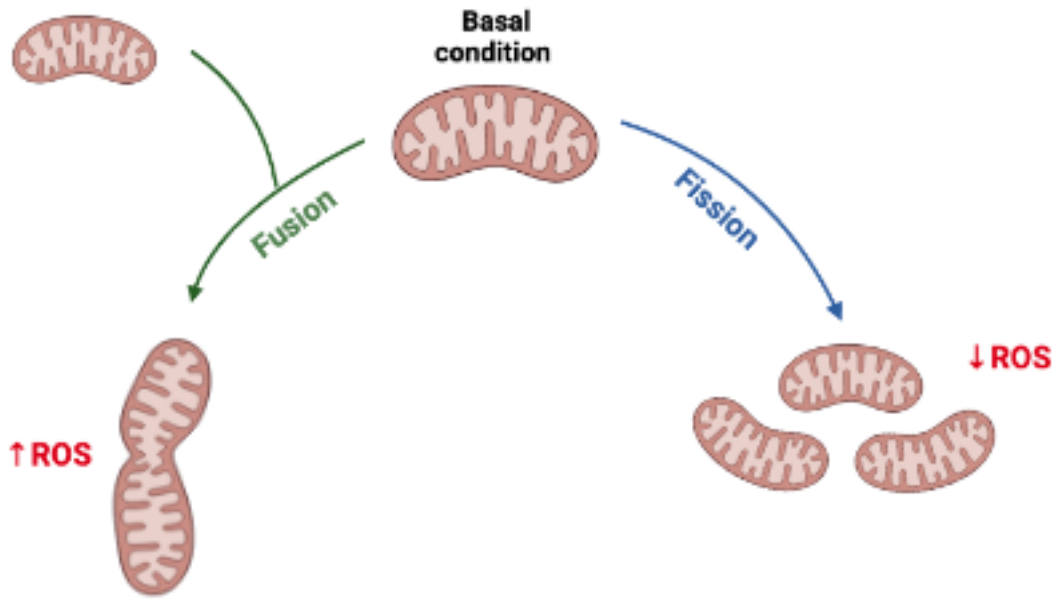


Figure 3. Mitochondrial dynamics: the process of fission and fusion.

Mitochondrial fission is the process where mitochondria separate from mitochondrial tubules. When there is a lower demand of energy, ATP synthesis is reduced as fission facilitates uncoupled respiration. In contrast, stress and energy demand lead to mitochondrial fusion which increases metabolic competence [39].

2.1.1.1 The role of mitochondria in eye diseases

Literature showed that the accumulated DNA mutations and secondary damage of the mitochondria have been involved in the aetiology of many ocular disorders [40]. The eye is an intensively energy demanding organ, especially vulnerable to mitochondrial damage as it is a major source of ROS generation and scavenging.

Diabetic retinopathy is one of the diseases that causes an activation of matrix metalloproteinase-2 (MMP2), a biomarker that mediates the process of mitochondrial membrane degradation and apoptotic machinery [41]. Thus, MMP2-mediated mitochondrial damage may be a therapeutic target for diabetic retinopathy. Primary congenital glaucoma has also been shown to be involved with increased mitochondrial-mediated trabecular damage and oxidative stress [42]. Noteworthy, glaucoma is the primary cause of visual impairment, which optic neuropathy is characterised with cupping of optic nerves densely packed with mitochondria, and with increasing vulnerability to a reduction in mitochondrial respiratory capacity [42].

2.1.1.2 The role of mitochondria in cancers

Several biological characteristics such as proliferative signalling, growth suppressors, cell death resistance, angiogenesis, replicative immortality, are acquired during human tumour development provide a simplified logical framework for comprehending the complexity of neoplastic diseases [43]. In the last decade, two emerging hallmarks, i.e. energy metabolism reprogramming, have also been introduced to facilitate the therapeutic development of human cancers [1].

The well-known Warburg effect refers to cancer cell's increased glycolytic activity and decreased mitochondrial respiration relative to healthy cells. It has been widely shown that mitochondrial DNA (mtDNA) mutations, dysregulated enzymes and/or tumour suppressors result in multiple deregulated cellular energetics processes [44, 45].

Literature showed that mtDNA is responsible for cellular bioenergetics that support the reprogramming of cancer cells metabolically, ROS or Ca^{2+} mediated tumour-promoting changes, or metabolites release, leading to mitochondrial dysfunction. There are many types of mtDNA alterations such as point mutations, extensive insertions, deletions, and copy number variations, shown to be cancer causing. It has be estimated that at least one point mutation has been identified in 66% of cancers [46]. For example, in prostate cancer cell line – PC3, a pathogenic mutation (T8993G) in the ATP synthase subunit 6 (ATPase 6) gene of mtDNA appeared to enhance tumorigenesis [47]. In addition, there are two-point mutations T1659C and G5650A transitions that have been linked to mitochondrial disorders, were widely identified in hepatocellular carcinoma tissues [48, 49]. As to clinical manifestation, a reduction in mtDNA copy number is likely correlated with tumorigenesis, tumour growth, metastasis, and a poor patient survival in liver cancer [50]. Regarding clinicopathological correlations with changes in the number of mitochondrial genomes per cell, mutations may be employed as a predictive biomarker in certain types of cancers. However, additional evaluations will be required to determine its contribution to cancer development. In addition, mitochondrial dysfunction caused by somatic mtDNA alterations shifts energy metabolism from oxidative phosphorylation to glycolysis, thereby promoting cancer progression [51]. And mtDNA mutations that generate ROS were found to promote the metastasis of tumour cells, indicating that mitochondrial dysfunction enhances tumour growth or promotes the progression of cancer via multiple mechanisms [52]. The expression of integrin α5 is increased by ROS-induced mitochondrial respiratory chain impairment in gastric cancer cells [53]. A wide variety of

integrins are closely linked to the progression of cancer including cell proliferation, angiogenesis, tumour metastasis, and carcinogenesis [54]. Cancer development and progression are significantly impacted by the microenvironment of the tumour and extracellular matrix (ECM) [55]. During initiation of tumour metastases, cells interreact with ECM and cause tissue stiffness, porosity and organisation which promote mitochondrial fragmentation, localisation and metabolic plasticity [56].

Enzymes involved in the tricarboxylic acid (TCA) cycle and mitochondrial deacetylases are demonstrated to be involved in mitochondrial dysfunction and cancer progression. For example, mutations of the heterotetrametric protein complex SDH (respiratory complex II) induce ROS production and thus, activate HIF, which then leads to genomic instability [57]. SDH deficiencies cause the accumulation of extra-mitochondrial succinate, thereby establishing a tumorigenic state [58]. Fumarate hydratase is a nuclear-encoded mitochondrial matrix enzyme in fumarase-deficient cells. It may arise from the stabilisation depending on the level of ROS and initiation of HIF-1, to decrease P53 stabilisation [59]. Similar to SDH, dysregulated fumarate hydratase tumorigenic effect results in an abnormal build-up of fumarate and an increase in the production of nuclear factor erythroid 2-related factor 2 (NRF2), a master regulator of several antioxidant genes and promotes tumour formation [60].

In cancers, altered oncogenes and tumour suppressor genes, such as HIF-1 and TP53, can inhibit mitochondrial respiratory function. Recent research has indicated that alterations in ROS or Ca2+ intracellular levels, and mitochondria-released oncometabolite are crucial for mitochondrial retrograde signalling to promote neoplastic transformation. HIF-1 is a heterodimeric protein and impacts on how normal and cancer cells respond to hypoxia [51]. HIF-1α is essential for angiogenesis, metastasis and cancer cell proliferation and invasion [51, 61, 62]. Numerous tumour types are associated with malignancy when HIF-1α is overexpressed [51, 63]. Furthermore, HIF-1α is essential for controlling both mitochondrial respiration and cellular metabolism [51, 64]. For example, LON protease is vital for the degradation of COX4-1 subunit of cytochrome c oxidase complex (COX), which process is induced by HIF-1α. It has been shown that substituting COX4-2 for COX4-1 in cancer cells promotes electron transfer to oxygen, respiration and reduces the generation of ROS when oxygen supply is limited [51, 65]. However, TP53 is widely known for its roles in apoptosis, cell cycle arrest and DNA damage response (DDR). As a result, TP53 abnormalities may be involved in the Warburg effect in tumours [51, 66].

Elevated ROS may cause non-specific damage to DNA, proteins, and lipids. This, in turn, can disrupt the ETC leading to mitochondrial dysfunction and cell death [51]. The generation of mitochondrial ROS can be promoted by hypoxia, activated oncogenes or deactivated tumour suppressors as well as induced mtDNA mutations or dysregulated enzymes [51, 67].

Overall, mitochondrial dysfunction greatly contributes to cancer progress and metastases. Therefore, targeting mitochondria may be a viable strategy for developing mitochondria-targeted anti-cancer therapeutics. Current anti-cancer agents targeting mitochondrial destabilisation include several classes: agents against Bcl-2 family proteins, hexokinase inhibitors, drugs targeting VDAC/ANT, thiol redox, ETC, the inner membrane, TCA cycle or mtDNA [68].

2.2 Mitochondria targeted therapies

Current anti-cancer agents targeting mitochondrial destabilisation include several classes: agents against Bcl-2 family proteins, hexokinase inhibitors, drugs targeting VDAC/ANT, thiol redox, ETC, the inner membrane, TCA cycle or mtDNA [68].

2.2.1 Hexokinase inhibitors

Hexokinase (HK) is an enzyme and plays a key role the phosphorylation of glucose bio-transforming into glucose-6-phosphate (G6P), which process is coupled with ATP generation. In tumour cells, it binds to both ATP and glucose, resulting in the production of G6P in high levels [69]. Literature has shown a correlation between the growth of carcinomas and HK activity [69]. Studies showed that several HK inhibitors can supresses tumour growth due to rapid depletion of ATP in animal models. Some HK inhibitors are candidate drugs evaluated in cancer clinical trials [70, 71]

2.2.2 Compounds targeting Bcl-2 family proteins

Recent research has unveiled that Bcl-2 involved in the process of mitochondrial biogenesis, anti-apoptotic and pro-apoptotic Bcl-2 family proteins; consequently become targets for mitocans. As an illustration, α-tocopherol succinate (α-TOS) inhibits the interactions of Bcl-2 and Bcl-xL with Bak protein, thereby inducing apoptosis and cell cycle arrest [72]. It has also been shown to interact with the ubiquinone. It is known that molecules disrupt the mitochondria ETC, are potent to interfere with the pro-apoptotic proteins [73].

2.2.3 Thiol redox inhibitors and VDAC/ANT targeting drugs

A combined class of mitocan is comprised of thiol redox inhibitors and VDAC/ANT targeting agents as they are involved in the redox environment of tumour cells. In comparison to normal cells, tumour cells exhibit higher intrinsic levels of ROS, which sensitise tumour cells oxidative stress [74]. Compounds such as arsenic trioxide have been shown to have favourable tumour selectivity by erupting homeostasis of cellular redox environment in leukaemia cells [75, 76].

2.2.4 ETC targeting drugs

ETC targeting drugs act on mitochondrial complexes. Given that ETC is the primary source of ROS due to its large electron flow, its superoxide by-product is not harmful but its deregulation may lead to permanent high level of ROS that predispose carcinogenesis [77]. For example, alpha tocopheryl succinate (α-TOS) is an apoptotic agent that has been modified by tagging with cationic triphenylphosponium (TPP^+). It shows potent anti-cancer activity and selectivity in the breast and colorectal cancer rodent models [78].

2.2.5 Lipophilic cations targeting the inner membrane

It has been reported that the altered mitochondrial bioenergetics is a cause of high trans-membrane potential in tumour cells as compared to that in non-malignant cells [79]. This feature of cancer cells dictates that the intracellular targeting lipophilic cations may increase trans-membrane potentials and promote mitocans to be more selective for malignant cells. In addition, many delocalised lipophilic cationic agents may be toxic to cancer cells [80].

2.2.6 Molecules targeting the tricarboxylic acid cycle

Another class of mitocan that relates to the ETC are molecules that target the tricarboxylic acid cycle (TCA). TCA is a source of electrons in the ETC, which drives the electrochemical proton gradient required for ATP generation [68]. There are a number of compounds targeting TCA cycle, including pyruvate dehydrogenase kinase (PDK) inhibitors. PDK is the enzyme that catalyses the reaction converting pyruvate to acetyl-CoA, a prerequisite of pyruvate to enter the mitochondria and the TCA cycle. PDK inhibition leads to the activation of pyruvate dehydrogenase and TCA cycle. For instance, dichloroacetate (DCA), a clinically used drug to treat patients with mitochondrial deficiencies, might be a potential anti-cancer agent as it shows selectivity in inducing cancer cell death by deactivating PDK [81].

2.2.7 Drugs targeting mtDNA

Compounds targeting mtDNA may also be considered to be anti-cancer agents. Mitochondria have their own genetic information encoded on mtDNA. To date, mtDNA targeted compounds may interfere the function and/or stability of mtDNA or impact on the activity of mtDNA polymerase- γ involved in mtDNA replication [68]. For example, mtDNA targeting agent vitamin K3 (menadione) can induced cell apoptosis through deactivating DNA polymerase- γ [82].

2.3. Mitochondria-targeted therapeutics in UM drug development

The rationale for drugs targeting mitochondria for therapeutic development in UM lies in mitochondria's pivotal role in the energy homeostasis, production of ROS and cell death. The potential therapeutic applications of mitochondria include but not limit to specific delivery of antioxidants to mitochondria; the inhibition of mitochondrial permeability transition (MPT) in diseases such as stroke and heart attack; and induce cell death by disrupting the activities of key signalling factors in cancer therapies due to distinct function of mitochondria in tumour cells compared to that in normal cells.

In healthy cells, mitochondria regulate the growth-death cycle, whereas in tumour cells, mitochondrial metabolism is dysregulated due to the high metabolism of rapidly proliferating cells [83]. Tumours are distinguished from normal cells by the evasion of cells and mitochondria-mediated cell death. The differences in mitochondrial function in tumours consequently inhibit oxidative phosphorylation, resulting in insufficient respiration and ATP production [84]. Under normal conditions, ROS production by mitochondria is deemed necessary; however, when cell death is induced in cancer cells, ROS leads to neoplastic transformation [85]. The modulated mitochondrial metabolic activity of tumour cells enables the selective effect of therapeutic agents in cancers [86]. Anti-cancer drugs are designed to selectively disrupt cancerous mitochondria by inhibiting glycolysis, depolarising membrane potential, and/or disrupting the mitochondrial permeability [87, 88]. The mitochondria-targeted anticancer therapeutics can be mainly classified into two categories: drug/toxic agents and drug-free agents [29].

