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Characterising the Effects of 1,4,5-Oxathiazinane-4,4-Dioxide (OTD) on Non-Neoplastic Cells and Primary and Metastatic Melanoma Cells *In vitro*

Thesis presented by
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for the degree of
Doctorate in Medicine (MD)

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26th September 2024

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Signed: .....

Stephanie O'Callaghan

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Poster Presentations

UCC Bright Futures, University College Cork, 2023

- O'Callaghan SK, Foley C, Redmond HP, The effect of a surgical adjuvant on human dermal fibroblast migration and inflammatory cytokine production (2023)

Thesis Abstract

Melanoma is an aggressive form of skin cancer, responsible for the largest proportion of skin cancer related deaths in Ireland (69.4%) despite only accounting for 9% of all skin cancers leading to the development of several adjuvant therapies aiming to reduce mortality. Tissue damage inflicted by surgical excision of tumours, can induce an exaggerated sterile proinflammatory immune response followed by immunosuppression, providing an optimal environment for residual tumour cells to differentiate, proliferate and propagate into surrounding and distant tissues resulting in cancer recurrence. Surgical adjuvants have the potential to induce cytotoxicity in residual tumour cells and reduce excessive inflammation, possibly preventing subsequent immunosuppression. The synthesised compound 1,4,5-oxathiazinane-4,4-dioxide (OTD) is a structural analogue of Taurultam derived from anti-inflammatory Taurolidine. OTD demonstrates anti-neoplastic actions against several cancers, however, its effects on inflammation and wound healing remain undetermined.

The overall aim of this study was to assess the potential of OTD as an adjuvant treatment following melanoma excision by investigating its effects on cells involved in wound healing (Human Dermal Fibroblast (HDF) cells), tumourigenesis (primary and metastatic melanoma cells) and the adaptive immune system (CD8+ T lymphocytes). The physiological and pathological functions of these cell types were assessed *in vitro* with five concentrations of OTD (0.25mM, 0.5mM, 0.75mM, 1mM and 1.25mM).

Given the critical role of wound healing in the post-operative period, this thesis initially assessed the effects of OTD on the wound healing capacity of HDF cells. At concentrations up to 0.75mM of OTD this study demonstrated that OTD, does not significantly impair HDF migratory function in a wound healing assay ($n=3$, $p>0.05$). Additionally, OTD effectively suppressed the release of pro-inflammatory cytokines, IL-6 and IL-8, in IL-1α stimulated HDF

cells at concentrations above 0.5mM (n=6, p>0.05). Moreover, OTD preserved HDF cell viability at all tested concentrations compared to untreated controls (n=6, p>0.05).

Further investigations aimed to determine the cytotoxic effect of OTD on primary and metastatic melanoma cells. Cytotoxicity was observed in primary (A375/WM3248) and metastatic (SK-MEL-2) cell lines at all OTD concentrations (n=6, p*<0.05). However, metastatic WM164 cells demonstrated resistance to OTD treatment with no observed cytotoxicity (n=6, p>0.05). Furthermore, cell death in primary (WM3248) and metastatic (SK-MEL-2) cell lines occurred via apoptosis (n=3, p*>0.05). Notably, instead of a dose-dependent effect, a phasic response was observed, with prominent cytotoxicity at concentrations of 0.5mM in WM3248 cells (p****<0.0001) and SK-MEL-2 cells (p** <0.01) followed by recovery of cell viability at higher concentrations. This response may be attributed to metabolic rewiring of melanoma cells in response to glycolytic inhibition, promoting tumour survival.

Immunosuppression post-operatively results in impairment of adaptive immune responses such as anti-tumoural CD8+ T lymphocyte function. Thus, the final aspect of this thesis explores the effect of OTD on CD8+ T lymphocyte viability and cytotoxic function measured by IFNγ release. Results indicated a negative effect on CD8+ T lymphocyte function with suppression of IFNγ release in cells stimulated with phorbol myristate acetate (PMA)/ionomycin or anti-CD3, at all concentrations of OTD, despite preservation of cell viability (n=3, p <0.05). Considering the infiltration of CD8+ T lymphocytes into the surgical site approximately one week postoperatively, our findings suggest that use of OTD might be best confined to the early postoperative period, although further studies *in vivo* are required to clarify this.

In conclusion, this thesis demonstrates that OTD is a potential surgical adjuvant in melanoma treatment. However, the findings also highlight important considerations for its clinical application including optimal timing and duration of therapy as well as potential combination

with alternative metabolic inhibitors to prevent resistance. Further studies are essential to fully characterise the effects of OTD and guide its potential clinical applications.

List of Abbreviations

7-AAD	7-Aminoactinomycin
ADC	Antibody-Drug Conjugates
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
BCG	Bacillus Calmette-Guerin
BFA	Brefeldin A
bsAB	Bispecific Antibody
CAR	Chimeric Antigen Receptor
CO_2	Carbon Dioxide
COX	Cyclooxygenase
CTLA-4	Cytotoxic T Lymphocyte Associated Protein 4
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cell
DFS	Disease Free Survival
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsufoxide
EC50	Effective Concentration midpoint
ECM	Extracellular Matrix
EMEM	Minimum Essential Eagles Medium
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum
FKBP51	FK506-binding protein 51
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HDF	Human Dermal Fibroblast
HER2	Human Epidermal Growth Factor Receptor 2
HIF-1α	Hypoxia-Inducible Factor-1α
HPA	Hypothalamic-Pituitary-Axis

IBTS	Irish Blood Transfusion Service
ICI	Immune Checkpoint Inhibitor
ICS	Intracellular cytokine staining
IFNγ	Interferon gamma
IL	Interleukin
ILC	Innate lymphoic cells
IV	Intravenous
mAB	Monoclonal Antibody
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
MDSC	Myeloid Derived Suppressor Cell
MFI	Median Fluorescent Intensity
MHC	Major Histocompatibility Complex
MIP-1α	Macrophage Inflammatory Protein-1α
MMP	Matrix Metalloproteinase
MRD	Minimal Residual Disease
MSD	MesoScale Discovery
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCRI	National Cancer Registry Ireland
NEDD8	Neuronal precursor cell-Expressed Developmentally Down-regulated protein 8
NET	Neutrophil Extracellular Trap
NF-κB	Nuclear Factor-κB
NK	Natural Killer
NLR	Neutrophil-Lymphocyte ratio
NO	Nitric Oxide
NSAID	Non-Steroidal Anti Inflammatory Drug
NSCLC	Non Small Cell Lung Cancer
OD	Optical Density

OS	Overall Survival
OTD	1,4,5-Oxathiazinane-4,4-dioxide
OXPHOS	Oxidative Phosphorylation
PAMP	Pathogen Associated Molecular Protein
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Solution
PD-1	Programmed cell death protein-1
PDL1	Programmed cell death ligand-1
PI	Propium Iodide
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear
PS	Phosphatidlserine
PSN	Penicillin/Streptomycin/Neomycin
RCF	Relative Centrifugal Force
RFS	Recurrence Free Survival
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
SCC	Squamous Cell Carcinoma
SEM	Standard Error of the Mean
SIRS	Systemic Inflammatory Response Syndrome
TGF-β	Transforming Growth Factor-β
Th	T helper
TLR	Toll-like receptor
TNFα	Tumour Necrosis Factor alpha

Chapter 1: Introduction

1.1 Surgery

The first known evidence of surgery in history is the leg amputation of a child in Borneo 31,000 years ago (Maloney *et al.*, 2022). Initial progress in the field was slow, with a high risk of morbidity and mortality. It was not until the 19th century that critical changes such as the introduction of anaesthesia in 1846 by William Morton (Andrew *et al.*, 2022) and the proposition of aseptic techniques by Joseph Lister in 1865 (Michaleas et al., 2022) improved the safety of surgery, shaping the field into what it is today, with over 300 million surgeries currently performed worldwide each year (Meara *et al.*, 2016).

Cancer surgery is often essential for a favourable patient outcome. It is estimated that approximately 58% of all patients with cancer require surgery (Perera *et al.*, 2021). However, despite high volumes of oncological surgery being performed, emerging evidence suggests a strong correlation between the initiation of surgery and the acceleration of tumour growth (Tohme et al., 2017b). Recurrence rates following curative resection are highly variable among cancer subtypes, yet they remain high with a rate of 6.8-28.8% in colorectal cancer (Nors *et al.*, 2024) and 25-30% in breast cancer (Courtney *et al.*, 2022).

1.2 Postoperative wound healing

Wound complications post oncological surgery pose a high risk of recurrence, these include infectious and non-infectious complications (Adwall *et al.*, 2021; Beecher *et al.*, 2018; Murthy *et al.*, 2007; O'Connor *et al.*, 2020; Pang *et al.*, 2021). There is a correlation between wound infection, chronic wounds, metastasis and poor prognosis compared with non-complicated wounds. This is demonstrated in colorectal (Aoyama *et al.*, 2017), gastric (Pang *et al.*, 2021),

breast (O'Connor *et al.*, 2020), oesophageal (Saunders *et al.*, 2020) and ovarian (Angeles *et al.*, 2022) cancers, among others.

Wound healing is a complex and dynamic process, involving several different cell types and mediators. In simple wounds, mechanical trauma initiates the wound healing cascade, consisting of four overlapping stages; haemostasis, inflammation, proliferation and remodeling (Schultz *et al.*, 2011).

Endothelial damage leads to platelet activation and results in the formation of a platelet plug. Platelets with the plug then release growth factors and cytokines which assist in the recruitment of inflammatory cells such as neutrophils, monocytes and lymphocytes (Sonmez and Sonmez, 2017). Neutrophils are typically the first inflammatory cell recruited during the inflammatory phase. Their antimicrobial functions include phagocytosis, degranulation and release of Neutrophil Extracellular Traps (NETs), which bind and remove pathogenic microbes (Rosales, 2018). Macrophages take over the primary phagocytosis function in the late inflammatory phase (Rodrigues *et al.*, 2019). Macrophage subtypes include M1, a more proinflammatory phenotype, or M2, a more anti-inflammatory phenotype (Krzyszczyk *et al.*, 2018).

Resolution of inflammation and initiation of proliferation are largely attributed to polarisation of macrophages to an M2 phenotype and are dependent on factors such as IL-4 and IL-13. This results in the secretion of anti-inflammatory cytokines such as IL-10 and upregulation of matrix metalloproteinases (MMPs) produced by fibroblasts in the proliferative phase of wound healing (Landén *et al.*, 2016). MMPs act to degrade the provisional matrix formed in the haemostatic phase while simultaneously depositing extracellular matrix (ECM) components, such as collagen, proteoglycans, hyaluronic acid, glycosaminoglycans and fibronectin (Landén *et al.*, 2016). Fibroblast differentiation to myofibroblasts produces contractile forces acting on the wound to assist in wound closure. This effect continues into the remodelling stage of wound

healing, increasing tensile strength by replacing collagen III with collagen I and reorganising newly synthesised tissue structures (Chitturi *et al.*, 2015).

Surgery however, can disrupt the natural progression of wound repair, leading to dysregulated wound healing and chronic wounds. This is often marked by an extended inflammatory phase, which delays the overall progression of wound repair (Wilkinson and Hardman, 2020). Perioperative factors such as the transfusion of blood products and administration of anaesthetic agents can enhance proinflammatory (McSorley *et al.*, 2020) and immunosuppressive (Kim, 2018) responses respectively. Furthermore, the degree of surgical invasiveness relates to the severity of inflammation and subsequent immunosuppression. Highly invasive open surgery, as opposed to minimally invasive laparoscopic surgery, results in greater immunosuppression, predisposing the patient to wound infection and in cases of cancer surgery, providing an optimal environment for tumour progression (Shi *et al.*, 2023).

1.2.1 Dermal Fibroblasts in Wound Healing

Dermal fibroblasts are often described as key mediators of the wound repair process. Their main function is in cell proliferation and wound closure, contributing to angiogenesis, granulation tissue formation and wound contraction. While described as the critical cells in the proliferative phase, fibroblasts are also capable of regulating the inflammatory response (Cialdai *et al.*, 2022).

During the inflammatory phase, dermal fibroblasts enhance the local immune response by reacting to damage associated molecular proteins (DAMPs) produced during tissue injury. They act to enhance the immune response via several mechanisms including the production of proinflammatory mediators such as tumour necrosis factor alpha (TNFα), interferon gamma (IFNγ), interleukin-6 (IL-6) and interleukin-12 (IL-12). Fibroblasts also secrete chemokines

such as interleukin-8 (IL-8), which recruit immune cells to the injury site and mobilise immune cells embedded within ECM to migrate to injured tissues (Correa-Gallegos *et al.*, 2021).

In addition to their pro-inflammatory function, fibroblasts also play a role in resolution of inflammation by upregulation of programmed cell death ligand-1 (PD-L1) which modulates macrophage polarisation and immunosuppression (X. Wang *et al.*, 2022). Thus, disruption of fibroblast function can lead to dysregulated wound healing with features including excessive inflammation, chronic wounds and increased morbidity.

1.3 Hallmarks of Cancer, Wound Healing and Surgery

Hanahan and Weinberg originally described the "Hallmarks of Cancer" in 2000 as six acquired capabilities common to most tumours. These included; sustained proliferative signaling, evasion of growth suppressors, resisting cell death, enabling replicative immortality, induction of angiogenesis and activating invasion and metastasis (Hanahan and Weinberg, 2000). In 2011 they revised their definition to include emerging hallmarks; deregulation of cellular energetics and avoidance of immune destruction as well as enabling characteristics such as genomic instability and tumour promoting inflammation (Hanahan and Weinberg, 2011).

More recently, Maccarthy-Morrogh and Martin (2020) described the similarities between the hallmarks of cancer and wound healing finding that each acquired capability and enabling characteristic showed relevance to both processes with the exception of two; the enabling of replicative immortality and genomic instability (***Figure 1.1)*** (MacCarthy-Morrogh and Martin, 2020). Similarities between wound healing and tumourigenesis are well described. In 1863, Virchow described the initiation of tumourigenesis at sites of inflammation, including chronic wounds (Balkwill and Mantovani, 2001). Dvorak expanded on this in 1986, defining tumours as wounds that do not heal (Dvorak, 1986). He revisited this definition in 2015, fortifying his

hypothesis with more recent advances in our understanding of the inflammatory response in tumourigenesis (Dvorak, 2015).

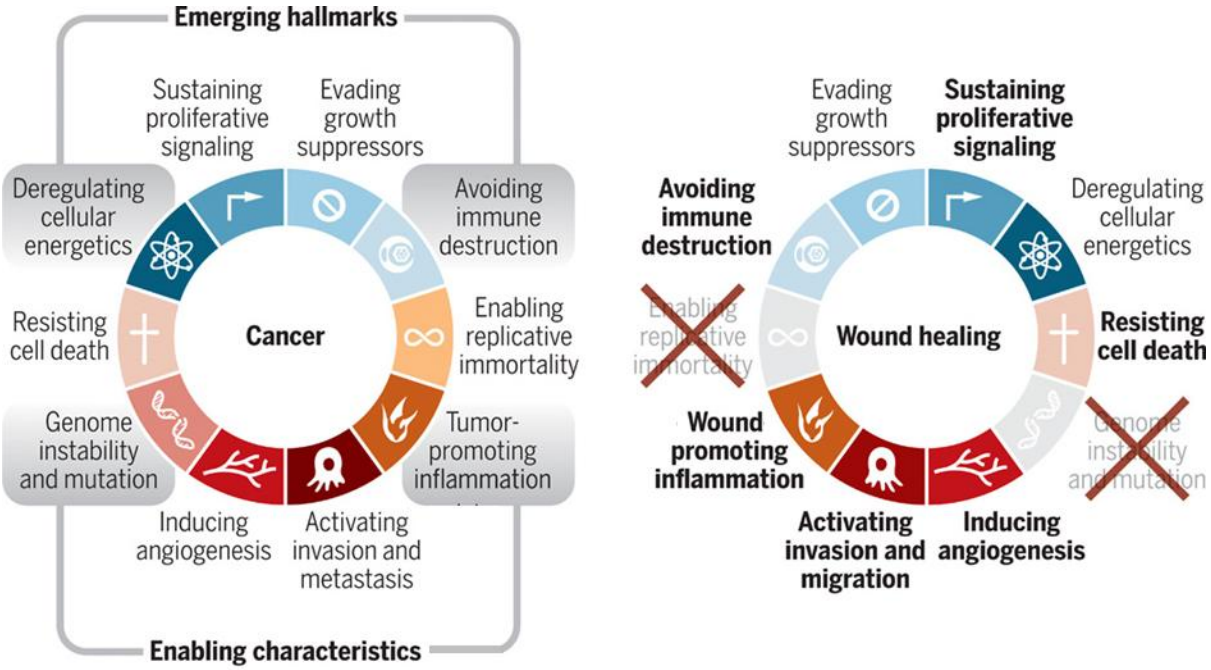

***Figure 1.1**: Hallmarks of Cancer and Wound Healing. Similarities between the hallmarks of cancer and the hallmarks of wound healing, Adapted from Maccarthy-Morrogh and Martin (2020)*

The cellular and molecular process of physiological tissue regeneration and tumour stroma generation are interlinked through physiological and pathological immune responses, cell proliferation, cell migration, tissue remodeling and cell death. Furthermore, aberrations in the wound healing process can lead to cancer metastasis and recurrence (Matsuo *et al.*, 2012; Murthy *et al.*, 2007; Walter *et al.*, 2011). The cellular and molecular parallels between cancer progression and dysfunctional wound healing involve sustained inflammation, excessive immunosuppression, sustained proliferative signaling, activation of invasion angiogenesis and evasion/resistance of cell death (MacCarthy-Morrogh and Martin, 2020).