ATP depletion, initiation of membrane permeation, deficit of mitochondrial membrane integrity, suppression of the mitochondrial respiratory pathway, and mitochondrial DNA damage are case-specific effects of oxidative stress, which may be considered as therapeutic targets to optimise the efficacy of mitochondria-targeted agents in cancer treatment. For instance, mitochondrial produces most ATPs and drives cancer cell proliferation. A reduction in ATP level, cell growth and cell viability has been reported in UM cells treated with calcium electroporation (CaEP) [89]. Besides, a study on oncogenic Gq/11 signalling demonstrated that it short-termly induces and long-termly maintains metabolic reprogramming in UM while blunting mitochondrial respiration and glycolysis [90].

An in vitro study on ocular neoplasms including UM uncovered the antioxidant and anti-inflammatory effects of polyphenol in inducing cell apoptosis. An increase of curcumin concentration resulted in mitochondrial-induced apoptosis and cell destruction [91, 92]. Such finding suggested that agents that directly inhibit tumour-specific mitochondrial function may be promising drug candidatures for the treatment of ocular cancers [40].

Studies have investigated small molecules that targeted the overexpressed growth factor receptors in UM. For instance, sorafenib is a known dual inhibitor of C-RAF and B-RAF. As a mitochondria-targeted anti-cancer agent, it impacts on the ATP synthesis and mitochondrial electron transport chain. Studies demonstrated there is a significant improvement in progression-free survival in the sorafenib treated UM patients compared to placebo [22].

2.3.1 Conventional mitochondria-targeted drugs

Mitochondria-targeted anticancer agents such as doxorubicin (DOX), camptothecin, and chlorambucil, have been explored in UM for their anti-cancer effects. These conventional agents exhibit great clinical limitations such as the development of drug resistance, dosage limit and undesirable side effects.

Doxorubicin binds specifically to the abundant phospholipid cardiolipin in the IMM and accumulates in mitochondria [93]. Doxorubicin-induced toxicity is mainly due to an increase of ROS associated with redox cycling in mitochondria. Chemotherapies, particularly doxorubicin have direct effects on increasing the production of ROS in many tissues such as the skeletal muscle [94]. The previous study found that UM responds to doxorubicin with

modulating the cAMP response element binding protein (CREB) [95]. However, doxorubicin results in dilated cardiomyopathy caused by the generation of ROS within myocardia tissue, as it interferes with coenzyme Q in electron transfer [96].

Camptothecin is a natural compound with potent inhibitory effect against DNA topoisomerase. However, gastrointestinal toxicity is a significant side effect of camptothecin treatment, as well as neutropenia occurred in 22% of cancer patients, and diarrhea appeared in 29% of cancer patients [97, 98]. A phase II trial of a camptothecin derivative has shown unsatisfactory outcome against UM [99]. Upon from that, there has been no further report on camptothecin and its derivatives in UM, although several semisynthetic derivatives of camptothecin depicted promising results in other cancers [100].

Chlorambucil is an alkylating agent that is clinically adopted to treat various cancers. It has been shown to adhere to DNA strands and prohibit its replication in cancer cells [101]. It is most effective in conditions that are associated with the proliferation of white blood cells, particularly lymphocytes, but is deemed ineffective because its prolonged treatment can increase the risk of acute leukaemia [102]. In relation to eye diseases, chemotherapeutic drugs such as chlorambucil have shown to be viable options to treat regional eye lymphoma, however a case report suggested that these drugs may induce papilledema. Although such side effect is reversible, their toxicities may be more pronounced in specific organs such as the eyes. Overall, the outcome of the above-mentioned mitochondria-targeted drugs is unsatisfactory and there remains an unmet need to develop novel and effective treatments for ocular diseases in particular UM [103, 104].

2.3.2 Molecules targeting mitochondrial metabolism, functions or proteins

The role of mitochondria in metabolism is essential for tumour growth, making this pathway an ideal target for cancer growth inhibition. Given that mitochondria produce ATP by oxidising glucose (Fig 3), substances that impede the respiratory chain likely induce oxidative stress and lead to mitochondrial dysfunction [105]. For instance, resveratrol, a naturally occurring histone deacetylase inhibitor derived from berries, inhibits tumour growth by reducing oxidative stress and increasing the level of mitochondrial superoxide dismutase (SOD2) [106]. Specifically, in a UM animal model, the previous study suggested that resveratrol treatment can inhibit the growth of tumour and induce apoptosis via modulating intrinsic mitochondrial pathway,

increase bioavailability of the agent as well as tumour regression. Given that, it is a plant derived polyphenol which can be found in grapes, berries and peanuts making resveratrol a less-toxic agent [107, 108]. Subsequently, in the UM cell lines studied, its anticancer activity is mainly by altering the intrinsic mitochondrial pathway [108]. When treated with resveratrol, the MMP rapidly decreases along with opening mitochondrial permeability transition pore (mPTP), mitochondrial cytochrome c and Smac/DIABLO are released, and then activating caspase-9 and -3. These events in the mitochondria trigger resveratrol-induced UM cell death. Therefore, considering the potent inhibition of UM cell growth and non-toxic nature of resveratrol, it may be a promising candidate drug for UM treatment [108].

Overexpressing mitochondrial proteins has been considered as a viable approach for the development of cancer-selective drugs. Chaperon tumour necrosis factor receptor associated protein 1 (TRAP1) is a member of the HSP90 family, which is abundantly expressed in cancer cells; It inhibits apoptosis and reorganises metabolism [109]. Literature showed that SMTIN-P01, a mitochondria-targeting HSP90 inhibitor, selectively inhibits mitochondrial TRAP1, resulting in a synergic inhibitory activity of mitochondrial and metabolism of glycogen in UM cells [110, 111]. Over the recent years, attempts have been made to design TRAP1-targeting drugs that do not impact on the activity of HSP90, thereby reducing overall cell toxicity. To deconstruct the dynamics of client interactions under various conditions, highly selective small molecules targeting TRAP1 are required but haven't been identified. Nevertheless, TRAP1 remains a promising target in cancer treatment including UM [112]. Moreover, other studies in relation to TRAP1 or HSP90 inhibition suggested that this can be a rational approach in treating metastatic UM, such that it progressed to phase II trial in 2018. The trial reported 59% of patients develop metastases in the liver and the result of this trial did not demonstrate clinical benefit, suggesting rapid development of resistance.

Similarly, imipridones (ONC201), an orally active small molecule antagonist of the G protein coupled receptor dopamine receptor D2 (DRD2), has been found to inhibit UM cell survival. Its anti-UM effect is accompanied with the decreased expression of succinate dehydrogenase complex, subunit A (SDHA) and subunit B (SDHB) in UM cells. It targets the metabolic vulnerabilities of metastatic UM through elevated mitochondrial oxidative phosphorylation (OXPHOS) and thus, controls cell growth. Additionally, the post treatment of imipridones showed a reduction of protein biosynthesis pathways and an alteration of lipid metabolism [113, 114].

2.3.3 Mitochondria-targeted peptides (MTPs)

Conventional target chemotherapies are important systemic treatment of cancers; however, their clinical application may be greatly limited due to acquired drug resistance in cancer cells, low target selectivity or common adverse effects. Thus, it is pivotal to circumvent obstacles and enhance the efficacy of chemotherapies [115, 116]. This may be accomplished as mitochondria-targeted drugs to be conjugated with mitochondria-targeting moieties like triphenyl phosphonium ion (TPP).

As the structure and function of mitochondria are distinct between healthy and cancerous cells, numerous therapeutic peptide drugs specifically targeting mitochondrial pathways have been widely explored. Such agents are featured with a high potency in inducing cell death, a low toxicity, high specificity, biodegradability, and ease of synthesis [117]. A peptide can move across the mitochondrial membrane if its positive charge and hydrophobicity are optimised [118]. It can be accomplished by incorporating lipophilic cations like TPP – a mitochondrial targeting ligand, or by incorporating positively charged and hydrophobic moieties in peptide design such as mitochondrial processing peptidases (MPP) - a class of peptides. MTPs may function as mitochondrial transport vehicles when conjugated with a variety of cargo molecules [118]. A platinum analogue covalently attached with MPP is favourable for the delivery of cisplatin (mtPt), which induces apoptosis in cancer cells with leaving their nuclear DNA untouched [119]. Considering that UM is one of the diseases with highest OXPHOS gene expression, conventional drugs have been designed in conjugation with TPPs to improve its mitochondrial targetability [120]. For instance, doxorubicin and TPP-linked cisplatin (Pt) can agitate the mtDNA, increase the level of mitochondrial ROS, which ultimately contributes to mitochondrial dysfunction [121]. The anti-cancer effect of certain conventional drugs is not readily achieved in mitochondria through the traditional delivery methods; however, with the help of TPP, prodrugs are able to selectively accumulate in the mitochondria [122].

2.4 Conclusion and future directions

Multiple strategies are available to achieve cancer-selective mitochondrial damage. Conventional mitochondria-targeted cytotoxic drugs, molecules that specifically target mitochondrial activities have demonstrated reasonable and promising cancer treatment efficiency.

The dysregulated cellular energetics may be associated with deficiencies in mitochondria. Mitochondrial dysfunction can accelerate cancer progression to invasive chemo-resistant and/or apoptosis-resistant phenotypes through many pathways. As cancer progresses and oncogenesis occurs, these modifications to the mitochondria may trigger cytosolic signalling pathways that connect the mitochondria to the nucleus, altering the expression of nuclear genes and ultimately resulting in neoplastic transformation [51].

In the recent years, mitochondria-targeted anticancer agents have been widely studied. Indeed, various lead molecules have been proved to be effective in managing a range of cancers. Although, conventional target drugs such as doxorubicin and camptothecin are promising, they will require further optimisation like conjugation with nanocarriers, in order to better improve the efficacy of drug delivery.

Overall, because mitochondrial metabolic functions are essential for tumour growth, selectively disrupting mitochondrial function in tumour cells is a promising approach in cancer treatment. The relationship between mitochondria and its involvement in tumour cell growth forms the basis of mitochondria-targeting anti-cancer drug development. The conventional cytotoxic agents are no longer preferred. It is anticipated that new mitochondria-targeted agents with high tumour selectivity can be investigated as they disrupt mitochondrial function and specifically impact on malignant cell growth and proliferation. Furthermore, it is highly desired that drug-free approaches targeting the mitochondria may also be widely considered in the drug development of cancers particular UM.

Chapter 3: Anti-cancer and antimetastatic effect of soluble epoxide hydrolase inhibitors in UM culture models

Soluble epoxide hydrolase (sEH) is an enzyme that has been proven to be a cross-functional target with multiple therapeutic potentials in hypertension, inflammation, and cancer [123].

3.1 SOLUBLE EPOXIDE HYDROLASE

In humans, sEH is a bifunctional enzyme encoded by the EPHX2 gene, widely distributed in organs including the liver, kidney, and brain. It plays a central role in the metabolism of bioactive lipid signalling molecules.

sEH consists of two functionally and structurally independent domains, which correlate to two different activities. While the N-terminal shows phosphatase activity, the C-terminal domain is in charge of hydrolase activity [123].

3.1.1 Cancer cell metabolism

In recent years, remarkable advances in cancer research have resulted in a paradigm shift to include metabolic reprogramming in cancer hallmarks. Since Otto Hendrich Warburg first proposed it, our understanding of cancer metabolism becomes more sophisticated. It is an adaptation mechanism to assist biosynthetic needs of rampant proliferation [124]. The identification of metabolic heterogeneity among the cancer types as well as differences in cellular metabolism between low- and high-grade diseases or between primary and metastatic tumours. It is generally recognised that tumourigenesis and progression require fatty acids and amino acids [125]. Tumour cells use metabolic reprogramming to sustain the need for biosynthesis, bioenergy and oxidation balance. The recent discoveries contributed to renewed interest in elucidating the diverse roles of fatty acid metabolism in cancers and better defining the nutrient pathways [2, 125]. Fatty acid metabolism is an essential cellular energy metabolism pathway, pivotal in cellular energy supply and oxidation balance [126].

Lipids are a broad range of hydrophilic organic biomolecules [127]. The main function of lipids is for long-term energy storage and also for insulation, lubrication and hormone precursors that

form cell membranes and chemical messengers [128]. Lipids require various types of helpful lipid fat to maintain physiological functions. One of the main lipids is fatty acids. In the human body, lipids include blood lipids, fat deposits and structural lipids in biological membranes [127].

Cancer cell biology influences fatty acid metabolism in many ways, particularly synthesising lipid building blocks for membranes and phosphatidates to aid mitogenic and/or oncogenic signalling [129]. Tumorigenesis and metastasis share a common feature: the modified metabolism of fatty acids. Cancer cells accquire fatty acids from various intracellular and extracellular sources [125].

Disruption in cancer cell metabolism directly results from the modulation of intracellular signalling pathways caused by mutated oncogenes and tumour-suppressor genes [130]. Thus, alterations in cell metabolism are vital factors that trigger tumorigenesis. Intracellular signalling pathways and mutations can be deemed as the chicken-and-egg situation, in which the modification of cell metabolism facilitates the tumorigenic process, and, in turn, mutations of oncogenes and tumour suppressor genes lead to altered cell metabolism [131].

Fundamentally, building blocks of nucleic acids, lipids and proteins are required by tumour cells, given that cancers are disorders of cell growth and proliferation. For instance, cellular proliferation is a common feature among all cancers, where tumour cells require fatty acids to synthesise membranes and signalling molecules [132]. Mammalian cells can acquire fatty acids through direct exogenous uptake or *de novo* synthesis. Moreover, when exogenous uptake of fatty acids occurs, the involved transporters possess increased expression in tumour cells, which generally correlate with poor prognosis in various cancers [133].

Although the mechanisms driving specific lipid phenotypes are still unknown, it is hypothesised that these are dependent on hypothesised tumour sub-classifications [133] (Fig 4). Therefore, fatty acid synthases are the key lipogenic enzymes which regulate lipid metabolism and proliferation in tumour cells. They include type I and II, which are multifunctional enzymes catalysing fatty acid biosynthesis. Epoxides of PUFAs are bioactive and can cause negative impacts on cells, leading to tumour cell proliferation and angiogenesis [134].

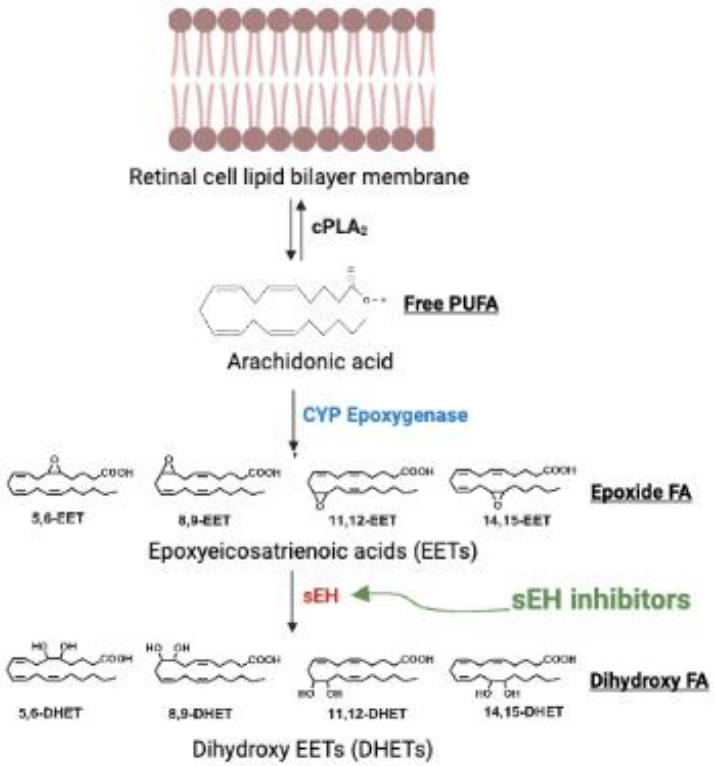


Figure 4. Overview of metabolism of polyunsaturated fatty acids (PUFAs).

In the breast cancer mouse model, PUFAs (i.e.. epoxides and diols) are active and contribute to tumour cell proliferation and angiogenesis [134]. Literature showed that, in general, EpFAs exhibit pro-survival roles in cancer models [26]. Several anticancer drugs have proven to decrease the expression of FANS and its transcriptional regulator, which has been associated with the pro-survival role of FAS. Specifically, it has shown killing efficacy in MUM2B cells, a hepatic metastatic UM cell line [26]. Moreover, the anti-inflammatory and antiangiogenetic EpFAs has been proposed to have great potentials in treating renal diseases like UM, through the inhibition of sEH [135]. Literature showed that in human retinal endothelial cells, an sEH inhibitor effectively blocked fundamental angiogenic properties and even synergised with anti-VEGF therapies in reducing laser-induced neovascularisation [136].

3.1.2 SEH as a potential therapeutic target

SEH is a major proinflammatory enzyme that is in charge of the infiltration of inflammatory mediators, hence sEH inhibition pronuncedly reduces the conversion of epoxyeicosatrienoic acids (EETs) to dihydroxyeicosatrienoic acid (DHETs). It highlights the role of sEH inhibition in addressing inflammation and carcinogenesis [123].

sEH inhibitors have been leveraged to address many diseases including hypertension, immunological disorders, cancer and eye diseases. They were studied in the co-administration with other inhibitors as combined therapies [123]. The dual inhibition and modulation of sEH have been examined over recent years. The inhibition of EET catabolism via sEH inhibitors produces significantly anti-inflammatory effects. In breast cancer, sEH have been shown to correlate with tumour size negatively, and sEH downregulation is correlated with aggressive cell behaviour; therefore, sEH is an ideal therapeutic target [123, 137]. Additionally, the correlation between sEH and its up-regulated activity in a choroidal neovascularisation (CNV) model has been reported, and sEH is considered to be a target for blocking CNV [138]. Potentially, sEH is an appealing target for local drug delivery in the eyes, as it shows considerable promise in the treatment of eye diseases [136].

3.1.2.1 Biochemical mechanism of sEH inhibition

sEH acts on the arachidonic acid cascade (Fig 4). The branch includes anti-inflammatory mediators known collectively as EpFA: epoxides of arachidonic acid (EETs), eicosapentaenoic acid (EEQs) and docosahexaenoic acids (EDPs). However, the degradation of EpFA, especially EETs, to corresponding proinflammatory diols is problematic [139]. In comparison to EpFA substrates, in some tissues, sEH has a higher abundance along with its high k_{cat} and low K_m value. While k_{cat} is the turnover number of how many substrate molecules will be converted to products per unit time by an enzyme; K_m value describes the substrate's affinities for the enzyme's active site. In order to limit the degradation of EpFA, a potent inhibitor with high target occupancy is needed; hence sEH inhibitors fit these criteria leading to the reduction of sEH and an increase in EpFA [140].

3.1.2.2 Physiological mechanism of sEH inhibition

It has been proven that when mitochondrial dysfunction and ER stress are involved, the administration of sEH inhibitors is beneficial as it allows the elucidation of EpFA's biological activities like anti-inflammatory, pro-resolving and antihypertensive [140]. sEH inhibition can block EpFA hydrolysis rapidly, which is found to include attenuating inflammation and can be beneficial in the treatment of stroke, sepsis, cardiac hypertrophy and cardiac contractile function [141]. Additionally, studies demonstrate that she inhibition stabilises EpFA, resulting in ER stress shifting back to stabilise the survival, against acute inflammation and/or cell death [142].

At the time of administration, sEH inhibitors stabilise whenever EpFA is released; since all EpFA is chemically unique, sEH inhibitors and their effects are anticipated to vary across patients depending on the composition of EpFA in the body, diet and physiological state; however, sEH inhibition can reverse the ER shift, stabilise and increase EpFA [140].

3.1.2.3 Clinically proven sEH inhibitors

A clinically proven sEH inhibitor is sorafenib, which stabilises the endogenous EpFAs anti-inflammatory, hypertensive EETs [143, 144]. Sorafenib is an oral multi-kinase inhibitor that was clinically approved in 2008. It impacts on the activity of pro-angiogenic kinases-RAF and vascular endothelial growth factor receptor-2 (VEGFR-2) and is also a potent inhibitor of sEH (Ki of ~17 nM) [145]. Sorafenib causes a reduction in acute inflammatory responses in inflammatory, cardiovascular, liver and kidney diseases [146-148]. Specifically, sorafenib is a first-line systematic treatment for hepatocellular carcinoma (HCC), a typical primary liver cancer [149]. Compared to other reported potent sEH inhibitors like t-AUCB and AUDA, sorafenib has a Ki value that is approximately 10-fold higher [145]. Sorafenib binds with sEH and bonded to the residues of Asp^{333}, Tyr^{381} and Tyr^{465} [150]. Noteworthy, sorafenib has been tested on UM cell lines combined with paclitaxel and carboplatin, it does not produce any objective response [26]. Hence the efficacy of such combination therapy appears to be very limited.

Another sEH inhibitor in clinical trial is EC5026. It mimics the activity of the natural mediators EpFA, which is known to reduce pain and anti-inflammatory, subsequently inhibits sEH activity at picomolar concentrations [140]. In clinical trials, EC5026 demonstrated effective pain reduction but are not consistently efficacious in cancer patients, implying the complexity and need for optimisation in treating cancers [140]. Similarly, GSK2256294 is a potent and selective sEH inhibitor. Its activity in inhibiting sEH is dose dependent [151]. It demonstrated synergistic anti-angiogenic and anti-cancer activity in preclinical models of endothelial cells [152].

Given that sEH metabolises pro-resolving EpFA into diols, it is a potential therapeutic target for diseases such as CNV in age-related macular degeneration (AMD) and other eye diseases. Substantial data showed that sEH overexpression at protein and mRNA levels implies that sEH

underlies the disease pathophysiology. The overexpression of sEH in retinal Muller cells led to an increase in retinal inflammatory exudates dihydroxydocosapentaenoic acids (DHDP), specifically 19,20-DHDP. Subsequently, highlighting the causative role of sEH in disease progression provides a context in developing pharmacotherapies in ocular diseases [136, 153].

3.2 PROJECT AIM AND SIGNIFICANCE

As mentioned above, all current treatments for UM – both primary and metastatic tumours, are non-pharmacological. None of the pharmacological therapies has shown satisfactory effects. Because the aetiology and biology of UM are very different from cutaneous melanoma and other cancers, the effective anticancer agents on the market are ineffective for UM. **The AIM of the current project** is to select sEH inhibitors that show efficacy in the treatment of UM. Given the efficacy of sEH as a therapeutic target in other cancers and eye diseases, we investigated the anticancer and antimetastatic effect of 11 epoxide hydrolase inhibitors available to us in UM cell models [154].

Although researchers have shown that reprogramming fatty acid metabolism has excellent potentials to treat various cancers, such an attempt has never been made in UM drug discovery. And sEH inhibition is a practical method to prevent inflammatory mediator invasion. Consequently, it is an effective therapy method for inflammation-associated cancers like UM [123]. Our study will be the first to explore such therapeutic strategy in UM. Ultimately, the long-term project built upon the current proposed study aims to discover novel candidate drugs that effective in the treatment of UM, through targeting sEH.

This project evaluated the anticancer and antimetastatic effect of the following compounds (Table 1) and selected the one with the highest potency to be molecularly characterised in further investigations. The tested 11 sEH inhibitors include some commercially available molecules like AUDA, as well as some novel compounds that are customised by our collaborators (IP protected).

Name	Mw (/mol)	sEH Ic50 (nM)
AUDA	392.6	3.2
1213	308.4	170.6
1659	368.6	3.9

1661	402.5	5.9
1663	418.5	7.3
SHH-5026-CTU	492.6	18.5
t-CTTU (UC2288)	481.8	9.0
PTUPB (21i)	543.6	0.9
TPPU (1770)	359.4	0.4
t-TUCB (1728)	438.4	0.4
t-CUPM (2278)	470.9	0.4
t-AUCB (1471)	412.5	1.5

Table 1*. The chemical structure of 11 sEH inhibitors tested in the current project.*

3.3 EXPERIMENTAL DESIGN

The initial drug screening was performed on four established UM cell lines. This student adopted the primary tumour derived MP46, C918 and 92-1 cell lines as well as the metastatic tumour derived OMM-1 cell line. Several UM cell lines were included in the study to recapitulate the genetic landscape of UM [155]. Additionally, non-tumour MIO-M1 and APRE cell lines along with primary cultured melanocytes were adopted in this study to illustrate the tumour selectivity of the tested compounds.

MTT and Alarma Blue cell viability assays were used in the compound screening as to the capacity of each compound in reducing UM cell viability. MTT assay measures the cell viability based on the mechanism at which metabolically live cells transform a water-soluble dye into insoluble formazan – an enzymatic conversion in mitochondria. Alarma Blue assay measures cell viability using a dye that incorporates an oxidation-reduction indicator (REDOX) and exhibits fluorescence and colour responding to a chemical reduction caused by cell growth [156]. MTT and Alarma Blue assays are both cell viability assays and both assays measure the percentage of surviving cells but does not have a direct measure of cell growth and death. It is used an initial approach to screen our compound library. Other molecular assays such as LDH assay will be needed to evaluate the impact of treatment on cell growth.

These assays provide an understanding of how many cells are alive or dead and, ultimately, the effectiveness of the agents studied [157]. Upon from those, LDH assay was performed to verify the finding. LDH is a cell death or cytotoxicity assay, which assesses the level of plasma damage caused by agents based on principles of leakage enzymes into the culture medium post-damage [158].

Following that, ROS and JC-1 assays were included to assess the impact of the test compounds on mitochondria, a critical cellular organelle for energy production and metabolism. In all these assays, controls were included, such as drugs under clinical trials in UM patients and a leading molecule FZ10 (self-synthesised, IP protected) shown superior anti-UM self-synthesised in our preliminary study. ROS assay is the specific method commonly used to evaluate the level of oxidative species in cells, which is an indication of mitochondrial function. JC-1 uses fluorescence-based measurement to determine mitochondrial membrane potential changes in cells through the discrimination of energised and deenergised mitochondria. A higher discrimination indicates a healthier mitochondrion, hence more healthy cells.

The compound UC2288 is the most potent candidate drug in reducing cell viability across all four cell lines and was then selected for further investigation. It showed better potency than the control drugs in UM clinical trials and a comparable cell killing effect to our in-house leading molecule FZ10. Its anti-cancer potency (IC_{50} values) was evaluated with cell viability assay in all four UM cell lines. According to the IC_{50} values obtained, the optimal concentration of UC2288 compound was selected for the following experiments.

Annexin-PI staining cell apoptosis flow cytometry was employed to further assess the cell death mechanism of UM cells upon UC2288 treatment. This assay detects whether cell death is due to apoptosis or necrosis [159]. Additionally, PI staining flow cytometry also assisted in evaluating cell populations at different stages of the cell cycle, a pivotal piece of information for the study of tissue repair and inflammation [160]. In this part of the study, molecular mechanism of anti-UM effect of UC2288 was investigated, which will assist in designing an optimal drug regimen later on [161]

The confirmatory experiment was performed on UM patient tumour-derived primary cultures, a unique resource in our laboratory, to validate findings in *in vitro* cell models. As to the approved human ethics, the UM primary cultures were all processed and maintained by A/Prof. Fanfan Zhou.

The wound healing assay is a simple and reproducible method that measures basic cell migration parameters and captures the cell-cell interaction over time intervals [162]. The colony formation assay demonstrates cell survival ability upon exposure to test compounds.

These two assays were implemented in the current project to demonstrate the antimetastatic effect of UC2288 [163].

3.4 MATERIALS AND METHODS

3.4.1 Cell viability assay

UM cells were cultured in 96-well plates (2-4 x 10^5 cells/well depending on the cell line) for 24h. Subsequently, treated with sEHIs (1μM in 0.1% DMSO) in RPMI or DMEM containing 1% FBS for 24h at 37C; Serum-free RPMI and DMEM were used as negative control. Following treatments, the cell viability was assessed using Alarma Blue (Thermo Fisher Scientific, NSW, Australia) and MTT (3-(4,5-Dimethlthiazol-2-yl) MTT (Thermo Fisher Scientific, NSW, Australia) according to the manufacturer's instructions. Equal amounts of the cell viability reagent were added to the 96-well plate after the medium was aspirated, incubated for 2h at 37C. Fluorescence was recorded using a reader (Model 680 Microplate Reader, BIORAD) at 550 nm for MTT assay and using a UV-Vis spectrophotometer (Perkin Elmer Victor) at 570 nm for Alarma Blue assay.

3.4.2 LDH assay

LDH assay was performed according to the manufacturer's instructions. UM cells were exposed to sEHIs (5μM in 0.1% DMSO) in RPMI or DMEM containing 1% FBS for a range of time, 24, 48 and 72h at 37C. Serum-free RPMI and DMEM were used as negative control. Readings were performed for supernatant using a reader (Model 680 Microplate Reader, BIORAD) at 490/655 nm.

3.4.3 ROS assay

ROS assay was performed according to the manufacturer's instructions. UM cells were exposed to sEHIs (5μM) in RPMI or DMEM containing 1% FBS for a range of time, 24, 48 and 72h at 37°C. RPMI and DMEM with 1% FBS and 0.1% DMSO were used as negative control. The 5uM dilution of stock solution was supplied to each well followed with an incubation of 30-60 minutes at 37C before being read with a UV-Vis spectrophotometer (Perkin Elmer Victor) at 485/525 nm.4

3.4.4 JC-1

UM cells are seeded on 96-well plates, and then treated with sEHIs (4μM in 0.1% DMSO) in RPMI or DMEM containing 1% FBS then incubated for 24h at 37C. JC-1 stock was thawed

and diluted 30-fold with serum free DMEM or RPMI then the placed into each well in near-dark conditions. Plates were incubated for 20min at 37C then centrifuge for 5min at 1250rpm and rinsed with warm PBS then re-centrifuge twice. Analysed using UV-Vis spectrophotometer (Perkin Elmer Victor) at 485/530 nm - monomer form (green fluorescence), 535/590 nm (red fluorescence).