1.4 Cancer Growth and Recurrence with and without Surgery

Cancer treatment involves a comprehensive, multimodal approach; combining first-line, adjuvant and neoadjuvant therapies such as surgery, chemotherapy and radiotherapy. More recently the armory of cancer treatments has evolved to include immunotherapy, stem cell therapy, targeted therapy and gene therapy (Debela *et al.*, 2021). Nonetheless, global figures estimate that approximately 58% of all patients with cancer require surgery at least once during the course of treatment (Perera *et al.*, 2021). In Ireland, approximately 43% of all invasive cancers in Ireland are treated surgically (NCRI, 2024).

1.4.1 Tumour Excision, Tissue Injury and Minimal Residual Disease (MRD)

Although a first-line treatment for many cancers, the downstream effects of surgical tissue trauma promote the differentiation and growth of cancer stem cells which remain in situ following curative treatment known as minimal residual disease (MRD). The affinity of neoplastic cells to sites of tissue injury is not a new concept, with Jones *et. al* reporting localisation of secondary tumours to sites of injury as early as 1914 (Jones and Rous, 1914). In fact, the biological mechanisms of several carcinogens, such as alcohol and smoking, attribute their effects to the causation of tissue injury. This is largely due to the production of oxidative stress through reactive oxygen species (ROS) and subsequent stimulation of inflammatory responses (Caliri *et al.*, 2021; Rumgay *et al.*, 2021).

In addition to the molecular mechanisms by which surgically induced metastasis occurs, it must also be recognised that dissemination of tumour cells may occur on a gross level due to tumour or lymph node manipulation, injection of analgesic agents or contamination of instruments including port sites (Coffey *et al.*, 2003). *In vivo* models have revealed the potential impact of surgical procedures and tissue injury on the rate of tumour growth, which may have implications for tumour biopsies, incomplete resections or the presence of MRD. In a murine model, a wound adjacent to the site of injected breast cancer cells resulted in accelerated tumour

growth in wounded compared to unwounded mice (Stuelten *et al.*, 2008). Likewise, in a Zebrafish model, repeated wounding promoted differentiation of preneoplastic cells into differentiated melanoma cells (Antonio *et al.*, 2015).

As reviewed by Coffey *et al.* (Coffey *et al.*, 2003) and more recently by Onuma *et al.* (Onuma *et al.*, 2020), the evidence in humans suggests that tumour excision may accelerate growth of MRD, resulting in perioperative tumour growth. In effect, the act of removing the tumour results in subsequent tumour growth.

1.4.2 Rate and Pattern of Cancer Recurrence with and without Surgery

Tumour dormancy and subsequent recurrence and/or metastasis represent a significant challenge in the treatment of all cancers. Metastasis can be considered in terms of the metastatic cascade, whereby cells adapt to their environments, invading both local structures and disseminating to select tissues through local invasion, intravasation, extravasation and colonisation (Boire *et al.*, 2019).

Cancer recurrence can be classified into three categories: local recurrence (occurring at the site of the original tumour), regional recurrence (affecting nearby lymph nodes or surrounding tissues), and distant metastasis (spreading to organs or tissues far from the initial tumour site). Local recurrence is often prevalent in cancers where achieving clear surgical margins is challenging. This is particularly evident in head and neck cancer (Sunkara *et al.*, 2023) and rectal cancer (Glynne-Jones *et al.*, 2017), where the complex anatomy makes complete tumour excision difficult, increasing the likelihood of residual cancer cells and subsequent local or locoregional recurrence. Distant metastasis occurs via two models, a linear model, whereby metastasis occurs late in the progression of disease or a parallel model, in which cancer cells disseminate at an earlier stage (Shi *et al.*, 2024). Sites of distant metastases vary among cancers, with several preferentially spreading to specific tissues. Breast metastases, for example, occur

in bone and lung tissue predominantly (Patanaphan *et al.*, 1988), whereas oesophageal cancer typically metastasises to liver, distant lymph nodes and lung tissue (Verstegen *et al.*, 2020). Sites may also vary with cancer subtype, with molecular subtypes predicting preferential sites of metastasis in breast cancer, such as hormone-receptor positive subtypes to bone and Human Epidermal Growth Factor Receptor 2 (HER2) positive subtypes to brain tissue (Gong *et al.*, 2017).

Treatment modality can also play a significant role in patterns of recurrence. Krall *et al.* describe the effect of the systemic response to surgery in promoting distant metastases in breast cancer mouse models whose tumour outgrowth was otherwise restricted by tumour-specific T cell responses (Krall *et al.*, 2018). In humans, Berg *et al.* compare surgical intervention with radiotherapy in non-small cell lung cancer (NSCLC), finding an increase in extrapulmonary metastasis in the surgical group compared with locoregional recurrence in the radiotherapy group (van den Berg *et al.*, 2015). Contrastingly, wound complications demonstrate a higher risk of local recurrence in soft tissue sarcoma in lieu of distant metastases (Dadras *et al.*, 2020).

Clinical studies comparing surgical interventions to non-surgical treatments are limited due to ethical implications of denying patients surgical treatment, however, the use of adjuvant techniques to enhance surgical outcomes is widely studied. Novel systemic therapies can fortify anti-cancer treatment regimens with neoadjuvant chemoradiotherapy followed by surgery, demonstrating a significant reduction in both distant and locoregional recurrences compared with surgery alone in oesophageal cancer (Liu *et al.*, 2020). In some cases, chemotherapy alone can produce subclinical responses, showing a higher risk of mortality in elderly colorectal cancer patients compared with surgery (Mehta *et al.*, 2017).

1.5 Surgery-Induced Proinflammatory Immune Response and Cancer Recurrence

Tissue damage inflicted by surgical excision of tumours, induces a sterile proinflammatory immune response, followed by immunosuppression and resolution of inflammation at the excision site and systemically (Margraf *et al.*, 2020; Tang *et al.*, 2020). The cellular and molecular mechanisms underpinning the transition from a proinflammatory to immunosuppressive state at the site of tumour excision are intended to promote tissue repair and wound healing. However, residual tumour cells and cancer stem cells hijack this physiological process to differentiate, proliferate, evade the immune response and propagate into surrounding and distant tissues, resulting in cancer recurrence (Hua and Bergers, 2019).

1.5.1 Mediators and Effector Cells of Postoperative Sterile Inflammation

1.5.1.1 Cytokines

Following haemostasis, injured cells release Damage Associated Molecular Patterns (DAMPs), hydrogen peroxide, lipid mediators and cytokines which assist in the recruitment of inflammatory cells, for example, neutrophils (Rodrigues *et al.*, 2019) DAMPs include reactive oxygen species (ROSs), mitochondrial DNA and heat shock proteins released due to cell damage. Together with Pathogen Associated Molecular Patterns (PAMPs), they play an important role in activating the inflammatory response. This effect is mediated by binding of DAMPs/PAMPs to pattern recognition receptors such as Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors and RIG-I-like receptors (Alazawi *et al.*, 2016).

Activation of these receptors initiates an inflammatory cascade via several pathways, including the nuclear factor-κB (NF-κB) pathway and mitogen-activated protein kinase (MAPK) pathway (Oeckinghaus *et al.*, 2011) ultimately resulting in the release of proinflammatory cytokines and mediators, several of which are summarised in ***Table 1.1***. Cytokine release is

also involved in tumourigenic pathways, with the release of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α stimulating the adhesion of circulating cancer cells.

Cytokine	**Principle Cellular Sources**	**Function**	**References**
Proinflammatory			
TNFα	Macrophages, natural killer (NK) cells, CD4+ Lymphocytes	Activation of NF-κB pathway	(Zelová and Hošek, 2013)
IL-1 (α and β)	Macrophages, monocytes	Activation of NF-κB pathway and upregulation of IL-6 and IL-8	(Mantovani *et al.*, 2019)
IL-6	Macrophages, T cells, endothelial cells, fibroblasts	Production of acute phase proteins	(Choy and Rose-John, 2017)
IL-8	Macrophages, epithelial cells, endothelial cells	Neutrophil chemoattractant	(Bernhard *et al.*, 2021, p. 8)
Macrophage inflammatory protein-1α (MIP-1α)	Macrophages, neutrophils, lymphocytes, fibroblasts	Chemoattractant for CD8+ T lymphocytes, macrophages and polymorphonuclear (PMN) cells	(Bhavsar *et al.*, 2015)

Anti-inflammatory			
IL-10	Monocytes, T cells, B cells	Inhibits production of pro-inflammatory cytokines/chemokines in monocytes, macrophages and dendritic cells. Suppression of antigen presentation in dendritic cells and antigen presenting cells via TLR/IFNγ interference	(Mittal and Roche, 2015, Ouyang and O'Garra, 2019)
Transforming Growth Factor (TGF-β)	T cells, monocytes	Suppression of inflammatory cytokine release from T cells, dendritic cells and fibroblasts. Polarization of macrophages from M1 to M2 phenotype	(Batlle and Massagué, 2019)

Table 1: *Summary of cytokines and their functions*

1.5.1.2 Neutrophils

Neutrophils are typically recruited to wounds within 4 hours of tissue trauma, reaching a maximum level between days 1 and 2 post-wounding, decreasing at day 5 (Kim *et al.*, 2008). Their antimicrobial functions include phagocytosis, degranulation and release of Neutrophil Extracellular Traps (NETs) (Rosales, 2018). NETs were first described by Brinkmann *et al.* as "structures composed of granule and nuclear constituents that disarm and kill bacteria extracellularly" (Brinkmann *et al.*, 2004). Neutrophils also demonstrate an immunoregulatory function through production of cytokines and inflammatory factors in response to stimulatory or inhibitory signals (Scapini and Cassatella, 2014). They contribute to the recruitment,

activation and programming of antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages, Langerhans cells and B cells, which act to process and present antigens to lymphocytes such as T cells (Nathan, 2006).

In addition to their physiological role in wound healing, neutrophils are well documented facilitators of tumour progression (Hedrick and Malanchi, 2022; Nolan and Malanchi, 2021; Wu *et al.*, 2020). Demonstrated in zebrafish models, tissue damage from melanoma biopsy revealed a persistent elevation in local neutrophil population in wounded tumour tissue compared with healthy wounds. This neutrophil influx was also associated with recruitment of pre-neoplastic cells, cell proliferation and poor prognostic outcome (Antonio *et al.*, 2015). In addition, recent reviews suggest that NETs could be a potential target for cancer therapy (Y. Chen *et al.*, 2022; Yang *et al.*, 2020; Zhao and Jin, 2022) as well as a predictor of cancer recurrence (Martinez-Cannon *et al.*, 2023). Moussett *et al.* suggest that targeting the IL-1β-NET-TGF-β axis involved in NET formation could reduce chemoresistance in breast cancer lung metastases using *in vivo* mouse models (Mousset *et al.*, 2023).

1.5.1.3 Macrophages

Macrophages primarily function in the late inflammatory phase, with levels in wounded tissue peaking at day 3 (Rodrigues *et al.*, 2019). They typically demonstrate pro-inflammatory behaviour early in the inflammatory process (M1 phenotype) and anti-inflammatory behaviour in later stages (M2 phenotype), coinciding with the suppression of the immune response and resolution of inflammation (Galli *et al.*, 2011).

Tissue-infiltrating macrophages refer to macrophages derived from infiltrating monocytes in response to different stimuli, such as cytokines or growth factors (Chaintreuil *et al.*, 2023). While tissue-resident macrophages are the first responders to injury, wound-macrophages are predominantly monocyte-derived, typically disappearing upon resolution of the inflammatory

phase (Brancato and Albina, 2011). In cases of prolonged inflammation, such as following an invasive surgery, monocyte-derived macrophages persist (Krzyszczyk *et al.*, 2018), providing a pro-inflammatory environment conducive of tumour growth. Loyher *et al.* also demonstrates that the presence of monocyte-derived macrophages correlates with tumour dissemination (Loyher *et al.*, 2018) fortifying a pro-metastatic environment.

Macrophages are crucial in the resolution of inflammation and progression to proliferative phases of wound healing, marked by expression of matrix metalloproteinases (MMPs). However, polarisation to an M2 phenotype can lead to overexpression of MMPs, promoting tumour growth and metastasis (Reunanen and Kähäri, 2013). Immunosuppressive mediators such as myeloid derived suppressor cells (MSDCs), M2 macrophages and regulatory T (Treg) cells are upregulated in inflammation resolution postoperatively and can be hijacked by tumour cells to enhance tumourigenesis.

1.5.2 Mediators and Effector Cells of Postoperative Immunosuppression

1.5.2.1 Myeloid Derived Suppressor Cells

Myeloid derived suppressor cells (MDSCs) are a subset of myeloid cells capable of suppressing the immune response inducing T lymphocyte suppression via programmed cell death protein-1 (PD-1) and cytotoxic T Lymphocyte associated protein (CTLA) engagement (Talmadge and Gabrilovich, 2013). In conjunction with T cell interaction, they also act to increase anti-inflammatory cytokine production, such as transforming growth factor beta (TGF-β) and IL-10 (Putra *et al.*, 2018). However, overstimulation of anti-inflammatory pathways can facilitate immune evasion in cancer cells with MDSC stimulation, promoting cancer growth in several cancers including breast (Zhang *et al.*, 2023) melanoma (Tengesdal *et al.*, 2021) and colorectal (H. Chen *et al.*, 2022) cancers.

1.5.2.2 Natural Killer Cells

Natural killer (NK) cells belong to a cytotoxic group on innate lymphoid cells (ILCs). NK cells are activated by stress signalling cytokines, in turn producing effector cytokines such as perforin, granzyme-B and IFN-γ which activate direct antitumoural responses of the adaptive immune system (Wolf *et al.*, 2023). IFN-γ promotes activation of dendritic cells (DCs), macrophages and T cells, however NK cells may also be influenced by inhibitory signalling, exhibiting cytotoxic effects against immature DCs, activated macrophages and activated T cells (Vivier *et al.*, 2008). Thus playing an integral role in regulating the immune response.

The compensatory immunosuppressive response to surgical inflammation leads to suppression of NK cell activity by increased activity of Tregs and MDSCs. This allows for both direct and indirect inhibition, facilitating immune evasion (Market *et al.*, 2021).

1.5.2.3 Tissue Infiltrating Adaptive Lymphocytes

Adaptive immune cells can be broadly classified into B and T lymphocytes, with both playing a role in the sterile inflammatory response to surgery. T lymphocytes can be subdivided into CD4+ (helper) and CD8+ (cytotoxic) T lymphocytes, which express both inflammatory and regulatory cytokines. Although studies on T lymphocyte levels in human wounds are limited, peak tissue levels of CD4+ and CD8+ T lymphocytes in mice are observed at days 5-10 and 7-10 respectively (Chen *et al.*, 2014). One human study conducted by Martin *et al.* demonstrated that T lymphocytes are typically peak in wounds between days 8 and 14 post-operatively, as indicated by the use of pan-T cell markers (Martin and Muir, 1990).

1.5.2.3.1 CD4+ T Lymphocytes

CD4+ Lymphocytes are often referred to as “helper” T lymphocytes consist of several distinct subsets, mainly functioning to promote B cell maturation to plasma cells or memory B

lymphocytes, activation of macrophages and activation of cytotoxic CD8+ lymphocytes (Luckheeram *et al.*, 2012).

Tregs are also a subset of CD4+ T lymphocytes which enhance immunosuppression by suppressing CD4 effector T cell function (So *et al.*, 2023) as well as cytotoxic CD8+ T cell function (Liu *et al.*, 2015), with levels of Tregs correlating to degree of surgical stress (Saito *et al.*, 2013). Increased levels have also shown an association with poor outcomes cancers, such as prostate (Cheng *et al.*, 2023) breast (Verma *et al.*, 2013) and colon with Jiang *et al.* demonstrating that regulating Treg function via neuronal precursor cell-expressed developmentally down-regulated protein 8 (NEDD8) targeting postoperatively can prevent metastasis in colorectal cancer patients (Jiang *et al.*, 2024). However the absence of Tregs can also be detrimental to wound healing, causing alterations in the cytokine microenvironment and dysregulated healing (Knoedler *et al.*, 2023).

1.5.2.3.2 CD8+ T Lymphocytes

Cytotoxic CD8+ T lymphocytes are considered to be the most prolific effector cells in anti-tumoural responses. They function as the adaptive counterparts of innate NK cells, exhibiting direct cytotoxicity through production of several effectors cytokines including IFN-γ, TNF-α, perforin and granzyme upon stimulation (Koh *et al.*, 2023).

Following surgery, levels of CD8+ T lymphocytes are reduced in response to immunosuppressive stimuli. Immune checkpoint expression is also increased of CD8+ T lymphocytes, enhancing immune evasion (Xu *et al.*, 2015). In addition, anaesthetic agents such as propofol and sevofluorane are capable of enhancing immunosuppression by decreasing CD8+ T lymphocyte levels and increasing Treg levels, respectively (Yamaguchi *et al.*, 2021). This ultimately provides an optimal environment for tumour recurrence, with Anath *et al.*

demonstrating that CD8+ T lymphocyte reduction following surgery predisposes to cancer recurrence in mice (Ananth *et al.*, 2016).