3.4.5 Would healing assay

Cells seeded on 12-well plates (density range from 3-5 x 10^4 cells/well depending on the cell line) for 24h or until 80-90% confluency. Three wounds were then created vertically of the well using sterile 200 μL pipette tip. Subsequently, medium was changed with sEHIs (5μM in 0.1% DMSO) in RPMI or DMEM containing 1% FBS then incubated for 24h at 37C; Serum-free DMEM and RMPI were used as negative control. Photographs of wound closure were captured at time 0 and 16h post-treatment using inverted microscope. The area of wound healing was measured using ImageJ Software version 1.53. Cell migration results are demonstrated as the percentage in respect to the control groups.

3.4.6 Colony formation assay

From the 12-well plates in wound healing assay, cells are re-cultured in sterile 12-well plates (100 and 200 cells/well) for 5-7 days depending on the cell line. On the day of analysis (approximately) 24h post-treatment, wells were washed with 100% methanol and then stained 0.5% crystal violet in 25% methanol; subsequently assessed for colony growth as the number of cells in clusters were counted.

3.4.7 Invasion Assay

UM cells were plated in 24 well plates with trans-well inserts. On the day of assay, Matrigel and consumables were kept on ice. Once cells reach confluency, a scratch was made in each well with a wound maker. Cells were washed twice gently with PBS and 45 μL of Matrigel was applied to the wells and incubated at 37 ^{o}C for 30 mins for Matrigel to set. Subsequently, fresh medium with or without treatment was added into the wells. And cell invasion was monitored, and images were taken using the Incucyte for 7 days.

3.4.8 Cell cycle analysis

UM cells were incubated with sEHIs (5μM in 0.1% DMSO) in RPMI or DMEM containing 1% FBS for 24h at 37C. Serum-free RPMI and DMEM were adopted as a negative control. Cells were collected and then washed by centrifuge at 1200g for 5 minutes and suspended twice

with cold PBS Then, fixed in ice cold 70% ethanol at 4^{o}C and washed with PBS and stained with PI for 30 min at 37C in the dark prior to analysis. Samples were processed with Guava easy®cyte flow cytometer within 48h (Merck Millipore, VIC, Australia).

3.4.9 Statistical analysis

A statistical analysis of the data was performed using multiple unpaired t-tests (GraphPad Prism 9.3.1 software, GraphPad Software Inc., San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant and significance levels were indicated * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

3.5 RESULTS

3.5.1 UC2288 is a potent anti-UM drug in 92.1, C918, MP46 and OMM1 cells

In the initial screening, the potency of 11 sEH inhibitors (Table 1) in reducing cell viability were assessed in four UM cell lines (92.1, C918, MP46 and OMM1). At the concentration of 1μM, several sEH inhibitors significantly reduced cell viability in these cell lines. Such concentration was adopted in the initial screening considering the plasma concentration of drugs are usually in the low micromolar range. Cell viability was assessed using two established assays, Alarma Blue and MTT to ensure consistent results. It was noticed that UC2288 demonstrated the relatively most potent effect in both assays, although the extent of influence on cell viability varies among four cell lines. Generally speaking, UC2288 performed preferentially in MP46 and OMM1, followed by 92.1 and C918 cell line. As shown in Figure 5 (MTT assay), UC2288-induced cell viability reduction ranged from 0.4 to 0.5 folds to control and in Figure 6 (Alarma Blue assay), the reduction was approximately 0.45-0.7 folds to control. As to these findings, UC2288 was then chosen to be further studied. Across all three cell lines except OMM1, most of the results were statistically significant with $p < 0.005$. Additionally, the IC_{50} values of UC2288 were then evaluated in all four UM cell lines, which were ranged from 1.99 – 3.77 μM (Figure 7, Table 2).

In accord with this, UC2288 was further investigated for its tumour selectivity in primary cultured melanocytes and non-tumour ocular cell lines (Figure 8). In non-tumour ocular cell lines of ARPE-19 (retinal pigment epithelium cells) and MIO-M1 (Müller cells), UC2288 exhibited good tumour selective activity with more than 0.9 folds cells retained in non-tumour ocular cell lines and 0.75 folds viability retained in primary melanocytes post treatment. In terms of statistically significant, only the primary melanocytes were significant with a p-value

< 0.05. Noteworthy, 3 µM of UC2288 was used in the tumour selectivity study (Figure 8), while 1 µM of UC2288 showed potent cell viability reduction in UM cell lines (Figure 3).

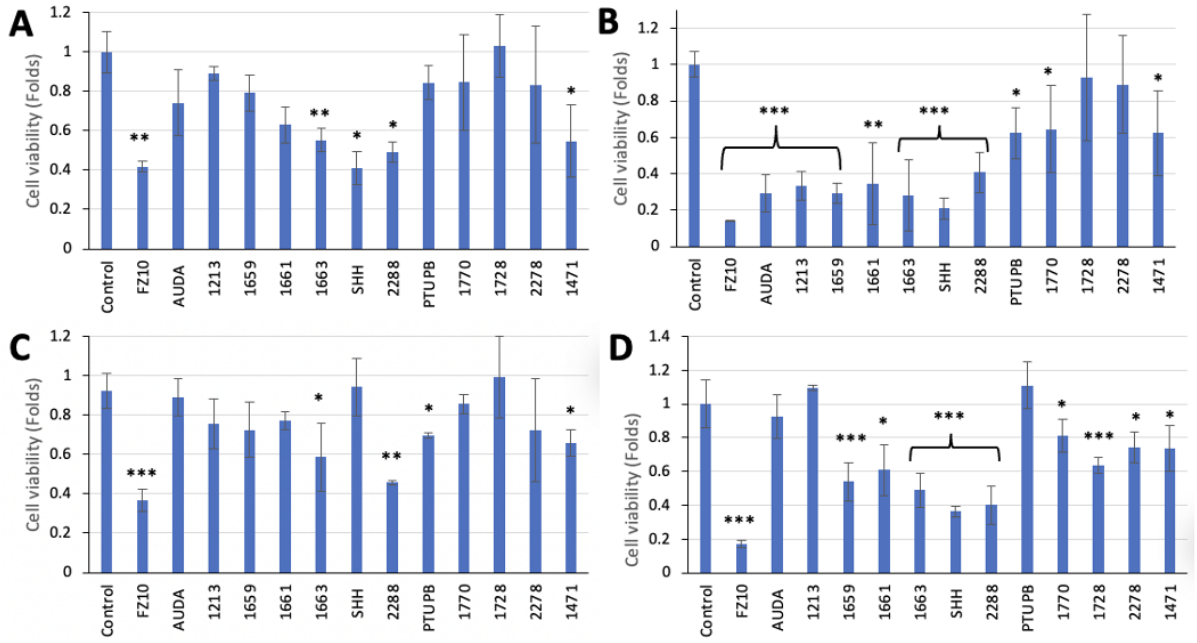


Figure 5. *Cell viability profile of 92.1, MP46, C918 and OMM-1 cell lines in response to sEH inhibitor using MTT assay. 92.1 (**A**), MP46 (**B**), C918 (**C**) and OMM-1 (**D**) cell line was treated with 1µM of individual compounds. DMSO containing medium was used as the negative control and FZ10 compound was the positive control. After 24 hr treatment, cell viability was evaluated with MTT assay. Data were presented as the percentage of the negative control group (mean ± SD). Experiments were repeated on one to two occasions (n=6 in each experiment).*

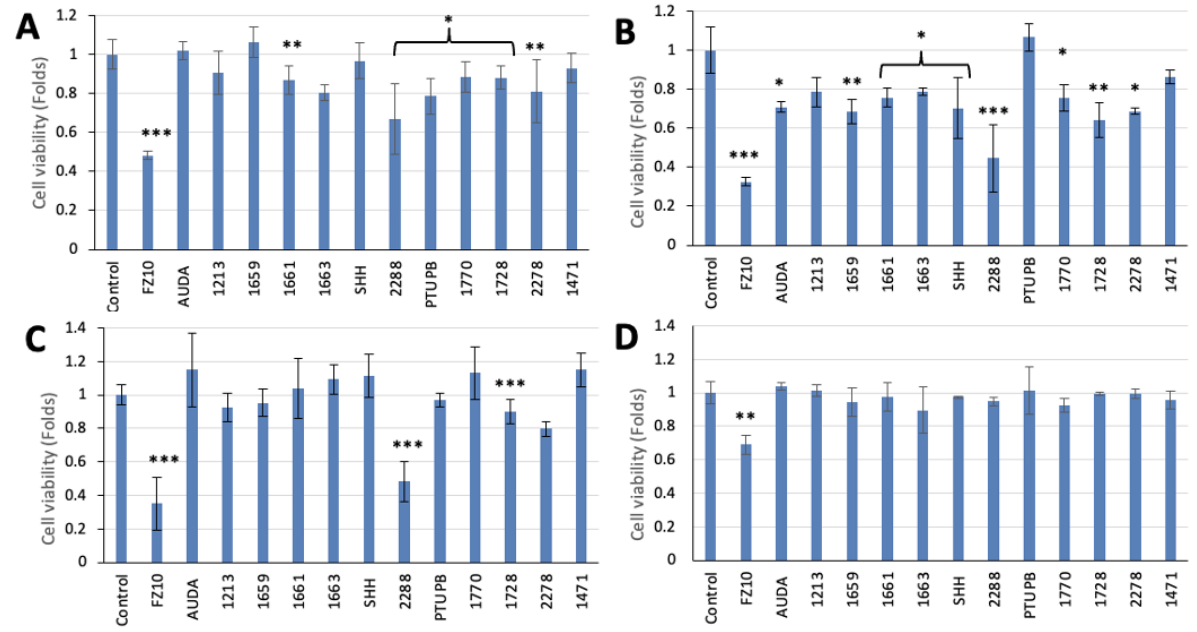


*Figure 6. Cell viability profile of 92.1, MP46, C918 and OMM-1 cell lines in response to sEH inhibitor using Alarma Blue assay. 92.1 (**A**), MP46 (**B**), C918 (**C**) and OMM-1 (**D**) cell line was treated with 1µM of individual compounds. DMSO containing medium was used as the*

*negative control and FZ10 compound was the positive control. After 24 hr treatment, cell viability was evaluated with Alarma Blue assay. Data were presented as the percentage of the negative control group (mean ± SD). Experiments were repeated on one - two occasions (n=6 in each experiment, * p < 0.05, ** p < 0.01, *** p <0.005).*

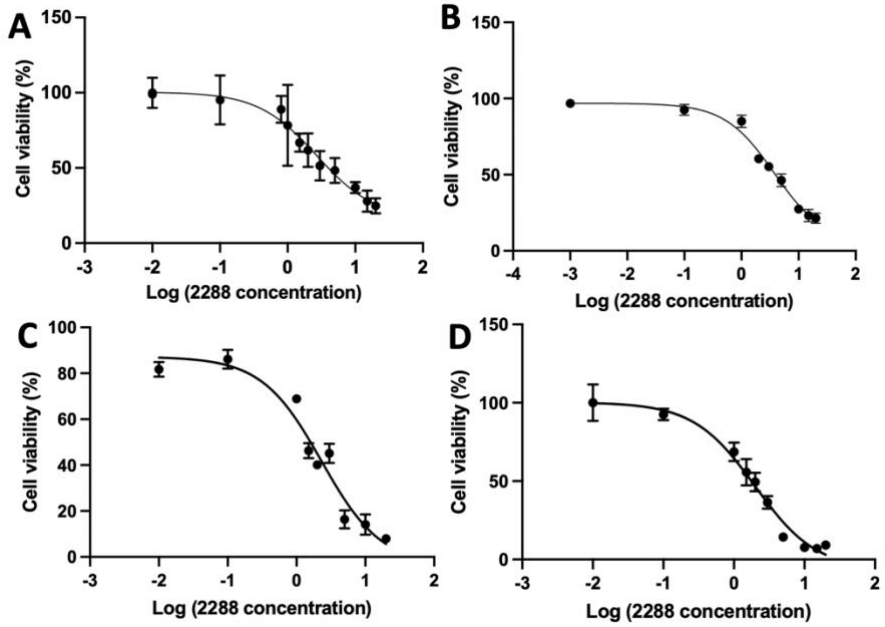


*Figure 7. IC_{50} evaluation of UC2288 in the four UM cell lines. 92.1 (**A**), MP46 (**B**), C918 (**C**) and OMM-1 (**D**) cell line was treated with different concentrations of UC2288 compound (ranged from 0 to 20 µM) for 24 hr. Cell viability was measured using MTT assay. DMSO containing medium was used as the negative control. Data were presented as the percentage of the control group (mean ± SD). n = 9 in the experiment. Data was analysed using GraphPad Prism 9.3.1 software.*

Compound	**UM cell lines studied**	**IC_{50} value (µM)**
UC2288	92.1	2.70 ± 3.19
	MP46	3.77 ± 3.89
	C918	2.31 ± 2.46
	OMM1	1.99 ± 2.04

Table 2. IC50 values of UC2288 in the four UM cell lines. IC50 values of UC2288 was estimated from the data in Figure 5 using GraphPad Prism 9.3.1 software.

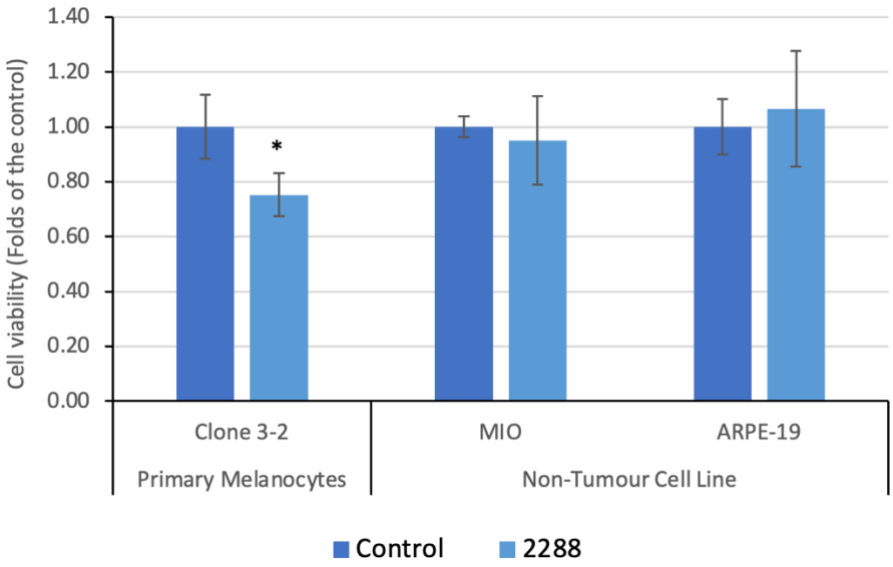


*Figure 8. The influence of UC2288 on cell viability of non-tumour retinal cells. Human primary melanocytes and non-tumour retinal MIO-1 and ARPE-19 cell lines were treated with UC2288 (3 µM, 24 hr) and cell viability was then measured using MTT assay. DMSO containing medium was used as the negative control. Data were presented as the percentage of the control group (mean ± SD). Experiments were repeated on two-three occasions (n=3 in each experiment, * $p < 0.05$).*

Subsequently, to determine the cytotoxicity of UC2288, LDH assay was performed in four UM cell lines upon the treatment of 3 µM of UC2288 for 48 hours. The most significant cytotoxicity was observed in 92.1 cells (Figure 9); while the effect was mild to moderate in the other three UM cell lines. These results are statistically significant given that C918 and 92.1 both have a p-value < 0.001, followed by p-value < 0.005 for MP46 and p-value < 0.01 for OMM1.