1.6 Surgical Adjuvant and Neoadjuvant Therapies

Neo-adjuvant and adjuvant therapy refer to therapies administered before and after primary cancer treatment, respectively. Typical treatments include chemotherapy, hormone therapy, molecular targeted therapy and radiation therapy (West and Jin, 2015). Hanahan and Weinburg, in their 2011 revision of the Hallmarks of Cancer, describe several adjuvant therapies used to target the Hallmarks of Cancer ***(Figure 1.2)*** (Hanahan and Weinberg, 2011)

Surgical adjuvants represent a promising area of research in combating excessive post-surgical inflammation (Redmond *et al.*, 2018) and thus immunosuppression. However, the timing of adjuvant therapy is of particular importance to prevent unintended negative effects (Cullinane *et al.*, 2021; Petrelli *et al.*, 2019).

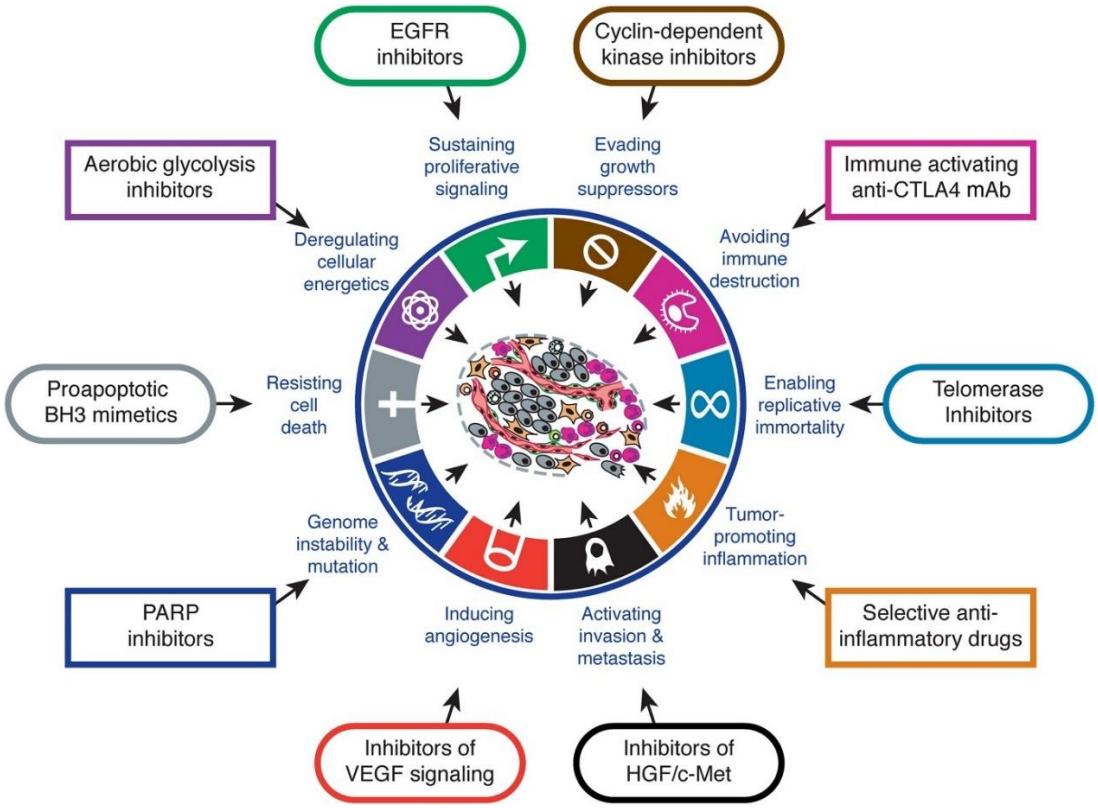

Figure 1.2: *Adjuvant therapies aimed at targeting the hallmarks of cancer, Hanahan and Weinburg (2011).*

1.6.1 Perioperative Immunotherapy

Perioperative immunotherapy has become the standard of care for several cancers, aiming to provide an optimal immune balance for long-term survival. Classifications of immunotherapeutic agents in use or in development include immunomodulators, targeted antibodies, adoptive cell therapy, cancer vaccines and oncolytic viruses (Ackerman *et al.*, 2022).

Immunomodulators include immune checkpoint inhibitors (ICIs), immunostimulatory cytokines and TLR agonists, among others. ICIs target immune checkpoints such as PD-1, PDL-1 and CTLA-4 to reduce immune evasion and thus surgically induced immunosuppression. PD-1 and CTLA-4 blockade using nivolumab or ipilimumab has been shown to reduce surgery-mediated immunosuppression while enhancing T helper cell type 1

(Th1) immunity in oesophageal cancer (Davern *et al.*, 2023). Immunostimulatory cytokines such as IL-2, granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α and IFN-α have been shown to reduce cancer recurrence and metastasis, in part, due to their role in regulating NK cell dysfunction postoperatively (Market *et al.*, 2018). Likewise, TLR agonists such as Bacillus Calmette-Guerin (BCG) therapy in bladder cancer induce apoptosis in tumour cells through activation of TLR7 (Yu *et al.*, 2015).

Targeted antibodies include unconjugated monoclonal antibodies (mABs), antibody-drug conjugates (ADCs) and bispecific antibodies (bsABs). mABs targeting host-tumour immunity, such as anti-CTLA-4 (ipilmumab) and anti-CD20 (rituximab), are currently used in metastatic melanoma and B-cell lymphoma, respectively to combat the immunosuppressive effect of surgery (Diakos *et al.*, 2014). ADCs, composed of a mAB attached to a cytotoxic drug, utilise the antigen specificity of mABs to deliver a direct cytotoxic effect. Their clinical use includes targeting of HER2 in breast cancer (Najminejad *et al.*, 2023) and nectin-4 in bladder cancer (Wong and Rosenberg, 2021). Finally, bsABs target two specific antigens in order to overcome emerging resistance to mABs. While their application began in the treatment of haematological malignancies, several trials have shown promise in the treatment of solid cancers such as gastric, breast and melanoma (Wang *et al.*, 2021).

Adoptive cell therapy involves the engineering of immune effector cells to recognise and eliminate cancer cells and includes chimeric antigen receptor (CAR) T cell therapy. CAR T-cell therapy consists of both CD4+ and CD8+ T cells engineered to function independently of major histocompatibility complex (MHC) activation. Together with PDL1 antibodies, they have been shown in mouse models to eradicate residual tumour cells following melanoma resection, therefore preventing tumour recurrence (Hanahan and Weinberg, 2011; Hu *et al.*, 2021).

1.6.2 Anti-Inflammatory Agents

Corticosteroids are routinely used in the perioperative period, mitigating the proinflammatory effects of surgical stress via the hypothalamic-pituitary-adrenal (HPA) axis, with dosing regimens correlating to the degree of surgical stress (Seo, 2021). The HPA axis relies on feedback interaction between the adrenal, hypothalamus and pituitary glands in response to sympathetic innervation from surgery, resulting in increased cortisol levels systemically. Physiologically, it functions to reduce systemic inflammation following the peak in local inflammation following surgery (Miller *et al.*, 2017). However, in pathophysiological scenarios such as non-resolving postoperative inflammation, the anti-inflammatory response is fortified with corticosteroid supplementation (Bain *et al.*, 2023).

Several non-specific anti-inflammatory drugs demonstrate potential in the prevention and therapy of cancer, including aspirin, celecoxib, dexamethasone, ibuprofen and piroxicam (Zappavigna *et al.*, 2020). Furthermore, targeted anti-inflammatory agents such as IL-1 antagonists, TNF blockers, anti-IL-6 agents and TGF-β inhibitors show great promise in reducing postoperative inflammation in cancer patients (Hou *et al.*, 2021).

Many natural phytochemicals also exhibit anti-cancer effects, including curcumin, which interferes with NF-κB to reduce inflammation and induce apoptosis in cancer cells without negatively impacting healthy cells (Wang *et al.*, 2012). Similarly, gingerol, derived from ginger, demonstrates anti-inflammatory properties in addition to antibacterial and antifungal properties, with several studies showing its therapeutic effects in cervical cancer (Zivarpour *et al.*, 2021).

Conversely, anti-inflammatory therapies may also negatively impact patient outcomes. Corticosteroids increase the risk of bleeding (Gallagher *et al.*, 2012), graft dehiscence (Jeong

et al., 2019) and infection secondary to immunosuppression (Youssef *et al.*, 2016). Long term pre-operative administration of corticosteroids increased the incidence of wound complications (Wang *et al.*, 2013) and suppression of the HPA axis via negative feedback mechanisms (Chen Cardenas *et al.*, 2022). Likewise, non-steroidal anti-inflammatory drugs (NSAIDs) are known to increase the risk of thrombotic events (Schjerning *et al.*, 2020), impaired bone healing (Al Farii *et al.*, 2021) and potentially increase the risk of postoperative bleeding, though evidence is conflicting in this regard (Bongiovanni *et al.*, 2021; Forsyth *et al.*, 2018).

1.6.3 Taurolidine and 1,4,5-Oxathiazinane-4,4-dioxide (OTD)

1.6.3.1 Taurolidine

In addition to anti-inflammatory properties, some drug therapies demonstrate concurrent anti-neoplastic properties, such as Taurolidine. Clinically, Taurolidine is used for catheter-related bloodstream infections and peritonitis (Alvarez Nadal et al., 2020; Savarese et al., 2024). Beyond its antimicrobial properties, Taurolidine is capable of reducing pro-inflammatory mediators such as IL-6 (Redmond *et al.*, 2018), IL-1 and TNF-α (Bedrosian *et al.*, 1991), suggesting an important role in modulating the immune response.

Preclinical studies furtherdemonstrate antineoplastic activity in several tumour types, including colorectal (Schubert *et al.*, 2019), melanoma (Sun *et al.*, 2007), pancreatic (Buchholz *et al.*, 2017) and liver cancer (Li *et al.*, 2018). However, its clinical application is limited by a short half-life of approximately 30 minutes (Gong et al., 2007), which restricts sustained therapeutic use.

1.6.3.2 1,4,5-Oxathiazinine-4,4-dioxide (OTD)

1,4,5-Oxathiazinane-4,4-dioxide (OTD), also known as substance GP-2250, is a structural analogue of Taurultam, the main derivative of Taurolidine. OTD has emerged as a promising anti-tumourigenic agent with a longer half-life (approximately 13.5 hours) than Taurolidine, allowing for potentially sustained systemic exposure and improved therapeutic efficacy (Buchholz et al., 2017). Preclinical studies demonstrate its antineoplastic effects in pancreatic

cancer (Buchholz *et al.*, 2017), melanoma (Gambichler *et al.*, 2023), peritoneal mesothelioma (Baron *et al.*, 2022) and breast cancer (Jinih *et al.*, 2021) while a clinical trial is currently underway evaluating the effect of OTD given in conjunction with Gemcitabine in patients with pancreatic cancer (Kasi and Iglesias, 2022).

1.6.3.2.1 Basic Structure and Pharmacological Properties

OTD is a small molecule oxathiazinane derivative, belonging to a heterogeneous class of compounds characterised by an aliphatic cyclic six-membered ring structure containing one oxygen, nitrogen and sulphur atoms. Structural-activity relationship studies carried out by Majchrzak-Stiller et al. demonstrated that methylation of the aliphatic six-membered ring structure at positions 5 and/or 6 resulted in the complete loss of anti-neoplastic and anti-bacterial activities (Majchrzak-Stiller et al., 2023).

The anti-neoplastic effects of OTD are largely dose dependent, demonstrating reduced tumour cell viability, migration and proliferation at higher doses (Buchholz et al., 2017; Jinih et al., 2021). Interestingly, this relationship is not universally linear across cell lines, with Baron *et al.* demonstrating improved cytotoxic function of OTD in malignant mesothelioma cells at lower concentrations followed by treatment resistance at higher concentrations (Baron et al., 2022) suggesting cell-line dependent cellular adaptation or resistance mechanisms.

Pharmacokinetic studies in preclinical models indicate that OTD possesses a prolonged half-life of approximately 13.8 hours (Buchholz et al., 2017) compared to Taurolidine (approximately 30 minutes) (Gong et al., 2007), allowing for more sustained systemic exposure. This extended half-life supported less frequent dosing and potentially improved therapeutic efficacy in vivo. Early toxicological studies using in vivo mouse models have reported good tolerability and low systemic toxicity to OTD (Buchholz et al., 2017). In addition to its antineoplastic potential, OTD may exhibit anti-inflammatory properties, though these effects have not been fully elucidated.

1.6.3.2.2 Proposed Mechanism of Action

The exact molecular mechanism of OTD remains undefined, however, Majchrzak-Stiller *et al.* propose that cytotoxic effects on pancreatic cancer cells are mediated by suppression of aerobic glycolysis via glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibition and downregulation of NF-kB transcriptional activity (Majchrzak-Stiller *et al.*, 2023).

Glycolytic inhibition results in the inability of products of glycolysis to enter mitochondria, resulting in mitochondrial dysfunction, oxidative stress and programmed cell death (Lazarev et al., 2020). Tumour cells predominantly rely on aerobic glycolysis, rather than more efficient metabolic pathways such as oxidative phosphorylation, due to their ability to rapidly generate energy to meet the heightened demands of tumourigenesis (D. Zhou et al., 2022). This reliance makes aerobic glycolysis a prime target for anti-cancer therapies.

The NF-κB pathway plays a critical role in regulating the inflammatory response, with dysregulation leading to chronic inflammation, oncogenesis and autoimmune disease. In cancer, this pathway is often hijacked by tumour cells to promote proliferation, survival, angiogenesis and invasive potential (Yu et al., 2020). In addition, the NF-κB pathway has emerged as a significant contributor to the development of drug resistance against chemotherapeutic agents, targeted therapies and immunotherapies (Li et al., 2024). By downregulating NF-κB transcriptional activity, OTD has the potential to not only reduce inflammation but also to overcome drug resistance when used in combination with other adjuvant agents.

1.6.3.2.3 Rationale for Choosing OTD over Other Agents

OTD was selected for investigation in this thesis over other anti-inflammatory and anti-neoplastic agents, including Taurolidine, due to several key advantages:

- **Improved Pharmacokinetics**

OTD demonstrates a significantly longer half-life (~13.8h) compared with Taurolidine (~30 minutes), allowing for more sustained systemic exposure and potentially more consistent therapeutic effects (Buchholz et al., 2017; Gong et al., 2007)

- **Cytotoxic Efficacy:**

  Preclinical studies show OTD induces cytotoxicity across multiple cancer types, including pancreatic, melanoma, breast and mesothelioma models (Baron et al., 2022; Buchholz et al., 2017; Gambichler et al., 2023; Jinih et al., 2021).

- **Mechanistic Novelty**

  Unlike conventional anti-inflammatory agents that target cytokine signalling, OTD acts through GAPDH inhibition and NF-κB modulation (Majchrzak-Stiller et al., 2023), thereby disrupting tumour metabolism while potentially attenuating inflammation and resistance pathways.

- **Favourable Toxicity Profile**

  Taurolidine is known to cause local irritation and discomfort at the site of administration, particularly with intraperitoneal or intravenous use. Kichko et al. compared the irritant potential of Taurolidine, Taurultam and OTD by examining the release of calcitonin gene-related peptide (CGRP), a key proinflammatory neuropeptide, in murine models. Their findings demonstrated that OTD induced a measurable CGRP release only at a high concentration of 10mmol/L, the response magnitude of which was approximately 50% and 25% of that elicited by Taurultam and Taurolidine, respectively (Kichko et al., 2016). These results indicate that OTD possesses a markedly lower irritant potential compared with its structural analogues.

  Complementary preclinical toxicology studies using in vivo murine models further support OTD's favourable safety profile, reporting good tolerability and low systemic

toxicity (Buchholz et al., 2017). Collectively, these findings underscore OTD's potential as a safer therapeutic alternative to Taurolidine.

- **Flexible Route of Administration**

  In contrast to Taurolidine, which typically requires systemic or intraperitoneal delivery, OTD has been formulated for both oral and topical delivery (Pfirrmann et al., 2024), expanding its potential for clinical application. The topical route, in particular, enables use in cutaneous, post-surgical or localised tumour settings, potentially reducing systemic exposure while maintaining local therapeutic activity. This route of administration also facilitates translational relevance to wound healing models, where topical exposure may better simulate real-world clinical use.

- **Clinical Translation Potential**

  The ongoing phase I/II clinical trial evaluating OTD in combination with gemcitabine in pancreatic cancer (Kasi and Iglesias, 2022) underscores its translational viability and relevance as a candidate for combination therapy.

1.6.4 Therapeutic window of adjuvant therapy

Optimal timing of neoadjuvant/adjuvant therapy in surgery is highly variable and largely dependent on the intended aims of the therapy. Neoadjuvant therapy is typically administered in order to reduce tumour volume, often enabling a less invasive surgical approach. Adjuvant therapy in contrast, is administered following surgical resection, targeting residual neoplastic cells and is often initiated 4-8 weeks after surgery to allow time for optimal wound healing (Biagi *et al.*, 2011). Pembrolizumab for example, is administered in advanced melanoma following resection, reducing immune evasion of residual melanoma cells via PD-1 inhibition, allowing the immune system to eliminate tumour cells and reduce recurrence (Rizzetto *et al.*, 2023). A rarer subset of adjuvant therapy is intraoperative therapy given during the surgery itself, with Layer *et al.* demonstrating that administration of intraoperative radiation and

immunotherapy in surgical resection of brain metastasis can lead to avoidance of delayed adjuvant therapy without impacting outcomes (Layer *et al.*, 2024).