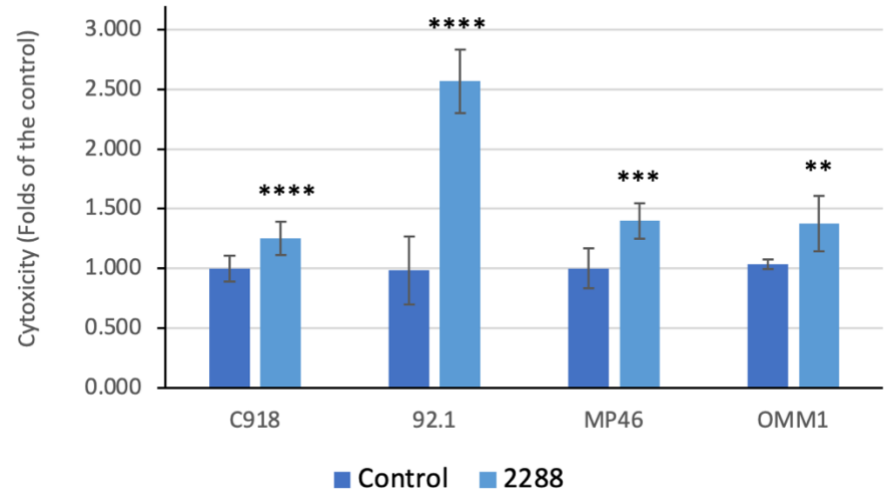


Figure 9. Cytotoxicity of UC2288 in the four UM cell lines (92.1, MP46, C918 and OMM-1). Each UM cell line was treated with 3 µM of UC2288 for 48h. DMSO containing mediums was

*used as the negative control. Cytotoxicity was evaluated with LDH assay kit. Data are presented as the fold of the negative control in cytotoxicity (mean ± SD). Experiments were repeated on two occasions (n=6-9 in each experiment). * p < 0.05, ** p < 0.01, *** p <0.005 and **** p < 0.001.*

As mentioned above, the chemical compounds tested in our study are sEH inhibitors, while sEH is the key enzyme involved in fatty acid metabolism in mitochondria. Therefore, it is desired that the mitochondrial health and activity should be evaluated upon the treatment of UC2288 in UM cells. Mitochondria is the main cellular organelle involved in the response of cells to oxidative stress. Therefore, the level of reactive oxygen species (ROS) is a good reflection of mitochondria function. And ROS production is a promising therapeutic target of cancers, since in cancer cells, mitochondrial genetic abnormalities often lead to an elevation of ROS. Interestingly, UC2288 significantly induced ROS elevation in two of three primary UM tumour derived cell lines (92.1 and MP46), moderately in C918 but not in the metastatic cell line OMM1 (Figure 10). The statistical significance in all primary UM tumour derived cell lines were p-value < 0.005.

To further examine the influence of UC2288 in mitochondrial health, the mitochondrial membrane potential (MMP) was then examined using JC-1 assay. UC2288 treatment significantly impaired MMP in all cell lines (Figure 11). With MP46 being the most aggregated and is also the only data set, which is statistically significant (p-value < 0.001).

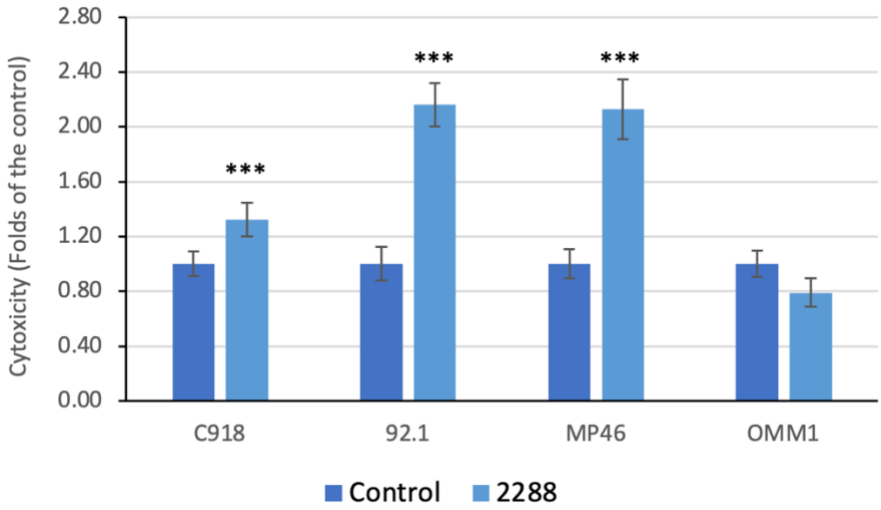


*Figure 10. The influence of UC2288 on cellular ROS levels in C918, 92.1, MP46 and OMM cell lines. Cells were treated with 3 μM UC2288 for 48 hr. DMSO containing mediums was used as the negative control. Data are presented as the % of control in ROS elevation (mean ± SD). Experiments were repeated on two occasions (n=9 in each experiment, *** p <0.005). The data was produced with the assistance of Ms. Janney Wang.*

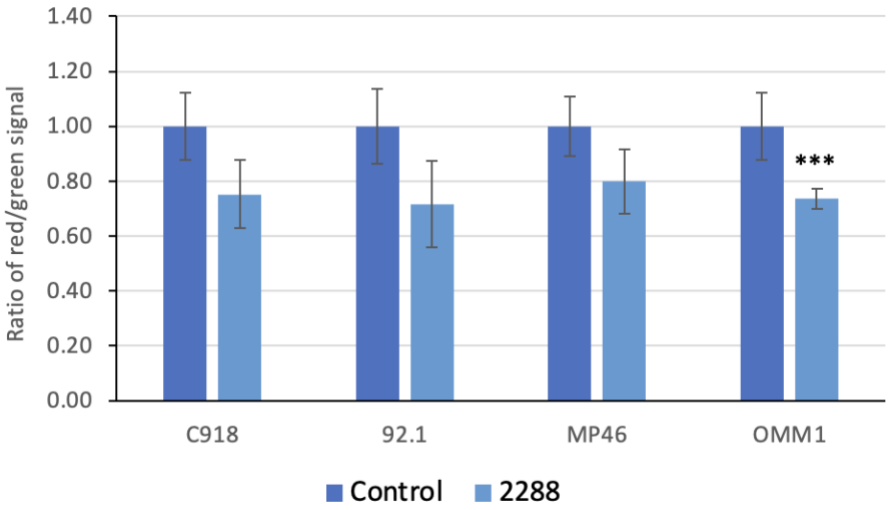


*Figure 11. Mitochondrial membrane potential of four UM cell lines upon the treatment of UC2288. Cells were treated with 3 μM of UC2288 for 48 hr. DMSO containing mediums was used as the negative control. The ratio of red signal (JC-1 aggregates) to green signal (JC-1 monomers) were compared across the treatment and control group. Data are presented as the percentage of the negative control (mean ± SD). Experiments were repeated on four occasions (n=3 in each experiment). ** p <0.005.*

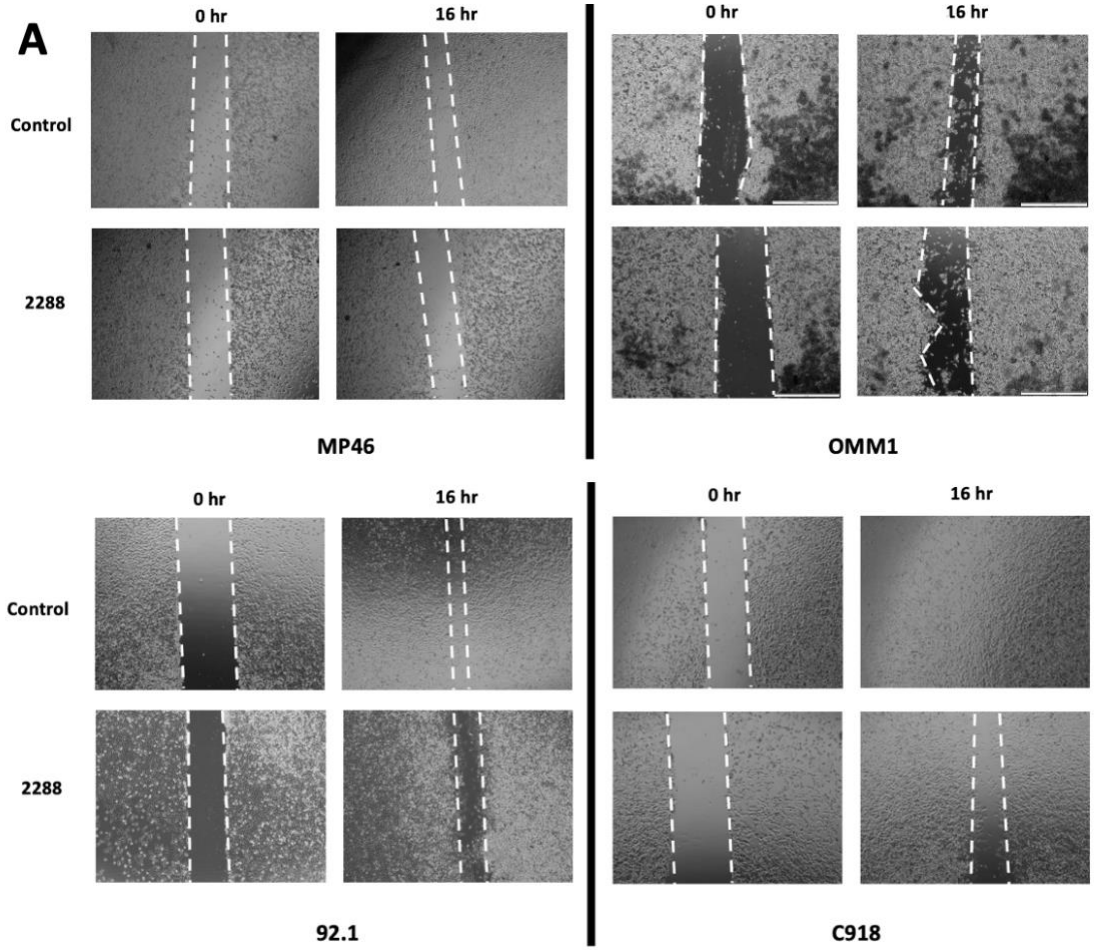
3.5.2 UC2288 impacts on cell migration and reproductive cell growth in 92.1, C918, MP46 and OMM1 cells

To evaluate the anti-metastatic effect of UC2288, cell migration, invasion and colony formation properties were assessed in four UM cell lines with or without the treatment of UC2288. Results demonstrated that UC2288 significantly decreased the rates of cell migration in all but MP46 cell line (Fig 12A). The quantification of the wound closure as shown in Figure 12B further confirms the visual representation of wound healing properties of each cell line. Such that, whether in the presence or absence of UC2288 treatment. MP46 has deficient cell migration properties with 80% of cells migrated. Whereas, in 92.1 and C918, about 60 percent of the cells migrated post-treatment of UC2288 and over 40 percent in OMM1. In these three cell lines, the results were statistically significant as p-value < 0.005, exemplifying the significant of effects observed in this experiment. The variable response of MP46 cell line to the treatment of UC2288 in comparison to the other tested cell lines could be attributed to its slow growth rate and for future study, extended treatment duration may be applied.

Additionally, the effect of UC2288 in reproductive cell growth was evaluated using colony formation assay. It was found that at UC2288 significantly suppressed the reproductive cell growth across all primary UM cell lines (Figure 13A). CV-stained cells produced visual

representation of colony formation which demonstrate that post-treatment of UC2288, the number of cells that were capable of forming colony reduced drastically, especially in MP46 (p-value < 0.005). The colony forming ability was moderate in C918 (p-value < 0.005) and 92.1 (p-value < 0.05) while inconclusive in OMM1 as it was statistically and visually insignificant.

The last property which was investigated was invasion in which OMM1 has the lowest folds of cells invading through the Matrigel, followed by MP46, C918 and 92.1 (Fig 14), all of which has a p-value < 0.005. Overall, it is evident that UC2288 reduced the rate of migration, invasion and inhibited the reproductive cell growth of UM cell lines. However, the rate and extend at which these reductions occur are different among four cell lines.



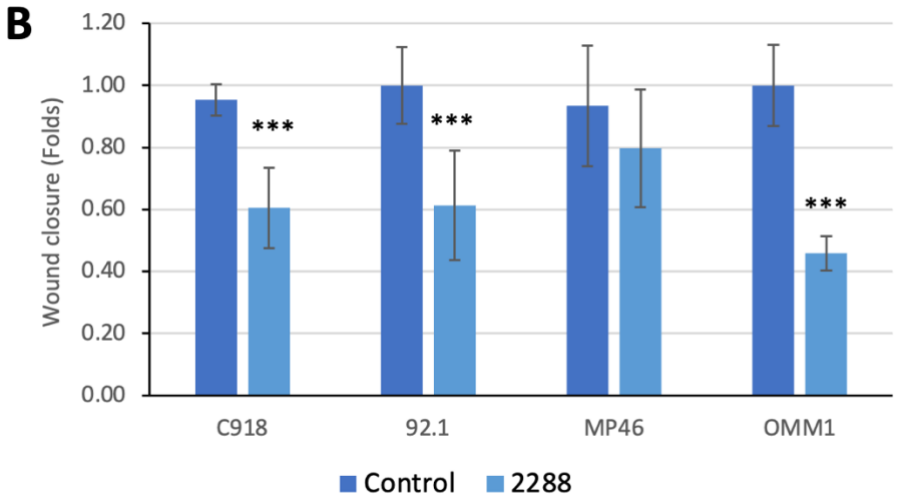
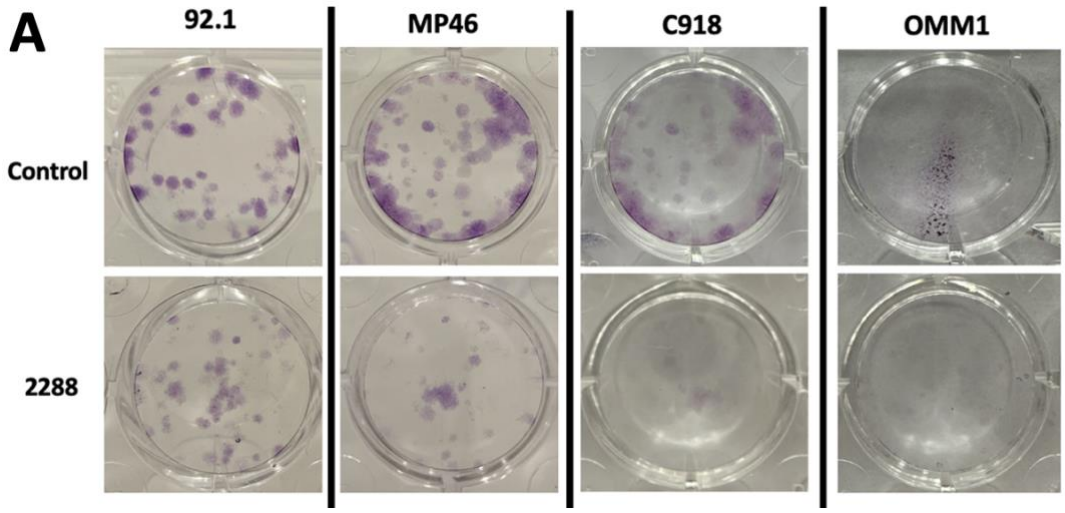


Figure 12. The influence of UC2288 on UM cell migration. Scratches were made on UM cell lines cultured on plates at t=0 hr. Then the cells were treated with 3 μM of UC2288 for 16 hr. DMSO containing mediums was used as the negative control. Cellular images were taken using Incucyte machine *every 2 hr.* ***(A)*** *Representative cell images at t= 0 or 16 hr.* ***(B)*** *Quantitative analysis of wound healing rate with or without UC2288 treatment. Data are presented as the percentage of the control (mean ± SD). Images were analysed using ImageJ. Experiments were repeated on 3 occasions (n=3-6 in each experiment). *** p <0.005. The data was produced with the assistance of Ms. Janney Wang.*



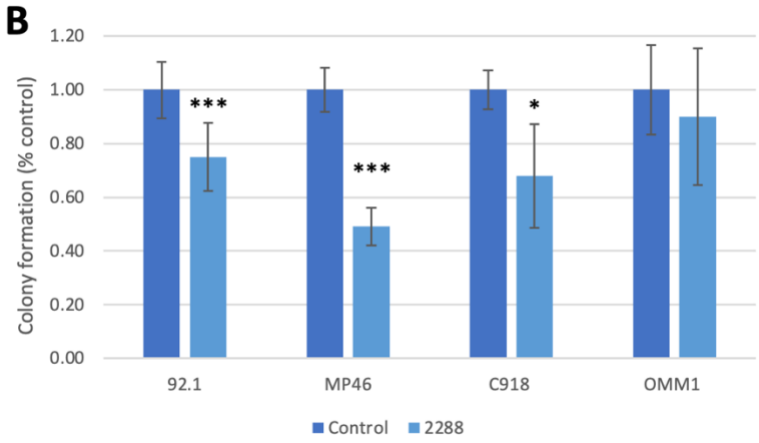
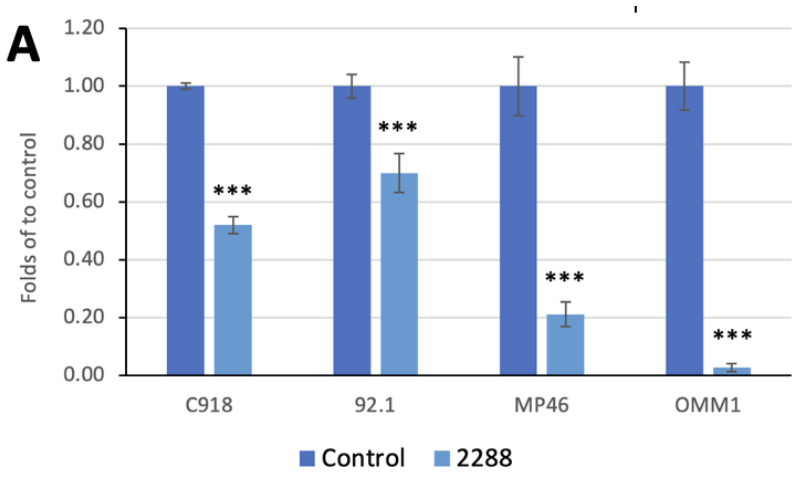


Figure 13. The influence of UC2288 on cell reproductive growth in UM cell lines. Cells were treated with 3 μM of UC2288 for 16 hr. DMSO containing mediums was used as the negative control. Cells were detached and cultured onto plates with 50-200 cells/well. Following 48-72 hr growth, the cell colonies formed on the plate were counted upon crystal violet staining. ***(A)*** *The representative images of stained cell colonies* ***(B)*** *Quantitative analysis of wound closure folds with or without UC2288 treatment. Data are presented as the percentage of the control (mean ± SD). Images were analysed using ImageJ. Experiments were repeated on 3 occasions (n=3-6 in each experiment). *** p <0.005. The data was produced with the assistance of Ms. Janney Wang.*



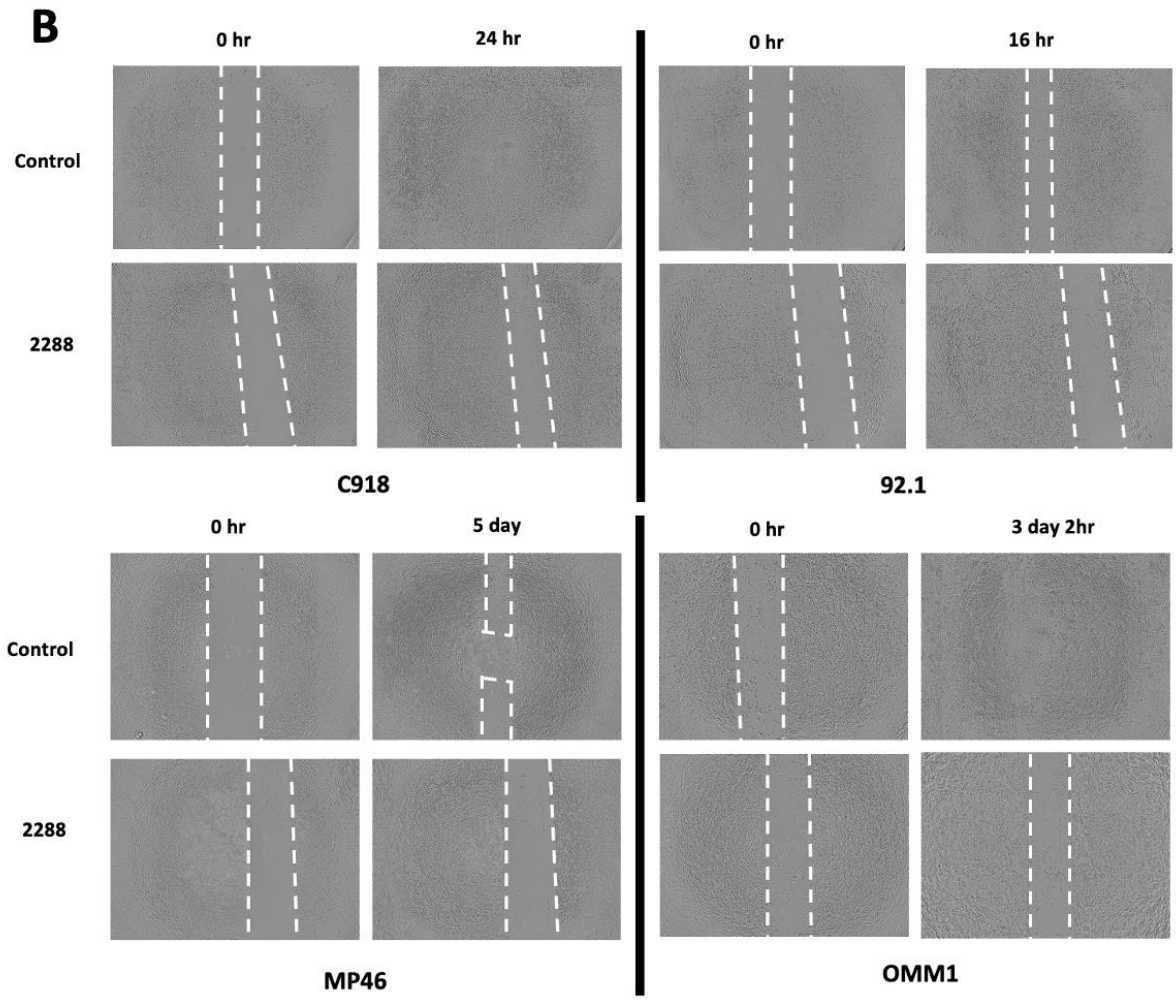


Figure 14. UC2288 attenuates invasion of uveal melanoma cancer cells. Cells were exposed to 3μM of UC2288 for 2-5 days depending on the specificity of each line and at various time-points cells that migrated through the inserted. ***(A)*** *Data shown represent as folds of wound closure to control (mean ± SD). Experiments were repeated on two occasions (n=5 in each experiment). *** $p < 0.005$.* ***(B)*** *Images show inserts treated at time 0 and corresponding time points. The data was produced with the assistance of Ms. Janney Wang.*

3.5.3 Cell cycle

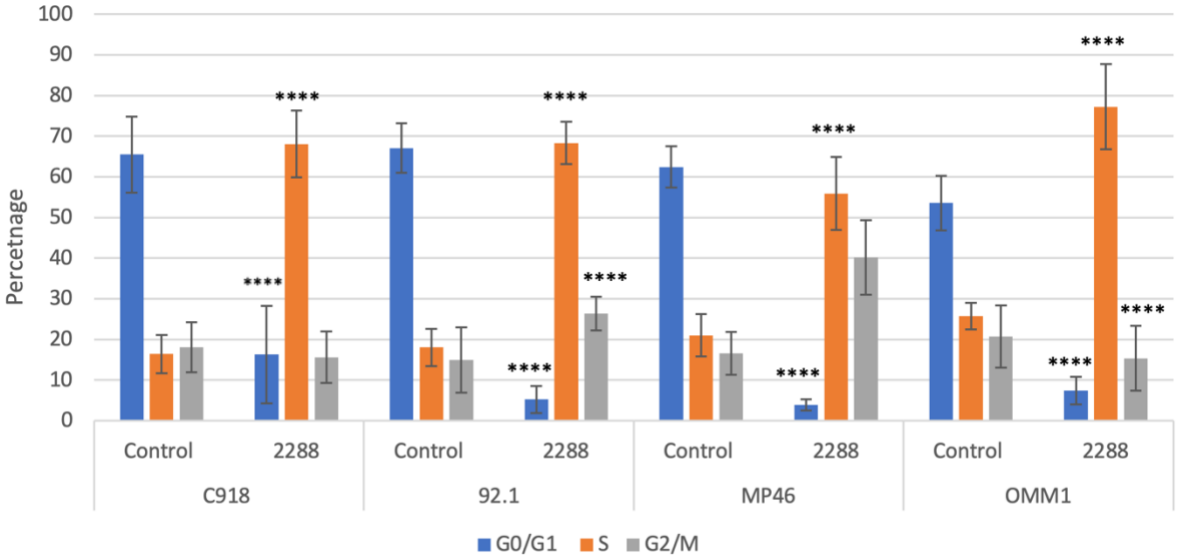


*Figure 15. Representatives of flow cytometry comparison between 3μM of UC2288 for 48h and negative controls which are non-treated negative serum-free (RPMI and DMEM) cells. Data are presented as percentage (mean ±SD). Experiments were repeated on two occasions (n=10-15 in each experiment). **** $p < 0.001$. The data was produced with the assistance of Ms. Janney Wang.*

Given that cell division is a highly regulated process, classically described as four sequential phases the progress from quiescence (G0) phase to proliferation (G1, S, G2 and M phases) then back to G0 phase. Results demonstrate that UC2288 is among other chemotherapeutic which work in S phase as the elevated percentage illustrate its activation when compared with control. The percentages range from 55 to 80 percent and most evidently in OMM1. The results in this experiment are all statistically significant, $p < 0.005$).

3.6 DISCUSSION AND CONCLUSION

UM has a poor prognosis. There are limited pharmacological treatments available for this deadly cancer. sEHIs have been shown to revolutionised therapies in other types of cancers like breast, hepatocellular and renal cell carcinoma. They target tumorigenic mechanisms to be functional, such as cell proliferation, survival, cell growth, motility, and metastases. Sorafenib is a novel multikinase inhibitor, which also has sEH inhibitory activity. It has been used to treat tumours through the inhibition of angiogenesis, and inhibits tumour proliferation [164]. Overall, sEHIs have been shown to be cross-functionally targeted and are known to be clinically useful in cancer treatment. However, it has not been investigated in UM.

This study demonstrated that there are potent anti-cancer effects of sEHIs in the treatment of UM. The preliminary drug screening demonstrates that several candidates among the eleven synthesized she inhibitor compounds, UC2288 was the most promising compound for further testing (Fig 5 & 6). UC2288 has potent anti-UM effects such that results are consistent across two assays (Alarma Blue and MTT). Both cell viability assays are widely used to evaluate cytotoxicity, cellular metabolic activity, and cell proliferation. While in Alarma Blue, an active and cell permeable ingredient – resazurin is responsible for the fluorescence of live cells which can be used to quantitatively analysis [165]; MTT is a quick colorimetric assay using the cleavage of MTT (3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide) by dehydrogenases in living cells to estimate viable cell number [166]. Although, there are varying cell viability across each cell line and assay, it is consistent that MP46 has the lowest number of cells viable post-treatment. Perhaps, MP46 is the most sensitive cell line to UC2288 and its molecular structure. Notably, UC2288 and 2278 are two similar compounds yet performed differently due to its functional groups, which might have suggested that although the parental group is pivotal in producing anti-UM effects, alteration in functional groups produce differing effectiveness. Potentially suggest that to further optimise UC2288, changes in functional groups might greatly impact the effectiveness of anti-UM effects. However, at this current stage, UC2288 is the most promising candidate for further investigation.