Anti-inflammatory therapy is intended to reduce excessive inflammation and prevent compensatory immunosuppression, however, a degree of inflammation is required to ensure adequate antimicrobial defence and wound healing. Tang *et al.* describe an immunological "window of opportunity" for clinical decision-making in the 6-month postoperative period, describing the cellular immune response to surgery (***Figure 1.3***) (Cheng *et al.*, 2022; Tang *et al.*, 2020). Due to the rapid initiation of inflammation postoperatively, anti-inflammatory therapies should likely be administered in the early postoperative period to reduce the production of proinflammatory mediators such as IL-6 and IL-8 and prevent impairment of cellular immunity characterised by lymphocyte and NK cell depletion. However, careful consideration should be given to preservation of physiological processes, including wound healing and potential adverse effects such as infection (Forsyth *et al.*, 2018) and bleeding (Gallagher *et al.*, 2012).

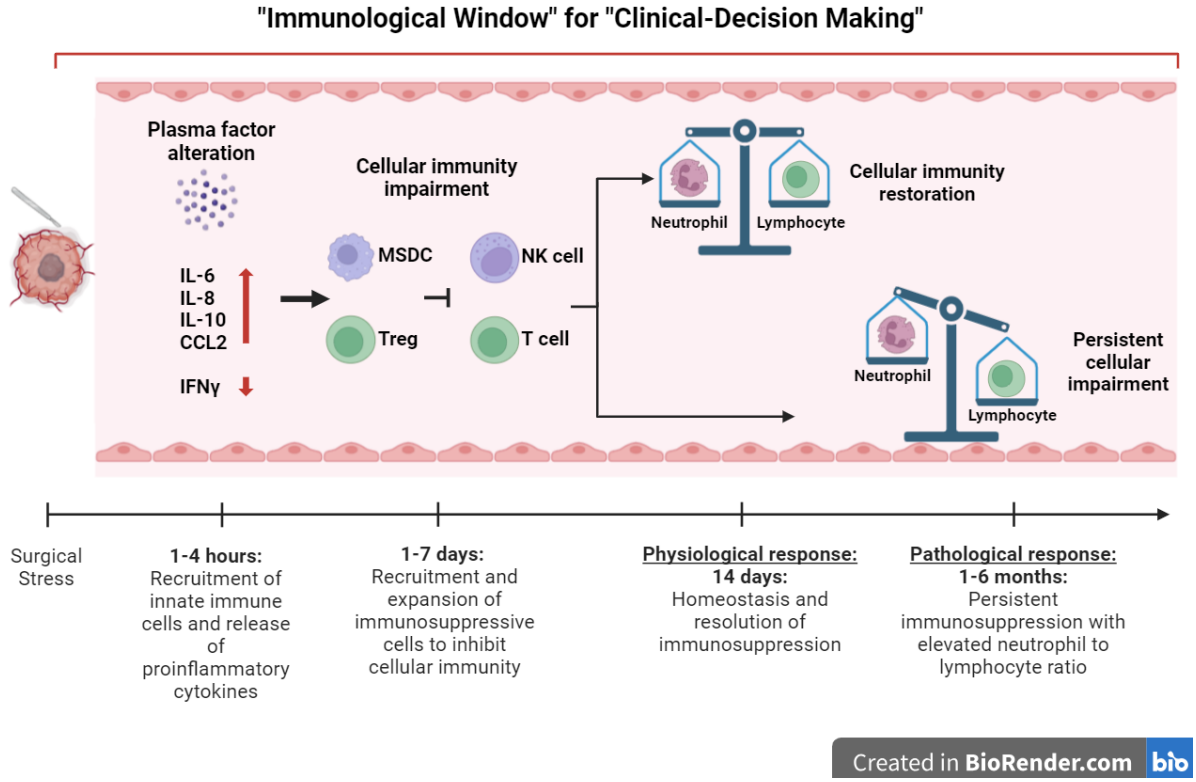

Figure 1.3: *Immunological window for clinical decision making, adapted from Tang et al. (2020) & Cheng et al. (2022). Surgical stress causes an initial proinflammatory immune response marked by recruitment of innate immune cells and release of pro-inflammatory cytokines within 1-4 hours. Pro-inflammatory cytokines stimulate the recruitment and expansion of immunosuppressive cells, causing impairment of cellular immunity within 1-7 days. In physiological scenarios, cellular immunity is restored within 14 days, however, in pathological scenarios, impairment may continue for up to 6 months.*

1.7 Melanoma

Cutaneous melanoma is an aggressive form of skin cancer, accounting for the majority of skin cancer-related deaths at 62.9% in Ireland, despite representing only 11% of all skin cancer cases (NCRI, 2025). The National Cancer Registry of Ireland (NCRI) estimates that by the year 2045, cases will reach over 1500 per year, a rise of 55% on figures from 2021 (NCRI, 2019). Irish mortality rates are significantly higher than the global average, with figures estimating that melanoma is responsible for 45.8% of skin cancer-related deaths worldwide (Global Cancer Observatory, 2025). This can be attributed to several factors, including fair-skinned population and suboptimal protection against ultraviolet (UV) radiation (Gibson *et al.*, 1997; WCRF, 2023).

1.7.1 Subtypes and Classification

Subtypes of melanoma include superficial spreading, nodular, lentigo maligna and acral lentiginous melanomas, with the superficial spreading form representing 50% of cases in Ireland (NCRI, 2025). Severity of melanoma is classified by four stages (I-IV), dependent on tumour-node-metastasis (TNM) staging with primary tumour depth (Breslow Thickness) and ulceration being the main prognostic indicators ***Table 1.2*** (Keung and Gershenwald, 2018).

T Category – Primary Tumour			
T Category		**Breslow Thickness**	**Ulceration Status**
Tx/T0/Tis		N/A	N/A
T1		≤1mm	Unknown/unspecified
	T1a	<0.8mm	Without ulceration
	T1b	<0.8mm	Without ulceration
		0.8 – 1mm	With or without ulceration
T2		>1 – 2mm	Unknown or unspecified

	T2a	>1 – 2mm	Without ulceration
	T2b	>1 – 2mm	With ulceration
T3		>2 – 4mm	Unknown or unspecified
	T3a	>2 – 4mm	Without ulceration
	T3b	>2 – 4mm	With ulceration
T4		>4mm	Unknown or unspecified
	T4a	>4mm	Without ulceration
	T4b	>4mm	With ulceration

N Category – Nodal Metastasis	
N Category	**Number of tumour-involved regional lymph nodes**
N0	No regional metastasis detected
N1	1 tumour involved node or in-transit, satellite and/or microsatellite metastases with no tumour-involved nodes
N2	2 – 3 tumour-involved nodes or in-transit satellite and/or microsatellite metastasis with 1 tumour-involved node
N3	≥4 tumour-involved nodes or in-transit, satellite and/or microsattelite metastases with ≥ 2 tumour-involved nodes
M Category - Metastasis	
M Category	**Anatomic Site**
M0	No evidence of distant metastasis

M1		
	M1a	Distant metastasis to skin, soft tissue including muscles and/or nonregional lymph node
	M1b	Distant metastasis to lung with or without M1a sites of disease
	M1c	Distant metastasis to non-CNS visceral sites with or without M1a/M1b sites of disease
	M1d	Distant metastasis to CNS with or without M1a/M1b/M1c sites of disease

TNM Staging

Clinical Stage Group	**T**	**N**	**M**
0	Tis	N0	M0
1A	T1a	N0	M0
1B	T1b/T2a	N0	M0
IIA	T2b/T3a	N0	M0
IIB	T3b/T4a	N0	M0
IIC	T4b	N0	M0
III	Any T	≥N1	M0
IV	Any T	Any N	M1

Table 1.2: TNM staging based on the American Joint Committee on Cancer (AJCC) 8th edition, adopted from Keung and Gershenwald, 2018

1.7.2 Biology of Progression and Metastasis

Recurrence and metastasis are common in melanoma, occurring rapidly in progression of the disease. Melanoma metastasis was previously explained by the Clark model whereby spread

occurs in a stepwise fashion in the later stages of disease progression (Clark *et al.*, 1984). Recent evidence however suggests the metastatic process can begin much earlier, arising from premalignant lesions or in parallel to disease progression, often remaining dormant for variable periods of time (Gofrit *et al.*, 2022; Shirley *et al.*, 2024). In addition, cases of melanoma metastasis without a discernible primary lesion account for approximately 3% of all melanomas (Del Fiore *et al.*, 2021).

1.7.3 The Melanoma Patient Journey

The most common clinical sign of melanoma is the development of a new pigmented lesion or progressive change in an existing naevus. Characteristic features are often summarised by the ABCDE criteria, assessing asymmetry, borders, colour changes, diameter and evolution over time. In addition, lesions appearing distinct from surrounding moles (ugly duckling sign) may also warrant investigation (Duarte et al., 2021).

Typically, suspicious lesions are found on self-examination or by primary care physicians, before referral is made to a dermatologist or plastic surgeon. Diagnosis is confirmed by excisional biopsy, involving surgical removal of the lesion, followed by histological analysis (HSE, 2025).

Staging and courses of treatment are typically determined by a multidisciplinary team (MDT), which may include specialists in dermatology, plastic surgery, medical oncology, radiation oncology, histopathology, radiology and/or specialist nurses. Factors including cancer type, stage and the patient's general health are taken into account to decide on the optimal treatment course (NCRI, 2025).

Melanoma staging is determined by TNM staging, which can require additional surgery (sentinel lymph node biopsy) and radiological testing. Patients with stage 1B to 2C melanoma are typically offered a sentinel lymph node biopsy to assess for lymphatic spread (NCRI, 2025).

National clinical guidance on radiological staging for distant metastasis recommends considering radiological staging for stages IIB and above, with options including whole body PET-CT with brain scan (MRI/contrast enhanced CT) and contrast enhanced CT TAP with brain scan (MRI/contrast enhanced CT) (HSE, 2024).

Surgery is the primary treatment of cutaneous melanoma. In addition to the previously mentioned excisional biopsy and sentinel lymph node biopsy, a wide local excision and may be required, depending on tumour thickness (Breslow Thickness). European guidelines for WLE margins dependent on Breslow Thickness are shown in ***Table 3*** (Garbe et al., 2025). Patients with lymphatic metastasis (stage III melanoma) may also undergo lymphatic dissection to remove affected nodes (NCRI, 2025).

Breslow Thickness	**Minimum Clear Margin Required**
0mm (confined to epidermis)	0.5cm
≤2mm	1cm
>2mm	2cm

Table 3 European recommended Wide Local Excision (WLE) minimum clear margin requirements based on Breslow Thickness, adopted from Garbe et. al., 2025

Medical adjuvant therapy, such as chemotherapy, immunotherapy and/or targeted therapy, may also be used in conjunction with surgical treatment. These options are expanded upon in Section 1.7.4.

Follow-up of melanoma after treatment is advised for at least 5 years, with visits typically including evaluation of reported symptoms, physical examination of scar and surrounding skin, physical examination of lymph nodes, full-body clinical and dermatoscopic examination, blood

testing and radiological surveillance (Garbe et al., 2025). In Ireland, radiological surveillance is considered in patients with stages IIB or higher. The typical frequency of surveillance is every six months for years 1 to 3 and yearly for years 4 and 5, however, patients with stage III disease that have not undergone complete lymph node dissection or patients with stage IIID or stage IV disease may undergo more frequent surveillance (NCCP, 2025).

1.7.4 Current Therapeutic Landscape – Adjuvant Therapy

The aggressive nature of melanoma has led to significant developments in adjuvant therapies, including immune checkpoint inhibitors, targeted therapy, radiotherapy and chemotherapy (Eljilany *et al.*, 2023; Faries *et al.*, 2013). The use of adjuvant therapies is typically reserved for late-stage melanoma and high-risk patients (Eljilany *et al.*, 2023). However, taking early dissemination into consideration, the use of adjuvant therapies in early-stage melanoma may be necessary.

1.7.4.1 Immunotherapy

Current adjuvant therapies for melanoma include immunomodulators such as immune checkpoint inhibitors (ICIs), which target immune checkpoints including programmed cell death protein-1 (PD-1) and cytotoxic T lymphocyte-associated protein-4 (CTLA-4) (He *et al.*, 2022). CTLA-4 inhibition enhances T-cell priming and activation in lymphoid tissues, while PD-1 blockade prevents T-cell exhaustion in the tumour microenvironment, enhancing anti-tumoural responses (Carlino et al., 2021).

Ipilimumab, a CTLA-4 inhibitor, was the first ICI shown to improve survival in patients with advanced melanoma (Hodi et al., 2010). Subsequent research identified PD1 as another critical target, with inhibitors such as Nivolumab and Pembrolizumab demonstrating superior efficacy compared with CTLA-4 blockade, resulting in improved survival and a favourable toxicity profile (Robert et al., 2019; Robert et al., 2015). Combination therapy with ipilimumab and

nivolumab has also been investigated due to their complementary modes of action. Long-term follow up from the CheckMate 067 trial demonstrated that five year survival was significantly higher in patients who received Ipilimumab and Nivolumab or Nivolumab alone compared wth Iplilimumab monotherapy (Larkin et al., 2019).

Ipilimumab, Nivolumab and Pembrolizumab are approved for clinical use in Ireland, either as monotherapy or combination regimens. While their use is typically restricted to advanced melanoma, Pembrolizumab monotherapy is also approved for select patients with stage II disease (NCCP, 2025). This follows evidence from the KEYNOTE-716 trial, which demonstrated a significant improvement in recurrence free survival in high-risk stage II melanoma (Luke et al., 2022).

1.7.4.2 Targeted Therapy

Targeted therapy is indicated for patients with melanoma harbouring actionable driver mutations, such as the BRAF V600 mutation which occurs in approximately 35 – 50% of melanomas (Pizzichetta et al., 2025). These therapies selectively inhibit oncogenic signalling pathways such as the mitogen-activated protein kinase (MAPK) pathway, inhibiting tumour growth and proliferation (Long et al., 2017)

Standard of care for BRAF-mutated melanoma in Ireland includes BRAF inhibitors (e.g. vemurafenib, dabrafenib) used as monotherapy or in combination with MEK inhibitors (e.g. trametnib, cobimetinib) (NCCP, 2025). BRAF inhibitors target BRAF kinase, while MEK inhibitors act on downstream kinases in the MAPK pathway producing more potent results when used in combination (Grimaldi et al., 2017; Proietti et al., 2020).

1.7.4.3 Radiotherapy and Chemotherapy

Prior to the introduction of immunotherapy and targeted therapy, radiotherapy and chemotherapy were the main adjuvant treatment options for advanced melanoma. While their

roles have diminished in recent years, both retain clinical applications, typically in palliative cases.

Dacarbazine (DTIC) was the first FDA approved chemotherapeutic agent for metastatic melanoma. It exerts its effect by alkylating DNA to induce apoptosis in rapidly dividing cells. Despite limited response rates, it remains the only recommended chemotherapeutic agent in Ireland (NCCP, 2025).

Melanoma is regarded as a radioresistant tumour, though certain applications of radiotherapy have proved beneficial. Stereotactic radiosurgery (SRS) and stereotactic body radiotherapy (SBRT) are effective in the treatment of brain metastases, providing local control and symptom relief (Minniti et al., 2017). Some studies have also explored the use of radiotherapy in combination with immunotherapy, suggesting that radiotherapy may synergise with checkpoint blockade, enhancing anti-tumour responses, though further clinical validation is required (Rafiq et al., 2025).

1.7.5 Mechanisms of Resistance and Metabolic Plasticity

Resistance to adjuvant therapy can occur frequently, with melanoma cells employing several evasion tactics including metabolic rewiring, intrinsic immune evasion and transcriptomic reprogramming (Johnson *et al.*, 2021; Zhang *et al.*, 2022). While most tumour cells typically rely on aerobic glycolysis as a form of energy metabolism, aggressive melanoma can demonstrate phenotypic heterogeneity, modifying its energy metabolism to alternative pathways, including oxidative phosphorylation and fatty acid metabolism in response to its environment and external stimuli such as adjuvant therapy to ensure survival (Falletta *et al.*, 2022).

Despite durable responses in many patients, between 40 and 55% of patients exhibit resistance to immune checkpoint blockade (Lim et al., 2023). Key mechanisms include defective antigen

presentation, interferon-γ pathway disruption and T cell exhaustion (Chow et al., 2022; Nielsen et al., 2022). Combination therapies have emerged as a potential strategy to overcome resistance to immunotherapy, combining ICIs with several modalities including chemotherapy, radiotherapy, targeted therapy, other ICIs and vaccines (Alsaafeen et al., 2025).

Resistance also remains a major limitation of targeted therapy, with mechanisms including reactivation of the MAPK pathway due to engagement of alternative signalling pathways and metabolic rewiring (Loftus et al., 2024; Wang et al., 2018) Several studies have explored methods of overcoming resistance to targeted therapy with promising results. Methods explored included the induction of mitochondrial dysfunction (Zhao et al., 2025), induction of metabolic rewiring (Redondo-Muñoz et al., 2023) and targeting of protein network alterations (Vasudevan et al., 2021).

1.7.6 Future Directions and Integration of OTD

The current therapeutic landscape for melanoma has evolved rapidly, with immune checkpoint inhibitors, targeted therapies and combination strategies significantly improving survival outcomes (Carlino et al., 2021; Long et al., 2017). However, therapeutic resistance remains a major clinical challenge leading to reduced tumour durability, tumour recurrence and treatment failure, particularly in advanced or metastatic disease (Lim et al., 2023).

OTD offers a distinct mechanistic profile with the potential to overcome several current therapeutic limitations. Through the downregulation of NF-κB transcriptional activity (Majchrzak-Stiller et al., 2023), OTD targets a signalling axis implicated in resistance to both immunotherapies and targeted therapies. NF-κB promotes melanoma cell survival, angiogenesis and immune evasion and is associated with reduced responsiveness to immune checkpoint blockade and BRAF/MEK inhibition (Li et al., 2024; Yu et al., 2020). By

suppressing this pathway, OTD has the potential to sensitise tumour cells to standard therapies, delay the development of resistance and improve treatment durability.

Another key feature of OTD is its potential for topical administration, in contrast to Taurolidine and most systemic therapies (Pfirrmann et al., 2024). Topical delivery may allow for high local drug concentrations at the tumour site while minimising systemic exposure, making it particularly advantageous for superficial primary or locoregional melanoma lesions, cutaneous metastasis and peri-surgical settings. This approach could complement existing systemic treatments, improving local disease control and reducing treatment-related toxicity.

Additionally, OTD's dual anti-tumour and potential anti-inflammatory properties may help modulate the tumour microenvironment, potentially enhancing immune cell infiltration and counteracting inflammatory signals that support tumour survival and progression.

Taken together, the integration of OTD into melanoma treatment strategies could offer a multi-faceted therapeutic approach, enhancing the efficacy of systemic therapies, reducing resistance and expanding local control options.

1.8 Hypothesis, Aim and Objectives

1.8.1 Overall Hypothesis

OTD reduces inflammation and exerts antineoplastic effects on primary and metastatic melanoma cells without adversely affecting non-neoplastic cells

1.8.2 Experimental Chapter 1

Aim: To investigate the effect of OTD on human dermal fibroblast migration, inflammatory cytokine secretion and viability in vitro.

Objectives:

1. Evaluate the impact of OTD on HDF migration
2. Assess the cytotoxicity of OTD on HDFs and primary melanoma cells
3. Characterise the effect of OTD on inflammatory cytokine release from HDF cells

1.8.3 Experimental Chapter 2

Aim: To compare the antineoplastic effects of OTD on primary and metastatic melanoma cell lines.

Objectives:

1. Evaluate the cytotoxicity of OTD on primary and metastatic melanoma cell lines
2. Characterise the mechanism of cell death in primary and metastatic melanoma cell lines – apoptosis or necrosis
3. Explore the effect of OTD on mitochondrial mass in primary and metastatic melanoma cell lines

1.8.4 Experimental Chapter 3

Aim: To determine the effect of OTD on viability and anti-tumoural cytokine release of CD8+ T Lymphocytes

Objectives:

1. Assess the optimal concentration of anti-CD3 required for stimulation
2. Explore the effect of OTD on IFNγ release in CD8+ T lymphocytes following anti-CD3 stimulation
3. Characterise the effect of OTD on IFNγ release in CD8+ T lymphocytes following Phorbol-12-myristate-13-acetate (PMA)/Ionomycin stimulation

Chapter 2: Materials and Methods

2.1 Cell culture

2.1.1 Dermal Fibroblasts Cell Line

Human Dermal Fibroblast (HDF) cell line, 106-05A was purchased from Sigma-Aldrich (St. Louis, MO, USA). HDF cells were cultured in Fibroblast Growth Medium 2 (Promocell, Germany) containing high glucose Dulbecco's modified Eagle's medium (DMEM) with stable 2mM L-Glutamine supplemented with 10% foetal bovine serum (FBS). Cells were seeded and passaged in culture flasks with a growth area of 75cm^2 at 37°C with 5% CO_2.

2.1.1.1 Rationale for Model Selection

Human dermal fibroblast (HDF) cells are a well-established in vitro model for studying wound repair and cellular responses to inflammatory mediators. They play a central role in extracellular matrix remodelling and cytokine secretion (Tracy et al., 2016; Wang et al., 2023). Their use in peer-reviewed studies has demonstrated reproducible and translationally relevant results for cutaneous healing models using the 106-05A cell line (Madsen et al., 2023). This study employs HDF cells to assess treatment-induced modulation of migratory function, cytokine profiles and viability.

2.1.2 Melanoma Cell Lines

Primary melanoma (A-375s and WM3248) and metastatic (WM164 and SK-MEL-2) melanoma cell lines were purchased from Sigma-Aldrich (St. Louis, MO, USA). A-375 cells were cultured using DMEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin/neomycin (PSN), WM3248 and WM-164 cells were cultured using basal medium and L-15 medium (containing L-glutamine) supplemented with 2% FBS and 1% PSN and SK-MEL-2 cells were cultured using Minimum Essential Eagles Medium (EMEM)

supplemented with 10% FBS and 1% PSN. Cells were seeded and passaged in culture flasks with a growth area of 75cm^2 at 37°C with 5% CO_2.

2.1.2.1 Rationale for Model Selection

Melanoma cell lines are widely recognised in oncology research as reliable and reproducible models for investigating anti-tumoural responses and evaluating therapeutic strategies (Marie Vincent and Postovit, 2017). The use of well-characterised cell lines, such as those chosen for this study, enables consistency and comparability across studies (Malka-Mahieu et al., 2016; Reigstad et al., 2022; Yang et al., 2021). In addition, both primary and metastatic melanoma cell lines were chosen to enable assessment of differential responses across tumour progression stages, increasing the translational relevance of the findings.

2.1.3 Peripheral Blood Mononuclear Cells (PBMCs)

2.1.3.1 Donors for PBMC Isolation

Heathy human buffy coat packs were obtained from the Irish Blood Transfusion Service (IBTS, St. James's Hospital, Dublin, Ireland). The IBTS provides clinically unsuitable blood components for research purposes from healthy, non-remunerated, anonymised donors following written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by standard density gradient centrifugation over Lymphoprep (Serumwerk Bernburg AG, Bernburg, Germany) and were then cryopreserved at -80ºC until further use. Ms. Amy Walsh isolated PBMCs from buffy coat blood and cryopreserved them.

2.1.3.2 PBMC Culture

Culture medium consisted of Roswell Park Memorial Institute (RPMI) medium 1640 (Sigma-Aldrisch, St. Louis, MO, USA) with stable 2mM L-Glutamine, supplemented with 10% FBS and 1% Penicillin/Streptomycin. Cryopreserved PBMCs with a density ranging from 5 x 10^5 –

8.22 x 10^6 cells/ml were thawed and rested overnight at 37°C in a humidified atmosphere with 5% CO2 before further use.

2.1.3.3 Rationale for Model Selection

PBMCs provide a physiologically relevant immune cell model, frequently used to evaluate cytokine production, immune activation and cytotoxic responses in vitro (Lawlor et al., 2021). This study uses an established model to evaluate the effects of OTD on immune cell function.

2.1.4 Cell Free DNA (cfDNA)

2.1.4.1 Sample and Patient Cohorts

Prospective patients presenting to the Skin Lesion Service at University College Cork, Cork University Hospital (UCC/CUH) in 2023, for elective surgery of potential melanoma lesions were screened for eligibility. Ethical approval for this project was granted by the Clinical Research Ethics Committee (CREC), Cork Teaching Hospitals (ECM 4 (p) 09/08/2022

& ECM 5 (10) 09/08/2022 & ECM 3 (l) 20/09/2022). Written informed consent was obtained from all patients who participated in this study.

Individuals aged between 18 and 85 were considered for inclusion. Eligibility criteria required an Eastern Cooperative Oncology Group (ECOG) performance status of 0-2, adequate organ and bone marrow function (as determined by peripheral blood sampling) and a melanoma probability score > 7. Patients meeting these criteria were invited to provide peripheral blood samples and consent for lesion photography.

Exclusion criteria included active or recent infection at the time of recruitment, surgery within 14 days prior to enrolment, a diagnosis of another malignancy within the past three years or any known diagnosis of autoimmune disease, diabetes mellitus or vascular disease. Additional exclusions were the use of glucocorticosteroids, immunomodulatory medications or antibiotic

therapy within 28 days prior to enrolment. The melanoma probability score was adapted from Ebell et al. to assess the likelihood of diagnosis (Ebell, 2008).

2.1.4.2 Blood Sample Collection and Processing

Peripheral blood samples were collected from patient (1) pre-operatively on the morning of surgery (pre-op), (2) between day one and day three postoperatively (early post-op) and (3) four weeks postoperatively (late post-op). Photographs were obtained of the lesion and subsequent wound at each time point to facilitate correlation of cfDNA levels with gross lesion characteristics and potential postoperative wound complications. The preoperative time point was representative of the patient's baseline systemic immune profile, the early postoperative time point aimed to capture the immediate postoperative immune response and the late postoperative time point was representative of the expected resolution of the postoperative immune response.

Peripheral blood samples (40ml) were collected at each time point in EDTA and STREK blood collection tubes. Samples were processed within one hour of collection. Tubes were centrifuged twice at 2469 RCF for 10 minutes, after which plasma was carefully isolated and aliquoted into 1.5ml DNA- and DNAse-free Eppendorf tubes. The buffy coat layer was similarly aspirated and transferred to separate tubes. All samples were cryopreserved at -80ºC for future analysis.

2.1.4.3 Rationale for Model Selection

Measuring cell-free DNA (cfDNA) in systemic circulation has emerged as a promising biomarker in oncology, reflecting processes such as cell death, immune activation and tissue injury (Bronkhorst et al., 2019). Plasma cfDNA levels have been known to rise following surgery, likely due to surgical stress, inflammation and tissue damage (Henriksen et al., 2020; Rosen et al., 2022). This study intended to perform next-generation sequencing on isolated cfDNA samples to characterise tumour-derived genomic alterations across the perioperative

timeline. This component of the study was discontinued prior to analysis due to logistical challenges, as outlined in Appendix A.

The pre-operative time point was intended to reflect the patient's baseline systemic immune and tumour genomic profile. The early postoperative time point (day 1-3) was selected to coincide with the peak of the acute inflammatory response, particularly neutrophil influx, which reaches maximum levels between postoperative days 1 and day 2 (Kim et al., 2008). The late postoperative time point (four weeks) was chosen based on previous findings demonstrating that cfDNA levels remain elevated for up to four weeks following surgical trauma before gradually returning to baseline (Henriksen et al., 2020).

Inclusion and exclusion criteria were designed to minimise confounding factors that could influence cfDNA levels, tumour genomic alterations or immune responses. Exclusion criteria therefore included previous cancer diagnoses, recent infection or surgery, autoimmune disease, immunomodulatory therapy, diabetes mellitus, vascular disease and glucocorticosteroid use, all of which are known to alter the inflammatory response or impair wound healing.

2.1.5 Treatments

2.1.5.1 1,4,5-Oxathiazinane-4,4-dioxide (OTD)

1,4,5-Oxathiazinane-4,4-dioxide (OTD) ultra-pure powder, provided by Geistlich Pharma AG (Wolhusen, Switzerland), was prepared to a 20mM stock concentration by dissolving in phosphate buffer solution (PBS) and filtering through 0.2µm syringe filters (Pall Corporation, Ann Arbor, MI, USA). Treatment concentrations of 0.25, 0.5, 0.75, 1 and 1.25mM OTD were prepared by diluting the stock solution in each cell line's respective medium.

2.1.5.2 Cytokine Stimulation

2.1.5.2.1 IL-1α stimulation

Interleukin-1α (IL-1α) was purchased from Peprotech (Rocky Hill, NJ, USA) and reconstituted in PBS to yield a stock concentration of 0.1mg/ml. Treatment concentrations of 10ng/ml were prepared by diluting in cell culture media and respective OTD solutions. Unstimulated and stimulated controls contained culture media/culture media with IL-1α respectively.

2.1.5.2.2 Anti-CD3 stimulation

Culture plates were coated with monoclonal anti-CD3 antibody in PBS (5ug/ml or 2ug/ml, Miltenyi Biotec, Cologne, Germany), wrapped in parafilm and stored at 4°C overnight. Anti-CD3 coated plates were transferred to 37°C for 2 hours prior to stimulation. Treated cells were transferred to the coated anti-CD3 plate and suspended in complete media containing 5ug/ml anti-CD28 (Biolegend, San Diego, California, USA) and 5ug/ml Brefeldin A (BFA) (Sigma-Aldrisch, St. Louis, MO, USA) for 5 hours at 37°C. Unstimulated samples were suspended in complete media with BFA alone.

2.1.5.2.3 Phorbol-12-myristate-13-acetate (PMA)/Ionomycin stimulation

Phorbol-12-myristate-13-acetate (PMA) and ionomycin were purchased from (Sigma-Aldrisch, St. Louis, MO, USA) and reconstituted in dimethylsufoxide (DMSO) to yield stock concentrations of 1mg/ml and 10mg/ml respectively. Final concentrations of 20ng/ml PMA and 1000ng/ml ionomycin were prepared by diluting in cell culture media and 5µg/ml BFA. Stimulation was applied for 4.5h at 37°C. Unstimulated samples were suspended in complete media with BFA alone.

2.1.5.3 Rationale for Treatment Selection

The treatment strategy for this study was designed to model both the tumour microenvironment and immune activation states relevant to melanoma progression and postoperative immune

responses. OTD was investigated for its potential immunomodulatory and cytotoxic effects, while cytokine and receptor-mediated stimulation strategies were employed to mimic physiologically relevant immune activation pathways

2.1.5.3.1 OTD Concentration Selection

The concentrations of 0.25, 0.5, 0.75, 1 and 1.25mM were chosen based on preliminary dose-response optimisation experiments (Section 3.2.1) and published data demonstrating biological activity within similar concentration ranges (Buchholz et al., 2017; Jinih et al., 2021; Majchrzak-Stiller et al., 2023). This treatment range was designed to capture both therapeutic and subtherapeutic effects and enable analysis of dose-response patterns.

2.1.5.3.2 IL-1α Stimulation

IL-1α was chosen to stimulate proinflammatory cytokine release in HDF cells, thereby modelling the real-world inflammatory response that occurs following tissue injury. IL-1α is rapidly released by damaged cells in response to wounding and acts on dermal fibroblasts to upregulate the release of key inflammatory mediators (Cordier-Dirikoc et al., 2022). In this study, IL-1α stimulation was used to stimulate the release of IL-6 and IL-8, two cytokines that are central to the postoperative inflammatory response. A treatment concentration of 10ng/ml was based on previously published protocols, including Huang et al., who demonstrated effective stimulation of inflammatory pathways in keratinocytes at that concentration (Huang et al., 2021).

2.1.5.3.3 Anti-CD3 Stimulation

Anti-CD3 stimulation, when combined with CD28 costimulation, provides a robust in vitro model of T cell activation that mimics physiological antigen-driven responses. Engagement of the T cell receptor (TCR) via CD3 initiates activation, however, in the absence of a concurrent costimulatory signal, such as CD28, T cells undergo apoptosis (Levine et al., 1997). The

addition of anti-CD28 enhances proliferation, cytokine release and effector function of T cells (Li and Kurlander, 2010).

Although the method is well established, optimal concentrations of anti-CD3 required for adequate stimulation of cytokine release are highly variable, with ranges varying from 1µg/ml to 20µg/ml and stimulation times varying from 6 hours to 4 days (Uhl et al., 2023; Biller et al., 2001; Gupta and Maecker, 2015; Machicote et al., 2018). Therefore, the concentrations used in this study were optimised in preliminary experiments (See Section 4.2.1) for adequate stimulation of IFNγ production.

2.1.5.3.4 PMA/Ionomycin Stimulation

Phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation is a widely recognised method for inducing maximal T cell activation in vitro. PMA activated protein kinase C (PKC) by bypassing the T cell receptor (TCR) complex, while ionomycin increases intracellular calcium levels through calcium ionophore activity. This combined activation stimulates downstream TCR signalling events, inducing robust cytokine production and proliferation (Abraham and Weiss, 2004).

This stimulation strategy typically elicits stronger responses compared with receptor-mediated activation, making it particularly useful for positive control experiments and for assessing maximal cytokine production in T cells. However, because it bypasses physiological receptor engagement, it does not accurately replicate antigen-specific or co-stimulatory signalling (Lee et al., 2023). In this study, PMA/ionomycin was used to elicit maximal stimulation of IFNγ production in CD8+ T lymphocytes within the PBMC population. The results of this analysis were then compared with those obtained following physiologically relevant anti-CD3 stimulation.

2.2 Wound healing assay

2-Well Silicone Culture Inserts, purchased from Ibidi (Martinsried, Germany) were attached to each well of a 24-well culture plate. HDFs were seeded in each compartment of the silicone inserts at a concentration of 5 x 10^5 cells/ml. 70µl of the cell suspension was added to each compartment. The plates were then placed in an incubator at 37°C with 5% CO_2 for 24 hours.

After 24 hours the silicone inserts were removed using sterile forceps, leaving a gap area of 500 ± 50 µm (according to the manufacturer). OTD treatments and control were applied to each well at a volume of 0.5ml. Microscopic images were obtained using a Leica DM IL inverted microscope equipped with Leica Flexacam C3 camera and Leica LAS X software (Leica Microsystems-Wetzlar, Germany) at nine timepoints starting immediately following application of control/OTD (0h, 3h, 6h, 12h, 18h, 24h, 30h, 36h, 48h). Each image was obtained at a 10X objective with a final magnification of 100X. Image J version 1.53t and a "Wound Healing Size Tool" (Suarez-Arnedo *et al.* 2020) were used to calculate wound area and percentage area with reference to a 250µm scale bar in each image.

2.2.1 Scratch Assay Optimisation

Prior to performing the wound healing assay using silicone inserts, a conventional scratch assay was carried out to optimise cell seeding density and experimental methodology. HDF cells were seeded in 24-well culture plates at concentrations of 3 x 10^5, 5 x 10^5 and 7 x 10^5 cells/ml and allowed to form a confluent monolayer over 24 hours. A uniform linear scratch was introduced into each well using a sterile 200µL pipette tip. All subsequent treatment and imaging conditions were identical to those used in the main wound healing assay, including OTD/control application, incubation, timepoints and image analysis.