According to the subsequent molecular analysis, UC2288 potently reduced cell viability across UM cell lines with the IC_{50} values ranged from 1.99 to 3.77 μM (Fig. 7 & Table 2). In comparison to sorafenib, a multikinase inhibitor that also has sEH inhibitor activity, UC2288 is much more potent to sorafenib such that the IC_{50} values ranged from 4.00 to 12.81 μM [167]. In accord with this finding, the IC_{50} range for UC2288 is much smaller than sorafenib, it presents as a promising candidate as it requires more than half the concentration to exert half of its maximal inhibitory effect. Sorafenib was in phase II study in 2016. It has achieved non-progression in 31.2% of patients at 24 weeks; however, 41.4% of patients underwent dose reduction due to toxicity [168]. Potentially, UC2288 can be a better alternative in treating anti-UM effects given that it is a potent compound yet demonstrated favorable tumour selectivity (Fig. 8). Primary melanocytes and non-tumour cell lines examined under same condition and UC2288 demonstrated that although there were reductions in cell viability, it is insignificant which validate that UC2288 does not target healthy cells but selectively target tumour. Specifically, in primary melanocytes clone 3-2, results were statistically significant ($p < 0.05$). Therefore, UC2288 may preferably inhibit cell proliferation rather than cell killing effect.

However, Fig. 8 suggests that UC2288 has mild to no inhibitory effect on cell viability of non-tumour retinal cell lines, which highlighted its tumour selectivity. Additionally, to further validate the results from the previous experiments, LDH assay was performed to examine cell death which confirms that UC2288 kills cancer cells across all four cell lines, most prominently in 92.1 ($p < 0.001$).

sEH is the key enzyme involved in fatty acid metabolism in mitochondria, it is plausible that UC2288 may impact on mitochondrial function and health. Elevated ROS level was observed in all UM cell lines excluding OMM1 (Fig. 10), which suggested that UC2288 induced cellular ROS accumulation, which can consequently lead to cell death via further damages to proteins, lipids, nucleic acids, membranes and organelles [169]. In specific, 92.1 and MP46 has the highest elevations of ROS and are statistically significant with p-value < 0.005. Given that tumours with mitochondrial genetic abnormalities (eg. copy number change and mutations) have upregulated ROS production in comparison to normal cells. Therefore, high levels of ROS suggest the trigger of apoptosis. Perhaps, the exception of OMM1 and inconsistent observation across four cell lines in ROS profile is due to the diverse responsiveness of UM cell lines to UC2288 treatment as to their different responsive potentials and/or cell growth rate. For future study, optimal time frame shall be established for each cell line. In addition, we also observed that UC2288 treatment potently disrupted the MMP of the UM cell lines except for the slight disruption observed in MP46 under the current experimental condition (Fig. 11). JC-1 is considered an indicator of mitochondrial membrane potential, which reflects mitochondrial health. With the current experimental condition, MP46 cell line exhibited significantly impaired mitochondroal health. The other three cell lines didn't show significant outcomes in this assay. Again, the discrepency of the results may be an outcome of diverse effective time frame of UC2288 in different UM cell lines. It is suggested that future experiments may need to include longer treatment duration in the UM cell lines that are not susceptible to UC2288. Slower growth rate corresponds to minimal or unchanged membrane potential and therefore smaller ratio between red and green signal. Overall, the findings indicated that UC2288 dysregulated mitochondrial function and health, which mechanism contributes to its effect in reducing UM cell viability. In addition, the diverse growth rate of UM cell lines may also contribute to the differential responsiveness in various molecular assays, which suggests that an optimal time frame shall be used for each UM cell line to yield expected treatment effect [108].

In UM, metastases are the primary cause of death in patients thus it is important to assess the anti-metastatic effect of UC2288. Interestingly, UC2288 demonstrated a significant inhibitory effect on UM cell migration in 92.1, C918 and OMM1 but not in MP46 cell line (Fig. 12A), which suggested that UC2288 significantly and selectively disrupt cell migration. MP46 is consistently not sensitive across several experiment which might be a result of MP46 slow growth rate. Evidently, MP46 has the least folds of wound closure compared to control and is also the only data set which was not statistically insignificant. For future research, to improve the quality of data, various time points should be adopted to determine the optimal treatment time frame for each cell line. It is determined that, MP46 performs best at around 72h time frame in other studies [108, 170]. Interestingly, UC2288 has a significant inhibitory effect on UM cell migration as well as induced reproductive cell death, specifically OMM1 ($p < 0.005$) which suggests its potential application in preventing UM metastasis. Furthermore, our data showed that UC2288 can suppress reproductive cell growth in all UM cell lines (Fig. 13A & 13B), mostly in MP46 and 92.1 ($p < 0.005$). However, given the non-statistically significant colony formation for OMM1, the anti-metastatic effects of UC2288 are inconclusive and require further investigation. Upon UC2288 treatment, the control and treatment group of OMM1 cells showed little cells, implying that there might have been an experimental error. So experimental repeats will be needed to account for the faulty.

In accord to the finding in anti-metastatic investigation, the study of invasion properties in UC2288 demonstrated that OMM1 has the least cells invaded across trans-well and 92.1 has the most invasion activity post-treatment. Inasmuch as, cell migration is the movement of individual cells from one location to another while cell invasion refers to 3-dimenstional migration of cells as penetration occur across an ECM. Results suggest that OMM1 is the most sensitive to UC2288 treatment ($p < 0.005$) in reducing cell ability to invade hence potentially decrease the capability of cancer cells metastasising [171]

Cell cycle analysis revealed that UC2288 is among other chemotherapeutic which cell cycle arrest was detected as enhanced S phase percentage across all four cell lines ($p < 0.001$). The S phase arrest in UM cells treated with UC2288 suggests that there might be DNA damage and thus, cells are unable to duplicate its DNA [26]. These results also suggested that UC2288 inhibits cellular proliferation. In summary, cell cycle analysis proved that UC2288 treatment induced S phase arrest in UM cell lines, in particular 92.1 and MP46 cell lines. This is consistent with the early results (Fig. 10) that these two cell lines display an increase in ROS

level, significant cell death (Fig. 9) and inhibition in cell migration (Fig. 12). Overall, it is evident that UC2288 inhibits UM cell growth, triggers cell death, suppresses cell migration, promotes reproductive cell death and induces S-phase arrest in UM cell lines, especially for primary tumour-derived types. However, cell death analysis and cell cycle data cannot indicate whether cell death is through apoptotic or necrotic pathway and flow cytometry may be required to address this.

UC2288 has a specific mode of action which was found to be a potent inhibitor of sEH and P21. There are sEHIs are in clinical use as anti-cancer agents, eg. sorafenib. Sorafenib is a multikinase inhibitor which is clinical used for the treatment of numerous cancers, including renal cell carcinoma through inhibition of VEGFR, PDGFR and Raf family kinases [172, 173].The unique thing about UC2288 is that it also inhibits p21 kinase . In a kidney cancer cell mode, UC2288 was found to decrease p21 mRNA expression and inhibits cell growth [174]. Additionally, from the results, it suggests that UC2288 inhibit cell migration and possibly act as an antimetabolite during cell division. Considering the other sEHIs showed less potent effect in UM cell lines compared to UC2288, it is likely that the anti-cancer effect of this lead molecule is largely due to its inhibitory effect on P21. Some preliminary study has been conducted to investigate the expression of P21 upon UC2288 treatment in UM cell lines. However, the result is not conclusive, so results was not included in this thesis.

Overall, experiments may be required to repeat the experiments with extended treatment duration and/or increased UC2288 concentration. Because the experiments conducted in this study were kept consistent across all four UM cell lines to establish the anti-UM property of UC2288 for the first time, it is beyond the scope of the current study to optimise the experimental condition for each UM cell line. Additionally, future study could examine UC2288 in UM patient tumour derived ex vivo models and xenograft UM mouse models that are available to our laboratory, to further evaluate the treatment effect of UC2288 in UM as performed in afatinib by our fellow lab members [167]

In summary, UC2288 has potent anti-cancer and anti-metastatic potentials in the treatment of UM based on the findings made in in vitro models. UC2288 may be applied to inhibit UM cell migration, invasion and suppressing cell productive growth and in turn, suppress metastases. sEH activity in UC2288 has emerged as a candidate drug that act as antimetabolites upon treating UM cells. Additionally, it has been proven to be effective in reprogramming fatty acid

metabolism in order to treat UM. UC2288 may be a novel candidate agent with potent therapeutic effect and selectivity in treating UM by inhibiting sEH activity.

Chapter 4: Validate the Anti-cancer Effect of UC2288 in the Three-Dimensional (3D) UM Culture Models

4.1 INTRODUCTION

Preclinical anticancerer drug testing is constantly evolving, while the two-dimensional (2D) *in vitro* cell model is commonly used in most studies. Recently, three-dimensional (3D) culture models have attracted attentions from pharmacologists as it better mimics human tissues, especially tumours. Consequently, 3D models have been considered as the enhanced biomimetic tissue models and are more clinically relevant compared to the traditional 2D models. 3D models can provide a more accurate simulation of tumour characteristics, such as hypoxia, possible drug resistance and anti-apoptotic features [175].

The 3D culture model simulates a tissue-specific pathophysiological microenvironment, specifically with cell-to-cell and cell-to-matrix interactions that cells rely on in order to proliferate, differentiate and aggregate [176]. Such unique feature favours the prediction of drug response in the treatment of diseases.

3D culture models can be applied in the early stage of drug discovery like "disease modelling, target identification and validation, screening, and drug efficacy and safety assessment" [177]. In recent years, research advances in cell biology enabled a wide range of technologies in establishing 3D culture models, ranging from multicellular spheroids, organoids, hydrogels and 3D bioprinting.

4.2 PROS AND CONS OF 3D CELL CULTURE MODELS

4.2.1 2D culture modes: a basic anticancer drug research experimental tool

2D cell cultures are still widely used because they are well-established, and economies of scale are much less expensive than other models. Since they have gained widespread acceptance and used in most cell culture-based research, researchers are very familiar with them [175]. However, cells cultured in 2D monolayers form exposed surfaces influencing cell behaviours, differentiation, gene expression and drug responses etc. Moreover, cell monolayers fail to replicate the gradients in nutrients, oxygen and molecules found in tumour microenvironment

(TME). In cancers, the variation of gradients determines tumour size and mass [178]. Therefore, findings obtained based on 2D culture models contribute to understanding the properties of anti-cancer candidate drugs but are not sufficient to reflect their actual performance in clinical settings.

4.2.2 3D culture models: advanced anticancer drug research platforms

The need to limit animal use in drug testing and minimise failures in clinical trials highlights the pivotal transition from 2D to 3D cell culture models.

Although 2D culture methods have been extensively used, there are numerous limitations due to their simplicity [179]. Thus, 3D culture models are highly desired to recapitulate the complex interactions between tumours and stromal cells, which contributes to better understanding the molecular mechanisms underpinning the pharmacological roles of anticancer drug candidates. 3D models present a more remarkable resemblance to cancer cells *in vivo* as they enable "cell-cell interactions, cell–ECM interactions, cell polarity, gene expression and signalling pathways" [175]. An emerging TME is a complex and continuously evolving entity where a tumour in the body is composed of a heterogeneous population of cells within the tumour and stromal tumour microenvironment, non-cellular and non-malignant elements like extracellular matrix (ECM) [179, 180]. TME is constituted by non-malignant cellular components and non-cellular elements. Research suggests that TME is pivotal in cancer development; therefore, taking TME into consideration may improve the outcome of cancer treatment.

3D cell culture models epitomise human tumour-stromal crosstalk by eradicating the existent cross-species incompatibilities found in patient-derived tumour xenografts [123, 181]. Moreover, in comparison to 2D models, cells in 3D models proliferate at a more realistic rate, thus they better reflect the long-term effect of anti-cancer molecules as cells can remain functionally stable for a more extended period [182]. 3D cell culture models also mimic the natural tumour architecture with two zones. These two zones are an external proliferating zone as well as an internal quiescent zone that has limited nutrient/growth factor distribution and oxygen [123, 183, 184]. In the studies of tumour metastatic progression promoted by a potent microenvironmental factor – hypoxia, 2D models fail to recreate critical aspects of tumour biology and, as a result, inaccurately demonstrate cell behaviours and drug responses [185]. Hence, the 3D model is more suitable for testing cellular drug responses with considering oxygen and pH gradients. More importantly, gene expressions found in 3D models are more

correlated with those in *in vivo* settings, implying that 3D models are also advantageous in identifying disease biomarkers [186].

Successful 3D models in other cancer types include models in non-small lung cancer (NSCLC). It is accounted for 80 percent of lung cancers which is characterised by morphological and clonal heterogeneity of tumours and multidrug resistance. NSCLC 3D model possesses certain molecular and physiological properties of tumours which can be successfully applied widely [187]. The main feature of organotypic spheroid is genetic heterogeneity which enable "personalised" medicine. Additionally, patient sample derived spheroids mimic cell composition and molecular profile of tumours. Given the highly heterogeneous nature of this cancer, 3D models are highly desired in therapeutic development prior to clinical trials to minimise risk and/or side effects [187]

On the other hand, 3D models may also have technical barriers compared to 2D cell models in drug discovery research. For example, due to its three-dimensional nature, it is challenging to visualise spheroids formed under microscopes, which largely impacts on data analysis. And 2D models are more cost-effective, while it lacks affordable and established methods with great reproducibility in the case of 3D culture model [188]. Several factors which attribute to unsuccessful 3D models include culture optimisation and assay variability as well as automated imaging and visualisation of 3D culture [189].

In this part of the study, the anti-cancer effect of UC2288 was validated using 3D UM cell models. Such pilot data forms the basis of future research.

4.3 MATERIALS AND METHODS

MP46, C918 and 92-1 are cell models derived from primary UM tumours; while the OMM-1 cell line is originated from subcutaneous metastatic UM tumours. These four cell lines were adopted in the current study. Additionally, two commercially available non-tumour ocular cell lines (ARPE-19 and MIO-M1) were chosen in this study to examine the drug safety and tumour selectivity. ARPE-19 is spontaneously transformed from retinal pigment epithelium, which is commonly used in pathological and pharmacology studies. MIO-M1 is a spontaneously immortalised human Muller glia cell line that exhibits indeterminate growth under adherent conditions; thus, it serves as a valuable instrument for examining the physiological and pathological processes that transpire within the retina in response to various stressors.

4.3.1 Generation of 3D cell culture models

UM or non-tumour ocular cell spheroids were generated using round-bottom 96-well plates (Corning Life Sciences) with the seeding density of 3 x 10^3 cells per well. Cells were cultured for 3 days to achieve uniform and compact spheroids. The spheroid cultures were incubated at 37C for the indicated time. Compact UM spheroids generated were ready for drug treatment on day 4.

4.3.2 Staining of spheroids

Additionally, after 72 h of treatment at 37 °C, spheroids were stained with 0.5% crystal violet (w/v in PBS) for 30 min in order to perform the supplemental quantification of spheroid size and compactness.

4.3.3 Determination of spheroid size and compactness

Cell spheroids were imaged on days 0, 2, 4 and 5 (n = 6) using Incucyte S3 Live-Cell Analysis (Sartorius) microscope at 4x magnification and analysed for growth determination using ImageJ Software version 1.53. The size of spheroids was estimated through calculating the cross-sectional area (μm^2) of spheroids via an ImageJ macro developed by Ivanov [190] determine the spheroid compactness. The density and cross-sectional area of spheroids in treatment samples were normalised to that of the untreated groups prior to treatment in arbitrary units (AU). For all cell lines, 3-6 spheroids were analysed in each experimental group.

4.3.4 Spheroid viability assay

3D cultured cells were treated with UC2288 (3μM in 0.1% DMSO) in RPMI or DMEM containing 1% FBS for 72h at 37 °C. Tumour cell viability (n = 6) was assessed using Alarma Blue assay (Section 3.4.1). Fluorescence signal was recorded using a UV-Vis spectrophotometer (Perkin Elmer Victor) at 570 nm.

4.3.5 Statistical analysis

The statistical analysis of data was conducted with a multiple unpaired t-tests using GraphPad Prism 9.3.1 software (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

4.4 RESULTS

4.4.1 Generation of UM and non-tumour cell spheroids

The use of round-bottom 96 well plate methodology resulted in successfully forming spheroids. The size, compactness and density of spheroids that formed vary among different UM as well as non-tumour cell lines. Cell spheroids varied in compactness, where 92.1 formed relatively larger and loosely packed aggregates that were ready for drug treatment at day 3 (Fig 16 & Fig 17). In contrast, OMM1 cells formed smaller but tight aggregates, posed as a challenge for quantification (Figure 17). The generation of UM required optimisation to determine the ideal duration for seeding period to treatment. The size of spheroids is different in each cell line.

4.4.2 Morphological, size and compactness changes of UM and non-tumour ocular cell spheroids upon the treatment of UC2288

The previous studies have shown that drug treatment can result in morphological changes of spheroid [191]. As shown in Figure 16, UC2288 treatment for 24 h induced changes of morphology in all the UM and non-tumour ocular cell spheroids.

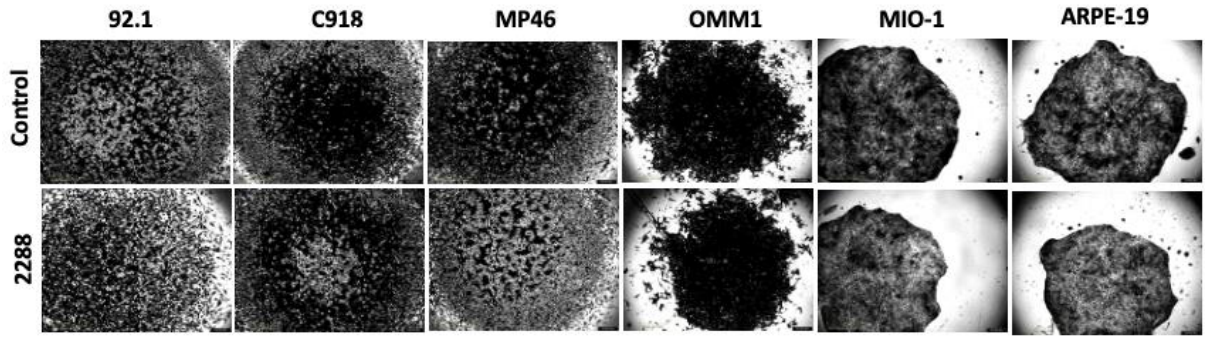


Figure 16. Representative cell morphology images of the spheroids of UM and non-tumour ocular cell lines. Cultured in round-bottom 96 well plates for 3 days then treated with 3μM of UC2288 for 72h before stained with 0.5% Crystal Violet and imaged using Essen Incucyte S3 instrument using spheroid mode at 4x magnification. Experiments were repeated on four occasions (n=6 in each experiment).

There were mild to moderate reductions in spheroid size and compactness in C918, OMM1, MIO-M1 and ARPE-19 cell lines (Figure 17). More significant reduction in spheroid size and compactness was observed in MP46, 92.1 and C918 cell lines (Figure 17).

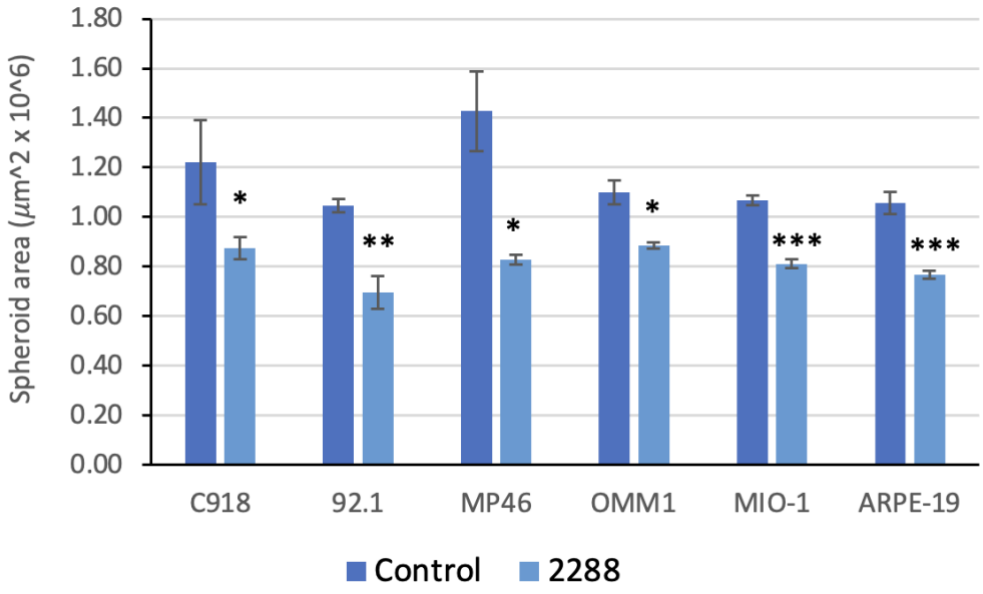


Figure 17. The spheroid size of UM and non-tumour ocular cells. Cells cultured in ultra-low adherence round-bottom 96 well plates for 3 days then treated with 3μM of UC2288 for 72h before stained with 0.5% Crystal Violet and imaged using Essen Incucyte S3 instrument using spheroid mode at 4x magnification. Image J software was used to estimate the size of spheroids. Experiments were repeated on four occasions (n=6 in each experiment).

4.4.3 Cell viability changes of UM and non-tumour ocular cell spheroids upon the treatment of UC2288

As a pilot study, we tested one single condition of UC2288 treatment in the UM or non-tumour retinal cell spheroids. UC2288 treatment reduced the viability of C918, 92.1 and OMM1 to around 0.3-0.45 folds of control ($p < 0.005$), whereas in OMM1 and non-tumours MIO-1 and ARPE-19 cells, the viability is moderately reduced to around 0. 8 folds of control ($p < 0.005$).

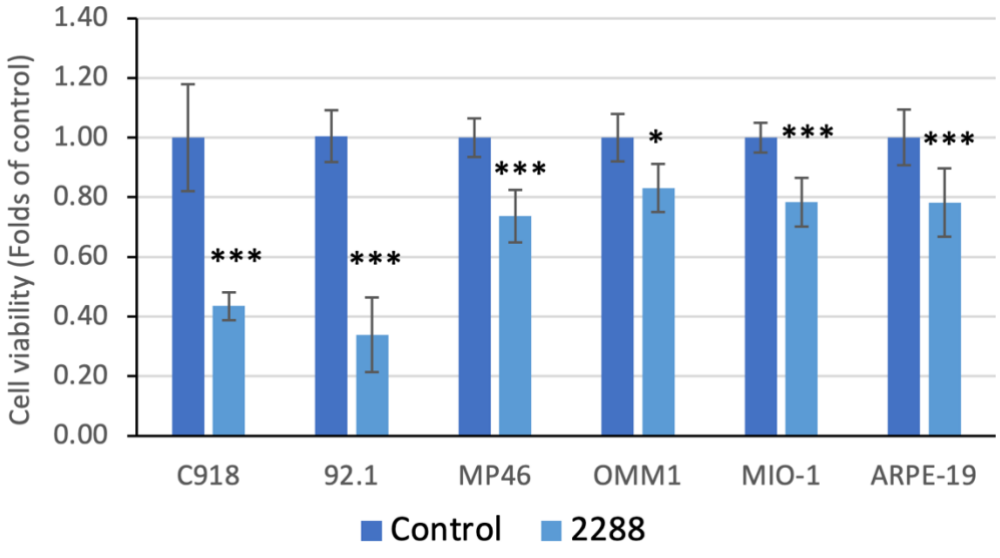


*Figure 18. Cell viability profile of 92.1, MP46, C918 and OMM-1 cells in response to UC2288 treatment. Cells were treated with UC2288 3 µM for 24 hr and cell viability was assessed with Alarma Blue assay in 3D culture model. DMSO containing medium was used as the negative control. Data are presented as the percentages of the control (mean ± SD). Experiments were repeated on four occasions (n=6 in each experiment). *** p <0.005*

4.5 CONCLUSION AND DISCUSSION

2D cell culture models have been widely used in cancer research. However, in recent years, researchers have been more preferred to assess drug responses in 3D cell culture models taking advantage of the inclusion of physiological TME, better mimicking tumour characteristics, etc. In this pilot study, 3D culture models were established using round-bottom 96 well plates, in which setting cell spheroids were formed after plates were spined in centrifuge. Round-bottom 96 well plate was the chosen method as it was readily available, simple and high reproducibility. Additionally, it is compliant with high-throughput screening and high-content screening. Other 3D models such as organoids, hydrogels and 3D bioprinting have been carried out internationally, however due to equipment shortage, similar experiment was not performed. Such that, organoids can be considered as it is a 3D cell culture technique proven to have patient specificity, in-vivo-like complexity as well as architecture. For instance, ultra-low attachment plates were used by a group in Germany, but it was not readily available for testing, but similar testing conditions were used [192]. Moreover, as previously discussed in Section 4.2, major limitations in this pilot study were optimisation and assay variability as well as automated imaging and visualisation of 3D culture. MTT assays and Alarma Blue were tested in 3D spheroid model, however, the removal of excess medium for analysis might have influence the amount of spheroid left over and its completeness. Although MTT was the better alternative, but not ideal for investigation as it require further optimisation. Moreover, given that the image analysis in this study was automated, spheroids were not centred hence images do not capture the whole spheroid, consequently, impact the quality of data.

Allowing the same culture duration, the resulted spheroids varied in size, compactness and density among all the UM and non-tumour cell lines. This is possibly due to the underlying characteristics of each cell line. Primary UM tumour-derived cell lines including 92.1 ($p < 0.001$), C918 and MP46 ($p < 0.05$) cells formed relatively less tightly packed spheroids and reached high confluency after three days of seeding. In contrast, metastatic UM-derived OMM1 cells generated dense and tightly packed spheroids for the same culture period ($p <$

0.05). Such spheroids of OMM1 might have impacted on drug penetration as only the inner core of the spheroids might have much reduced accessibility to drugs (Fig 17). The two non-tumour ocular cell lines formed relatively uniform size and quite dense packed spheroids. Both the representation of cell morphology (Fig 16) and spheroid cross-sectional area (Fig. 17) are consistent with cell viability change of UM (Fig 18), with slight variation in MP46. However, the variation in MP46 were consistent across the study hence statistically bias. OMM1 and two non-tumour retinal cell formed relatively uniformed spheroid hence lower penetration ability as evident in the higher cell viability (Fig 18). In accord to this information, C918 and 921 were loosely packed, UC2288 was able to penetrate through the gaps within spheroid. However, given the variation in compactness of spheroid, it possesses as a challenge to conclusive state that UC2288 has the anti-UM effects in 3D model. Additional experiments are highly desired to investigate the cytotoxicity of UC2288 in ARPE-19 and MIO-M1 cells, which would indicate the tumour selectivity of this compound in UM. Furthermore, interactions between the cell surface and extracellular matrix may influence drug efficacy and penetration. These aspects were not covered in the discussion but may need to be considered when selecting endpoint assays for drug development and screening [193].

Multiple cell types that represent the metastatic TME may be considered in the future studies on UM 3D cultures. Utilising hydrogels as scaffolds to encapsulate cells or as mechanical and biochemical cues that more accurately represent the composition of the tumour extracellular matrix and the sequestration of biomolecules within. Developing UM 3D models also requires a thorough comprehension of its TME composition, stiffness, and surface chemical properties, amongst other factors. Although the development of such complex model is time consuming, challenging and expensive, they will ultimately facilitate a greater understanding of UM biology and drug evaluation [193].

As mentioned above, 3D models have been applied in other cancer types. For instance, successful spheroid models have been constructed in lung cancer, which are useful in drug testing. The optimisation of preparation procedure includes angle adaptor incubation, which allows uniform spheroid formation. Such method has been proven to be adaptable. However, the limitation of this application is the cost of consumables and the space required to position the angle adaptor inside the incubator. Another limitation is the number of organoids generated for 384- or 1,536-well format [194].

Moreover, 3D models have been increasingly used in UM studies as cells cultured on ultra-low attachment (ULA) plates considering such method is an established method to obtain spheroids with uniform dimension. Most UM cell lines can be extracted from the ULA plates for subsequent assays [193]. This is pivotal for the uniform and compact formation of spheroids. Additionally, Ivanov and Grabowska demonstrated that agarose moulds necessitate the removal of tumour spheroids from the wells for histological staining, as opposed to the creation of spheroid tissue microarrays of large 3D tumour cell spheroid sample pools [193, 195].

3D culture model is highly desired in UM drug discovery and development, especially when exploring combined treatment. An investigation shows promising yet clinically practicable results of combine treatment in UM 3D spheroids [196]. It is anticipated that combined treatment of UC2288 with other anti-UM molecules or electrochemotherapy and tumour irradiation (pre or post drug treatment) could be investigated to expand the clinical applications of UC2288 in UM.

Future studies in testing sEH may adopt other 3D UM culture methods. For example, hydrogels form the scaffolds to support the cells or to better mimic the composition of the tumour extracellular matrix and biomolecule sequestration within the cells. Moreover, new and improved imaging modalities such as Citation 5/7 (BioTek), may offer higher quality images of spheroids and be suitable for further analysis of cellular events in 3D cell culture models. These modifications will ultimately shorten the gap of personalised medicine.

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Conclusion

In UM, UC2288 can potently induce the reduction of cell viability in 2D and 3D UM models. It has a significant impact on mitochondrial function in UM cells. Furthermore, it significantly inhibits cell migration and suppressed reproductive growth in certain UM cell models. Cell apoptotic and cell cycle assays showed that UC2288 induces a reduction in cell proliferation and an increase of cell death. All these evidence supports that UC2288 is a potent anti-UM candidate molecule, which shall be further studies for its clinical potentials in the treatment of UM.

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