2.2.2 Rationale for Technique Selection

The wound healing assay is a well-established and widely accepted in vitro model to assess cell migration and tissue regeneration. It allows for quantitative evaluation of HDF cell motility under treatment conditions. The incorporation of silicone inserts into the design protocol enhanced repeatability of the experiments, forming a uniform gap area and requiring fewer cells upon seeding compared with the scratch method (Jonkman et al., 2014).

2.3 Assessment of cytokine release in HDF cells

HDF cells were seeded in a 96-well culture plate at a concentration of 2 x 10^5 cells/ml supplemented with Fibroblast Culture Media. The plates were then placed in an incubator at 37°C with 5% CO_2 for 24 hours. After 24 hours, the remaining media was carefully removed and OTD stimulated and unstimulated treatments were applied with respective controls. The plates were then replaced in the incubator at 37°C with 5% CO_2 for 24 hours. After 24 hours, the solution was carefully aspirated from each well and frozen at -20°C.

For cytokine quantification, samples were thawed at room temperature and centrifuged for 30 seconds at 14’000 RCF. Electrochemiluminescence studies were carried out using a custom Human ProInflammatory Cytokine Panel Kit (MesoScale Discovery, MSD, Rockville, MD). measuring IL-6 and IL-8 levels. Analysis of MSD plates was carried out using MS2400 imager (MSD, Rockville, MD). The assays were performed according to the manufacturer’s instructions, with all standards and samples quantified in duplicate. All samples were diluted at 1:5 following preliminary experiments, preparing samples at a 1:2 dilution, due to higher proportion of stimulate and analyte concentrations present in culture samples.

2.3.1 Rationale for Technique Selection

IL-6 and IL-8 were selected due to their key role in the pro-inflammatory immune response to surgery. IL-6, along with other proinflammatory mediators such as tumour necrosis factor

alpha, interferon gamma and IL-12 is produced by several cell types, including dermal fibroblasts, in response to damage-associated molecular proteins (DAMPs) released following tissue injury. Fibroblasts also secrete IL-8, a potent chemokine which mediates immune cell recruitment to the site of injury (Correa-Gallegos et al., 2021). Elevated postoperative levels of IL-6 and IL-8 are associated with complications including wound infection, sepsis and bleeding (Pecqueux et al., 2023; Rettig et al., 2016). Moreover, elevated pretreatment levels of IL-6 and IL-8 are linked to poor prognosis and immunotherapy resistance in melanoma (Levati et al., 2024; Y. Wang et al., 2022), further supporting their clinical relevance.

Electrochemiluminescence immunoassays were used to quantify IL-6 and IL-8 levels due to their high sensitivity, low background signal and capacity for multiplex analyte detection, offering improved performance compared with conventional ELISA (Bolton et al., 2020). In addition, plate-based electrochemiluminescence assays provide a more reliable assessment of proinflammatory cytokine quantification than bead-based platforms, particularly in low-concentration samples (Dabitao et al., 2011). This method was therefore chosen to provide precise and reproducible measurements of proinflammatory cytokines released by human dermal fibroblasts following IL-1α stimulation.

2.4 Assessment of cell viability– MTT assay

All cell lines were seeded in 96-well cell culture plates at a density of 1 x 10^5 cells/ml. Cells were incubated for a minimum of 12 hours to facilitate adherence prior to exposure to OTD treatment for periods of 24, 48 or 72 hours. 10 µl of 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltertazolium bromide (MTT) was added at a concentration of 5mg/ml and incubated for a further 2 hours at 37ºC. Test media was discarded and formazen crystals were dissolved by adding 100µl of DMSO to each well and shaking at 225 RPM for 15 minutes. Absorbance for each well was measured using a Micro-titre Plate Reader (Dynex Technologies Inc., USA)

at 570nm wavelength. Cell viability was expressed by converting optical density (OD) to a percentage of the control.

2.4.1 Rationale for Technique Selection

The MTT assay is a colorimetric technique that measures cell viability via the reduction of yellow tetrazolium salt 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltertazolium bromide (MTT) into insoluble purple formazan crystals by mitochondrial dehydrogenase enzymes present in metabolically active cells (Mosmann, 1983). As this reaction only occurs in viable cells with intact mitochondrial function, the amount of formazan produced provides an indirect but reliable measurement of cell viability and overall metabolic state.

The MTT assay is widely used in cytotoxicity studies due to its simplicity and reproducibility. It has also been employed in previous studies evaluating the cytotoxic potential of OTD (Buchholz et al., 2017; Jinih et al., 2021), supporting its suitability for assessing treatment effects in the current experimental model. In this study, MTT was used to quantify the cytotoxic effects of OTD on fibroblast and melanoma cell lines, providing an indirect measurement of cell viability and overall metabolic state.

This method however, is limited by the potential inclusion of quiescent cytostatic cells in the non-viable cell proportion due to inference of viability from metabolic activity in lieu of a direct measurement of cell death (Ghasemi et al., 2021). To address this, MTT results were validated by Annexin/PI apoptosis assay, which provided additional clarity on the mechanism of cell death.

2.5 Flow cytometry

Following respective staining protocols, relative fluorescent intensities were examined on a BD Accuri C6 flow cytometer and further analysed using FloJo software. Flow cytometry gating strategies are detailed in ***Supplementary Figures 2-4***.

2.5.1 Cell necrosis and apoptosis

WM2348 and SK-MEL-2 cells were seeded in 96 well culture plates at a density of 1 x 10^5 cells/ml for a minimum of 12 hours prior to 24 hours of OTD treatment. Cell apoptosis and necrosis were measured using the annexin V/FITC and propidium iodide (PI) assay kit (Biolegend, San Diego, California, USA). Annexin V binds with phosphatidylserine translocated from the inner to the outer membrane leaflet in apoptotic cells, whereas PI intercalates with intracellular DNA following necrotic or late apoptotic loss of membrane integrity. Cell apoptosis and necrosis were detected by flow cytometry using a BD Accuri C6 flow cytometer and further analysed using FloJo software. Annexin V^-/PI^- cells were considered viable, Annexin V^-/PI^+ cells were considered necrotic, Annexin V^+/PI^- were considered early apoptotic and Annexin V^+/PI^+ cells were considered late apoptotic. Cell apoptosis and necrosis were expressed as percentages of the overall cell population.

2.5.1.1 Rationale for Technique Selection

Annexin V and propidium iodide staining is a well-established and peer-reviewed approach to discriminate between early apoptosis, late apoptosis and necrosis (Vermes et al., 1995). Its high sensitivity and reproducibility make it a standard technique for cytotoxicity studies. The assay has also been employed in previous studies evaluating the cytotoxic potential of OTD in other cell types, supporting its suitability for assessing cell death mechanisms in the present study (Baron et al., 2022; Jinih et al., 2021).

2.5.2 Mitochondrial mass

WM3248s and WM164s were resuspended at a density of 1 x 10^5 cells/ml and seeded in 96-well culture plates. Cells were incubated for a minimum of 12 hours prior to application of OTD treatment for 24 hours. 6 x 10^4 cells were collected for each treatment condition and washed with PBS. Cells were then incubated for 30 minutes at 5% CO2 and 37°C in staining

medium with 20nM MitoTracker™ Green FM (Thermo Fisher Scientific, M7514) to assess mitochondrial mass. Cells were then washed with PBS to remove unbound staining solution and re-suspended in 1 µg/ml 7-Aminoactinomycin (7-AAD) (Biolegend, San Diego, California, USA) to eliminate dead cells from sorts and analyses.

2.5.2.1 Rationale for Technique Selection

Quantification of mitochondrial mass using Mitotracker Green is an established indicator of mitochondrial biogenesis and metabolic state (Serasinghe et al., 2018). In addition, Fontana et al. demonstrate that mitochondrial mass correlates positively with "stemness" or metastatic potential in melanoma cell lines by assessing mitochondrial content in melanospheres compared with adherent melanocytes (Fontana et al., 2024). Building on this evidence, this study employed this technique to investigate whether OTD influences mitochondrial biomass and therefore, the metastatic potential of melanoma cells.

2.5.3 Intracellular staining & cytokine production in PBMCs

Following respective stimulation periods with anti-CD3 or PMA/Ionomycin, cells were washed in dPBS and stained with GloCell Fixable Live/Dead-APC (StemCell Technologies, Inc., Vancouver, BC, Canada) and surface CD8 antibody-FITC (Biolegend, San Diego, California, USA) for 30 min at 4ºC. Cells were washed and fixed with 1X Fixation Buffer (Biolegend, San Diego, California, USA) for 20 min at room temperature before washing with dPBS and 1X permeabilisation buffer (Biolegend, San Diego, California, USA). Intracellular anti-IFN-γ-PE (Biolegend, San Diego, California, USA) was added in permeabilisation buffer for 30 min at 4ºC. After two further washes in dPBS, cells were resuspended in 300µl fluorescence-activated cell sorting (FACS) buffer for flow-cytometric evaluation using a BD Accuri C6 flow cytometer and FlowJo software.

Our gating strategy was as follows; lymphocyte populations were gated and doublets were removed by selecting single cells. Dead cells were excluded, CD8 cells were gated and of these cells the proportion producing IFN-γ+ cells were selected, these were CD8+/ IFN-γ+ cells.

2.5.3.1 Rationale for Technique Selection

Intracellular staining by flow cytometry is a validated method for characterising immune cell activation and effector function at a single-cell level (Menon et al., 2014). Staining with surface CD8 antibody enabled positive selection of CD8+ T lymphocytes, while live/dead staining facilitated for exclusion of dead cells. Subsequent anti-IFNγ staining allowed for the isolation of live CD8+ T lymphocytes expressing IFNγ. Previous studies have validated this method to evaluate T cell effector responses (Badovinac and Harty, 2000).

2.6 Statistics

Excluding optimisations, each experiment was carried out with a minimum of three biological replicates or four independent donors, with all data expressed as mean and standard error of the mean (SEM) shown in the figures. GraphPad Prism (version 9.5.0) was used to carry out statistical testing. Values detected as significant outliers ($p<0.05$) using Grubb's outlier test were excluded ***(Supplementary Tables 1-6)***. One-way and two-way ANOVAs were used to test for significance with a 95% confidence interval ($p<0.05$). Should overall significance be achieved, multiple comparisons were carried out using Bonferroni or Dunnett post hoc testing.

2.6.1 Rationale for Technique Selection

Dose-response data were expressed as mean and standard error of the mean (SEM) for each treatment condition to reflect both central tendency and variability across biological replicates. Mean ± SEM is widely used in experimental and biomedical research to provide a precise estimate of the mean response across replicates, accounting for expected variability in repeated

measures. Previous studies examining the effects of OTD express data in a similar fashion (Buchholz et al., 2017; Sofia et al., 2024).

A minimum of three biological replicates, each consisting of three technical replicates, were analysed for each experimental condition to ensure adequate statistical power and reproducibility. Biological replicates capture variation between independent experiments, while technical replicates reduce measurement error within experiments, thereby increasing the reliability of the data (Blainey et al., 2014).

A Grubb's test for outliers was applied to all datasets prior to statistical analysis to identify and exclude extreme values that may have arisen from technical variability or experimental error, ensuring summary statistics accurately reflected the treatment response (Grubbs, 1969). Depending on whether one or two independent variables were assessed, a one-way or two-way ANOVA was used to evaluate the significance of treatment effects and interaction between factors. Bonferroni post hoc testing was applied for all datasets to account for type I errors while maintaining statistical power. An exception was made for the MTT assay, assessing viability on human dermal fibroblast (HDF) cells, for which Dunnett testing was applied as a less stringent approach to elucidate whether any significant treatment effects could be detected. No significant effects were observed.

Chapter 3: *In vitro* characterisation of the effect of OTD on Human Dermal Fibroblasts

3.1 Introduction

The dermal fibroblast population consists of several heterogeneous and functionally diverse subtypes, which, under stress conditions such as surgical wounds, participate in the regulation of inflammation and cell proliferation (Philippeos *et al.*, 2018; Stunova and Vistejnova, 2018). Dermal fibroblast dysregulation, however, can lead to excessive inflammation, scarring, chronic wounds and increased morbidity (Sen, 2021; Theocharidis *et al.*, 2020). Wound complications such as scarring and chronic wounds are associated with the development of skin malignancies. Although squamous cell carcinoma (SCC) represents the majority of cases, melanoma cases are also documented (Kelahmetoglu *et al.*, 2018; Kowal-Vern and Criswell, 2005; Łakomska *et al.*, 2023).

Tissue damage following surgical trauma initiates the wound healing process, which can be divided into four overlapping stages; haemostasis, inflammation, proliferation and remodelling. Dermal fibroblasts play a crucial role in the latter three stages (Cialdai *et al.*, 2022). During the inflammatory phase, dermal fibroblasts enhance the immune response by producing proinflammatory cytokines in response to damage associated molecular protein (DAMPs) secreted systemically following tissue injury (Cordier-Dirikoc *et al.*, 2022; Relja and Land, 2020). Then, in turn, produce proinflammatory mediators including tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-6 (IL-6) and interleukin-12 (IL-12) as well as C-C and C-X-C chemokines such as interleukin-8 (IL-8), which recruit immune cells to the injury site (Correa-Gallegos *et al.*, 2021).

Elevated systemic levels of pro-inflammatory cytokines such as IL-6 and IL-8 post-operatively are associated with postoperative complications, including wound infection, sepsis and bleeding (Pecqueux *et al.*, 2023; Rettig *et al.*, 2016). Ghazizadeh *et al.* also describe the role of IL-6 signalling in keloid scarring, reporting enhanced IL-6 expression in human keloid tissue samples, as well as increased deposition of extracellular matrix (ECM) components in IL-6-expressing keloid fibroblasts *in vitro* (Ghazizadeh *et al.*, 2007). In melanoma, elevated pre-treatment levels of both IL-6 and IL-8 systemically are associated with poor prognosis and immunotherapy resistance (Levati *et al.*, 2024; Y. Wang *et al.*, 2022). Additionally, elevated systemic IL-6 and IL-8 levels in both pre-treatment and post-treatment patients are associated with the accumulation of immunosuppressive myeloid-derived suppressor cells (MDSCs) and poor median overall survival outcomes (Tobin *et al.*, 2019). *In vitro,* Srivastava *et al.* demonstrate that IL-8 is a key mediator of FK506-binding protein 51 (FKBP51)-driven pathological processes in melanoma. Inhibition of IL-8 resulted in decreased tumour growth, clonogenicity, migration, and invasion in melanoma cell lines. Furthermore, their study demonstrated that neutralising IL-8 markedly diminished the pro-angiogenic effects of FKBP51-expressing cells, as demonstrated by a reduction in capillary-like structure formation in endothelial cells treated with culture medium from IL-8-silenced melanoma cells (Srivastava *et al.*, 2015).

During the proliferative phase, dermal fibroblasts aid in the formation of granulation tissue to replace the provisional wound matrix formed in the haemostatic phase. This process is mediated by the production of matrix metalloproteinases (MMPs) from fibroblasts to degrade the provisional matrix while simultaneously depositing ECM components, such as collagen, proteoglycans, hyaluronic acid, glycosaminoglycans and fibronectin (Landén *et al.*, 2016). Myofibroblasts are formed by fibroblast differentiation to produce contractile forces, facilitating wound closure. This effect continues into the remodelling stage of wound healing,

aiding in the replacement of collagen III with collagen I to increase tensile strength and reorganise newly synthesised tissue structures (Chitturi *et al.*, 2015).

The effect of drug therapies on physiological cells is often under-investigated *in vitro*, with adverse effects often observed in the clinical stages of development. Yet under-reporting of adverse effects is frequently observed, with Hazell *et al.* observing a 94% rate in under-reporting across 37 randomised control trials (Hazell and Shakir, 2006) and Seruga *et al.* finding that between 39% and 58% of adverse drug reactions were not reported at the clinical trial stage (Seruga *et al.*, 2016). While adjuvant and neo-adjuvant therapies have become increasingly popular in oncological surgery, acting to potentiate anti-cancer therapy, several therapies can adversely affect physiological cells. For example, Bevacizumab, used in several cancers, including metastatic colon cancer has been shown to cause significantly delayed wound healing (Kim *et al.*, 2022; Zhang *et al.*, 2016). As a result, treatment must be ceased 6-8 weeks prior to surgery and only reinstated after a period of 28 days (Gordon *et al.*, 2009).

1,4,5-Oxathiazinane-4,4-dioxide, also known as OTD, is a structural analogue of Taurultam (TRLT), the main derivative of Taurolidine. Taurolidine is clinically used in catheter-related bloodstream infections (Savarese *et al.*, 2024) and peritonitis (Alvarez Nadal *et al.*, 2020) as well as demonstrating anti-neoplastic and anti-inflammatory functions in studies on colon (Redmond *et al.*, 2018), liver (Li *et al.*, 2018), melanoma (Imhof *et al.*, 2011) and pancreatic (Hotz *et al.*, 2014) cancer. Its clinical application, however, is limited by its half-life of approximately 30 minutes (Gong *et al.*, 2007) compared with 13.8 h in OTD (Buchholz *et al.*, 2017).

OTD has similarly emerged as a potential anti-tumourigenic substance with studies showing antineoplastic effects on pancreatic (Majchrzak-Stiller *et al.*, 2023), breast (Jinih *et al.*, 2021), squamous cell carcinoma (Barras *et al.*, 2023) and malignant peritoneal mesothelioma (Baron

et al., 2022) *in vitro* (***Figure 3.1***). In fact human trials are currently in phase 1/2 of development for its application in combination with gemcitabine in advanced pancreatic cancer (Kasi and Iglesias, 2022) OTD has been shown to exert its antineoplastic effect via delays in cancer cell migration, proliferation (Barras *et al.*, 2023) and exertion of anti-inflammatory function via inhibition of the nuclear factor-κB (NF-kB) pathway (Majchrzak-Stiller *et al.*, 2023). Its effect on physiological cells such as dermal fibroblasts, however, has yet to be investigated.

Our present study aims to evaluate the migratory, inflammatory and cytotoxic effects of OTD on human dermal fibroblasts.

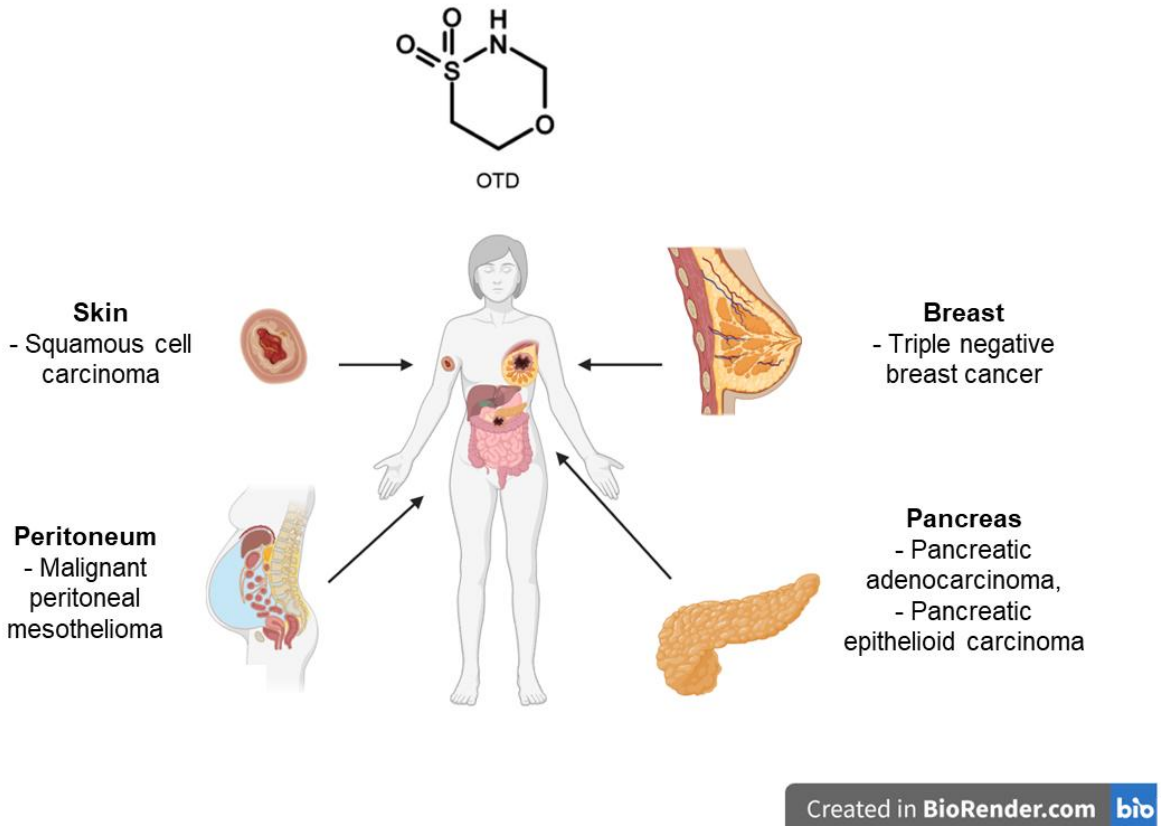

Figure 4.1*: 1,4,5-Oxathiazinane-4,4-dioxide (OTD) demonstrates antineoplastic effects on skin, breast, peritoneal and pancreatic cancer*

3.2 Results

3.2.1 Optimisation of Experimental Conditions

Prior to conducting the main experiments, preliminary optimisation studies were performed to determine the optimal parameters for the wound healing assay, cell seeding density, ImageJ analysis settings and sample preparation for cytokine detection. These optimisations were undertaken to ensure experimental reproducibility, achieve adequate signal intensity and facilitate experimental analysis, thereby enhancing the reliability and interpretability of the main experimental results.

Statistical testing was not carried out on optimisations because these studies were exploratory in nature and involved an insufficient number of biological replicates to support adequately powered statistical analysis.

3.2.1.1 Optimisation of Wound Healing Assay Method

Initial experiments employed a conventional scratch assay to assess wound closure. However, this approach produced substantial variability between replicates, making reliable quantification difficult. To improve reproducibility, silicone inserts were tested as an alternative. Both 2-well and 3-well inserts were evaluated. Although 3-well inserts allowed for the assessment of two wound areas within the same well, they were technically difficult to handle and frequently produced non-uniform wound gap areas due to challenges during removal. In contrast, 2-well inserts were easier to manipulate and provided more uniform and reproducible wound gaps. For this reason, they were selected for subsequent experiments ***(Figure 4.2A,B)***.

Treatment concentrations chosen for initial optimisation experiments were found to be too low to model the full range of OTD effects on fibroblast migration (0.1, 0.2, 0.5 and 1mM).

Therefore, a new treatment range of 0.25, 0.5, 0.75, 1 and 1.25mM was chosen for subsequent analyses.

For image acquisition, our initial approach used a measuring tape as an external reference to align plates on the microscope stage at predefined points, aiming to ensure imaging of the same wound region at each time point. However, this method lacked the precision required for consistent positioning between time points. To improve accuracy, the protocol was refined to include marking a uniform reference line on the outer surface of the culture plate prior to seeding, indicating the correct position for silicone insert placement and ensuring consistent wound positioning along the horizontal plane. This modification enabled precise and repeatable alignment of the wound region across all timepoints.

3.2.1.2 Optimisation of Cell Seeding Density

To determine the optimal seeding density for uniform monolayer formation, concentrations of 3×10^5, 5×10^5 and 7×10^5 cells/ml were tested using the scratch assay. At the highest density (7×10^5 cells/ml), a clumping effect was consistently observed at the wound edge, compromising assay uniformity. This was not evident at the lower two concentrations. The intermediate seeding density (5×10^5 cells/ml) was selected as it provided a confluent monolayer without clumping, while also ensuring sufficient cell numbers to accommodate potential downstream analysis.

3.2.1.3 Optimisation of ImageJ Analysis

Accurate wound area quantification was optimised using ImageJ software. Initial analyses focused on measuring wound gap width at three predefined points ***(Figure 4.2B)***, however, this approach was replaced with total wound area measurements to improve precision and reproducibility. Scale bars were incorporated into the original images using Leica LAS X software and used as a reference for calibration during image analysis. Adjusting image

contrast significantly improved wound edge definition, allowing for more precise delineation of the wound boundary ***(Figure 4.2C,D)***. Two mapping techniques were assessed, polygon selection and freehand tracing. Freehand tracing proved to be more flexible and efficient, particularly for irregular wound margins and was therefore adopted for all subsequent analyses ***(Figure 4.2E,F)***.

3.2.1.4 Optimisation of Sample Preparation for Cytokine Detection

Initial cytokine quantification was performed using under-diluted culture supernatants (1:2), resulting in most values falling outside of the assay's detection range. Upon further review of the assay results and manufacturer guidelines, a 1:5 dilution was identified as optimal to account for high analyte concentrations present in stimulated samples and to bring samples within the limits of detection. In addition, air bubbles were observed during the final step where the reading buffer was added, which can interfere with signal detection. To address this, reverse pipetting was incorporated into the study protocol to minimise bubble formation and improve assay consistency.

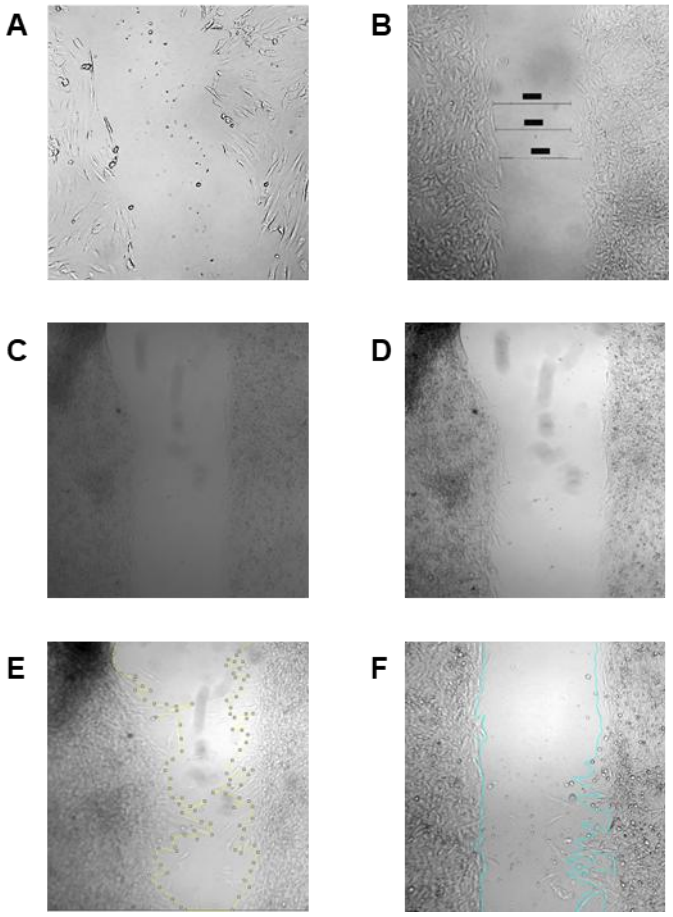

Figure 4.2: *Optimisation of wound healing assay and image analysis methods. Representative light microscope images of wound healing assays to detect HDF migration via* ***(A)*** *conventional scratch assay and* ***(B)*** *2-well silicone inserts. Contrast enhancements of wound healing images demonstrating* ***(C)*** *original image resolution and* ***(D)*** *enhanced contrast resolution. ImageJ analysis refinements depicting* ***(E)*** *polygon and* ***(F)*** *free-hand tracing methods for wound area delineation.*

3.2.2 OTD treatment does not affect fibroblast migration at concentrations of 0.75mM OTD and below

Human dermal fibroblast (HDF) cells were treated with different concentrations of OTD to assess its effect on fibroblast migration using a wound healing assay. Dermal fibroblast migration was not affected at concentrations of 0.25, 0.5 and 0.75mM, with no significant difference between treated samples and untreated control within 24 hours. Treatment at concentrations of 1 and 1.25mM for 24 hours resulted in a dose dependent arrest in fibroblast migration, greatest at 3-6h. At 3h an increase in percentage wound area of 6% and 8.5%

observed in the 1mM and 1.25mM groups, respectively, compared with an 11% decrease for the control group. At 24 hours, a significant delay in wound migration persisted at 1mM ($p < 0.05$) and 1.25mM ($p < 0.001$) with a remaining percentage wound area of 14.5% and 25% observed at 24 hours respectively, compared with 2.34% in the control ***(Figure 3.3)***. Observation was continued up to 48 hours, which revealed wound resolution at 36 hours in the 1mM group and persistence of delayed migration beyond 48 hours in the 1.25mM group ($p<0.01$) ***(Supplementary Figure 1)***.

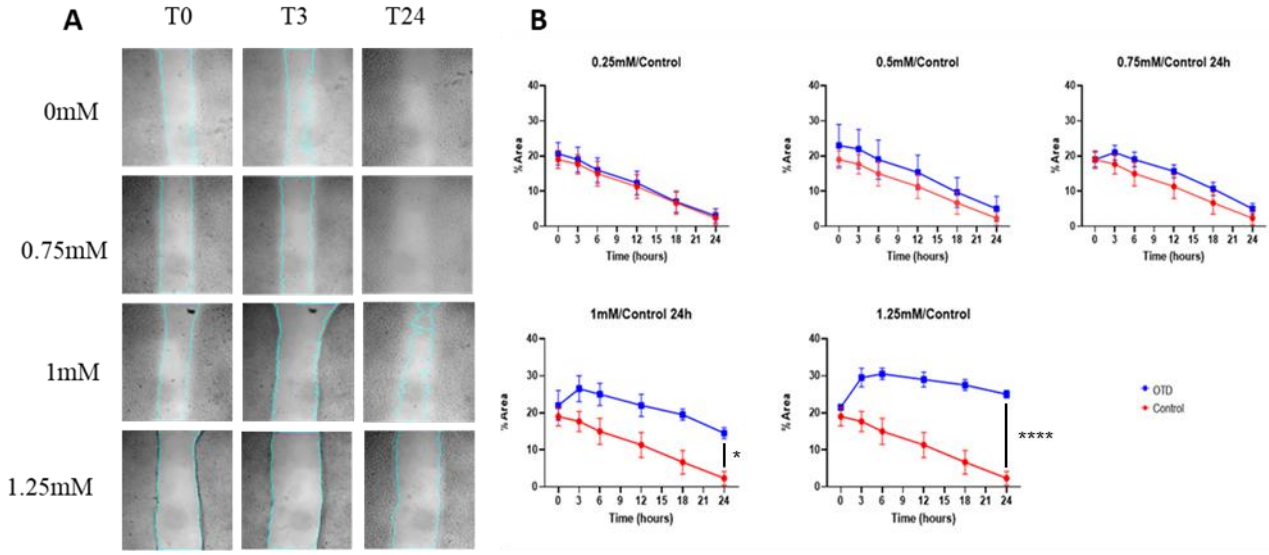

Figure 3.3 *: Effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) treatment on Human Dermal Fibroblast (HDF) cell migration measured by in vitro wound healing assay.* ***(A)*** *Representative light microscope images of wound healing assays to detect HDF migration after OTD treatment. Wound area was evaluated using Image J version 1.53t and a wound area calculator* ***(B)*** *Percentage wound area was quantitatively analysed (n=3) over time for each concentration of OTD compared to control. Two-way ANOVA followed by Bonferroni's post hoc test to correct for multiple comparisons were used to calculate the p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), and data are presented as mean values ± SEM of three independent experiments in triplicate.*

3.2.3 OTD reduces secretion of proinflammatory cytokines by HDF cells

To assess the effect of OTD on inflammatory cytokine secretion by HDF cells, OTD treatments were applied with and without IL-1α stimulation for 24 hours before quantifying IL-6 and IL-8 levels in their respective media. A suppression in proinflammatory cytokine production to baseline levels was observed in both the IL-6 **(*Figure 3.4A)*** and IL-8 **(*Figure 3.4B)*** groups. IL-6 and IL-8 levels in stimulated samples showed no significant difference compared to the unstimulated control group (OTD^{-}/$IL\text{-}1\alpha^{-}$) at concentrations of 0.5mM OTD and above. Similarly, cytokine levels were compared between stimulated ($IL\text{-}1\alpha^{+}$) and unstimulated ($IL\text{-}1\alpha^{-}$) samples at each OTD test concentration. There was no significant difference between stimulated and unstimulated samples above 0.75mM OTD for IL-6 ***(Figure 3.4C)*** and above 0.5mM OTD for IL-8 ***(Figure 3.4D)***.

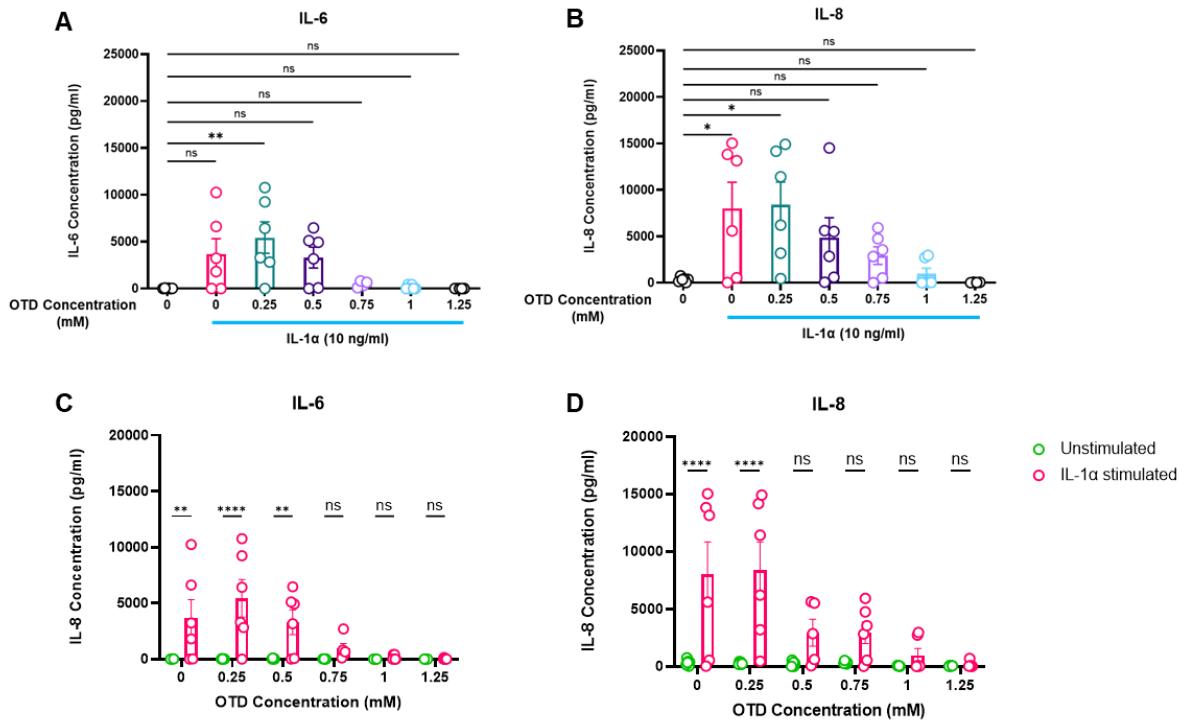

Figure 3.4: *Quantification of inflammatory cytokine release in Human Dermal Fibroblast (HDF) cells treated with 1,4,5-Oxathiazinane-4,4-dioxide (OTD). IL-1α stimulated samples were compared to the unstimulated control group for* ***(A)*** *IL-6 (n=6) and* ***(B)*** *IL-8 (n=6) levels in culture media after 24 hours. IL-1α stimulated samples were compared to unstimulated samples at each treatment concentration for* ***(C)*** *IL-6 (n=6) and* ***(D)*** *IL-8 (n=6) finding a suppression in finding a suppression in cytokine levels above 0.75mM and 0.5mM, respectively. Cytokine levels were quantified using a multi-spot assay obtained from MesoScale Discovery (MSD). One-way ANOVAs followed by Bonferroni's post hoc test to correct for multiple comparisons were used to calculate the p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), following removal of significant outliers, using a Grubbs Test* ***(Supplementary Table 1).*** *Data is presented as mean values ± SEM of six independent experiments in triplicate.*

3.2.4 OTD does not affect cell viability of Human Dermal Fibroblasts

Treatment of HDF cells with OTD at all concentrations did not affect cell viability. Following 24 hours of OTD treatment, cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Relative optical density (OD) value, corresponding to cell viability, was compared for each concentration against the control (culture-media treated cells), demonstrating no significant reduction in viability within 24 hours following statistical analysis using one-way ANOVA ***(Figure 3.5A)***. Similarly, when cell viability was analysed over time (24h, 48h, 72h) there was no significant change in viability within 72 hours **(*Figure 3.5B*)**.

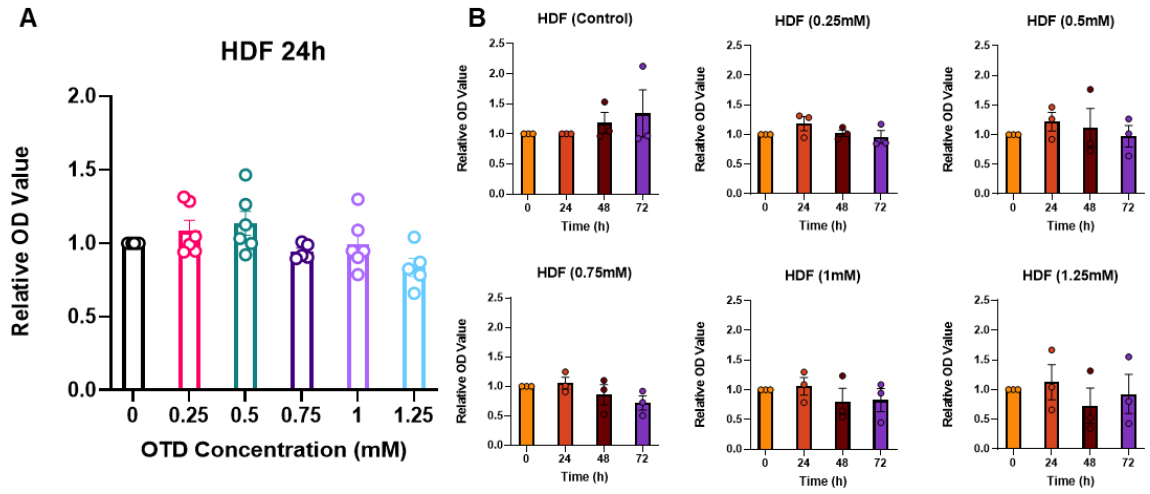

Figure 3.5: *Effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) treatment on cell viability in Human Dermal Fibroblast (HDF) cells. HDF cell viability for each concentration of OTD was compared to control* ***A)*** *at 24 hours (n=6) and* ***(B)*** *over time (n-3) up to 72 hours. One-way and Two-way ANOVAs followed by Dunnett's post hoc test to correct for multiple comparisons were used to calculate the p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), following removal of significant outliers, using a Grubbs Test* ***(Supplementary Table 2).*** *Data is presented as mean optical density values ± SEM of at least three independent experiments in triplicate.*

3.3 Discussion

Adverse effects of adjuvant therapies on physiological cells often remain undetected until the clinical stages of drug development. In this study, we evaluated the effects of the anti-neoplastic agent, OTD, on a HDF cell line, demonstrating that at an optimal concentration of 0.75mM OTD suppressed proinflammatory cytokine release while preserving migratory function and cell viability. These findings support the use of OTD in an oncological setting with careful attention given to optimal dosing.

In vivo mouse studies demonstrated adequate tolerability of OTD with acute and chronic toxicity demonstrated at concentrations of 2000mg/kg body weight and 1000mg/kg body weight, respectively (Buchholz *et al.*, 2017). A human trial, assessing the effect of OTD in combination with gemcitabine, is currently underway, utilising a dose escalation method to determine the optimal treatment dose with test doses ranging between 250mg and 30g of OTD (NIH, 2024). While our study demonstrated an optimal concentration of 0.75mM *in vitro*, *in vivo* concentrations may differ significantly due to metabolic bioavailability considerations (Chow, 2014).

Our study firstly assessed migratory function using a wound healing assay. This showed that at concentrations below 0.75mM, OTD does not affect wound healing. Treatment with higher concentrations of 1mM or 1.25mM significantly delayed wound closure with an increase in percentage wound area seen at 3h of 6% and 8.5% respectively and non-closure observed at both concentrations within 24 hours. Total percentage wound area decreased by 85.5% at 1mM and 75% at 1.25mM compared with 97.66% in the control group. Other studies demonstrate similar findings in cancer cell lines, with OTD reducing migration in SCC cells at lower concentrations of 0.1 and 0.2mM, with closure of 26.93 - 52.74% at the maximal concentration compared with 93.64 – 99.03% in the control group (Barras *et al.*, 2023).

We further determined the effect of OTD on proinflammatory cytokines by applying OTD treatments to stimulated (IL-1α^{+}) and unstimulated (IL-1α^{-}) HDFs and quantifying IL-6 and IL-8 levels. IL-1α is typically released by injured cells following wounding, acting on dermal fibroblasts to induce proinflammatory cytokine release (Cordier-Dirikoc *et al.*, 2022) and was used in this study to stimulate the production and release of IL-6 and IL-8.

This study demonstrated that concentrations of OTD exceeding 0.5mM effectively reduce IL-6 and IL-8 to baseline. Reducing the secretion of inflammatory cytokines may enhance outcomes in melanoma patients, as elevated levels of IL-6 and IL-8 have been shown to promote immunosuppression, tumour growth, metastasis, and angiogenesis, ultimately leading to an unfavourable prognosis (Martinović *et al.*, 2024; Srivastava *et al.*, 2015; Y. Wang *et al.*, 2022). Majchrzak-Stiller *et al.* propose that the antineoplastic effects of OTD are linked to its inhibitory effects on proinflammatory NF-kB signalling pathways (Majchrzak-Stiller *et al.*, 2023). Likewise, Taurolidine has demonstrated an attenuation in perioperative inflammation in metastatic colon cancer through a reduction in pro-inflammatory cytokine release (Redmond *et al.*, 2018) as well as similarly regulating NF-kB signalling in animal models (Lv *et al.*, 2022).

Excessive proinflammatory signalling and release following surgery can create a supportive environment for tumour growth and metastasis by promoting a compensatory immunosuppressive response (Tang *et al.*, 2020). Inflammation is required for adequate anti-microbial function. Therefore, further studies are required to ascertain the impact of OTD on immune cells, to ensure preservation of physiological immune function.

We further analysed cell viability in HDF cells following OTD treatment. Our results showed that OTD does not significantly affect HDF cell viability at all concentrations within 72 hours. Previous studies have demonstrated a cytotoxic effect of OTD on pancreatic cancer (Majchrzak-Stiller *et al.*, 2023), breast cancer (Jinih *et al.*, 2021), squamous cell carcinoma

(Barras *et al.*, 2023) and malignant peritoneal mesothelioma (Baron *et al.*, 2022) cells. However, the exact molecular pathways involved are undetermined. Majchrzak-Stiller *et al.* suggest that cytotoxic effects on pancreatic cancer cells are mediated by inhibition of energy metabolism and downregulation of NF-kB transcriptional activity. (Majchrzak-Stiller *et al.*, 2023) Our results indicate that the mechanism of OTD's cytotoxic action may be specific to cancer cells, an optimal characteristic of adjuvant therapy, however, further studies are required on alternative cell types.

In conclusion, this study demonstrates that treatment with OTD *in vitro* reduces proinflammatory cytokine production in HDF cells while preserving migratory function and cell viability at a concentration of 0.75mM or less. Our data suggests that there are no cytotoxic effects of OTD at a concentration of 0.75mM or less on HDF cells. This led to our next hypothesis, which was the effect of OTD at a concentration of 0.75mM on melanoma cancer cells.

Chapter 4: Examination of the antineoplastic effects of OTD in primary and metastatic melanoma *in vitro*

4.1 Introduction

Melanoma accounts for the largest proportion of skin cancer related deaths in Ireland at 69.4% despite only accounting for 9% of all skin cancers (NCRI, 2017). Incidence is currently on the rise, with 1211 cases reported nationally in 2021 compared with 987 cases in 2015, with an expected further increase of 55% by the year 2045 (NCRI, 2019). While previously demonstrating a higher incidence in women, rates among men have shown a significantly more dramatic increase of 8.83 to 21.78 cases per 100,000 compared with 13.9 to 21.21 cases per 100,000 in women from 1994 to 2021 (NCRI, 2017) (***Figure 4.1***). This increase is reflected on a global scale, however, incidence in Ireland is significantly higher than the global average due to several factors, including fair-skinned population and suboptimal protection against ultraviolet (UV) radiation (Gibson *et al.*, 1997; WCRF, 2024).

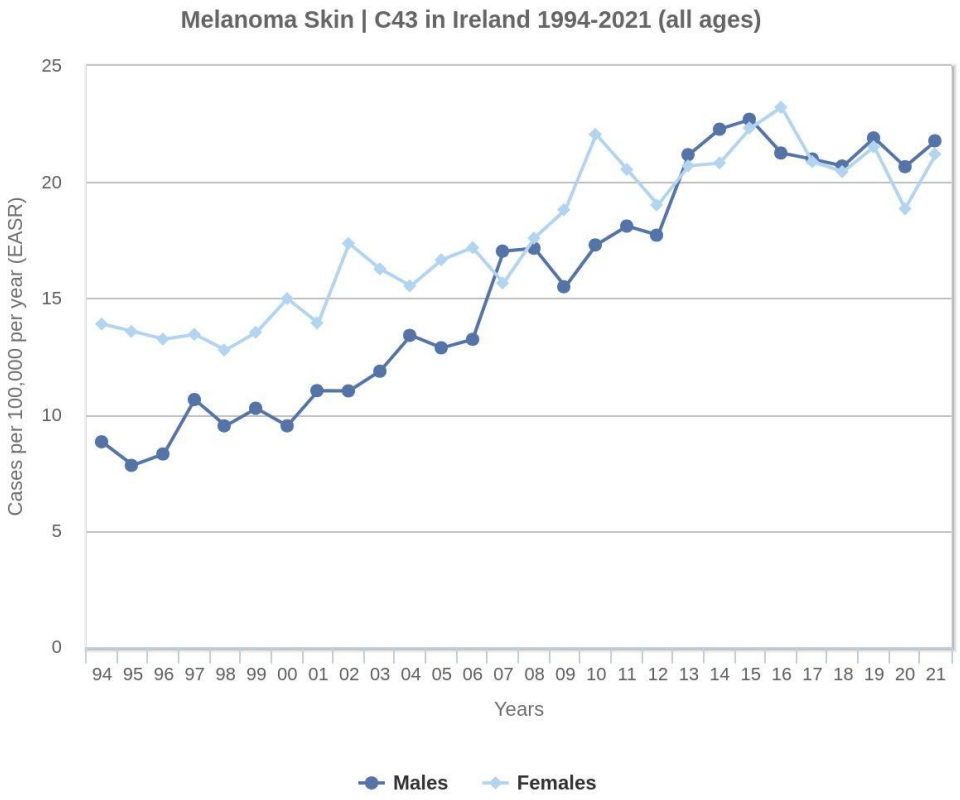

Figure 4.1: *Melanoma incidence among males and females in Ireland 1994 - 2021 (NCRI)*

Melanoma is a particularly aggressive disease with a high propensity for recurrence and metastasis, leading to significant developments in adjuvant therapies, including immune checkpoint inhibitors, targeted therapy, radiotherapy and chemotherapy (Eljilany *et al.*, 2023; Faries *et al.*, 2013). Metastasis in melanoma is classically explained using the Clark model, relying on a linear progression whereby metastasis occurs late in the progression of disease (Clark *et al.*, 1984). However, recent evidence has suggested that the metastatic process may begin much earlier in some cases, progressing in parallel to that of the primary tumour (Shain and Bastian, 2016). Therefore, the use of neoadjuvant and adjuvant treatments to prevent metastasis may be necessary in early melanoma, despite currently being reserved for high risk patients (Eljilany *et al.*, 2023).

Current adjuvant therapies in melanoma target immune checkpoints such as programmed cell death protein-1 (PD-1) and cytotoxic T lymphocyte associated protein-4 (CTLA-4) (He *et al.*, 2022) and mutation-dependent pathways such as the mitogen-activated protein kinase (MAPK) pathway activated in BRAF-mutated melanoma (Long *et al.*, 2017). While improved outcomes and prolonged survival have been demonstrated with adjuvant therapy in melanoma, frequent instances of resistance have been observed, necessitating the development of alternative or combined treatment options (Wu and Singh, 2011). Mitochondrial dysfunction has emerged as a key facilitator of resistance (Avagliano *et al.*, 2020) as well as accelerating tumour progression and metastasis (LeBleu *et al.*, 2014). In fact, melanoma stem cells, involved in tumour dormancy and metastasis, have demonstrated a high mitochondrial biomass compared with non-stem melanoma cells *in vitro* (Fontana *et al.*, 2024).

1,4,5-Oxathiazinane-4,4-dioxide, (OTD) is an antitumourigenic substance with studies showing antineoplastic effects in several cancers, including melanoma (Gambichler *et al.*, 2023). Human trials are currently in phase 1/2 of development for its application in combination with gemcitabine in advanced pancreatic cancer (Kasi and Iglesias, 2022). OTD has been shown to exert its antineoplastic effect via delays in cancer cell migration, proliferation (Barras *et al.*, 2023) and targeting of the glycolysis pathway via glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibition (Majchrzak-Stiller *et al.*, 2023). Glycolytic inhibition results in the inability of products of glycolysis to enter mitochondria, resulting in mitochondrial dysfunction, oxidative stress and programmed cell death (Lazarev *et al.*, 2020). However, the direct effects of OTD on mitochondria have yet to be studied.

Our present study aims to evaluate the antineoplastic effects of OTD on melanoma cells *in vitro*. Emerging evidence supports the initiation of metastatic processes in parallel with tumour progression in certain melanoma cases (Shain and Bastian, 2016). Therefore, for application as

a surgical adjuvant, it was important to evaluate both primary and metastatic cell lines due to inherent biological differences between them.

4.2 Results

4.2.1 Optimisation of Experimental Conditions

A series of preliminary optimisation and validation experiments were conducted to refine experimental conditions for viability and flow cytometry assays. These steps were designed to address cell line-specific handling issues, improve assay sensitivity and ensure data accuracy and reproducibility across subsequent experiments.

4.2.1.1 Optimisation and Validation of Cell Line Models

During MTT viability assays using the WM164 melanoma cell line, cells demonstrated poor detachment from the culture plate despite increasing trypsin volumes and incubation times. Mechanical scraping was attempted but yielded a very low cell recovery. The issue was traced to cell culture media and preparation of fresh media resolved the problem, enabling consistent detachment and viable cell recovery.

Similar challenges arose when handling WM164 cells for the Mitotracker assay, where a marked reduction in cell viability was observed after detachment, despite cells appearing healthy on microscopic inspection of the culture flask. Troubleshooting measures included testing different trypsin concentrations (0.05% and 0.25%), which did not produce significant differences in detachment efficiency. To enhance trypsin neutralisation, the ratio of media to trypsin was increased from 1:1 to 2:1, compensating for the lower Foetal Bovine Serum (FBS) concentration in WM164 media. Trypan blue was also filtered to assess whether this would improve cell counting accuracy. These adjustments did not improve yield and instead, fresh cell stocks were thawed for subsequent experiments, resulting in more consistent outcomes.

4.2.1.2 Optimisation of Annexin/PI Assay Method

Early Annexin V/PI assays produced atypical results and a high degree of variability between biological replicates ***(Figure 4.2)*** prompting re-examination of the experimental protocol. This effect was attributed to experimental error due to discarding of the cell supernatant prior to flow cytometry analysis and inadvertently discarding non-viable cells. The analysis was subsequently repeated to include analysis of the cell supernatant, ensuring full representation of the cell population. This refinement improved the accuracy of apoptosis and necrosis measurements, particularly at higher treatment concentrations where non-adherent, dead cells comprised a larger fraction of the population.

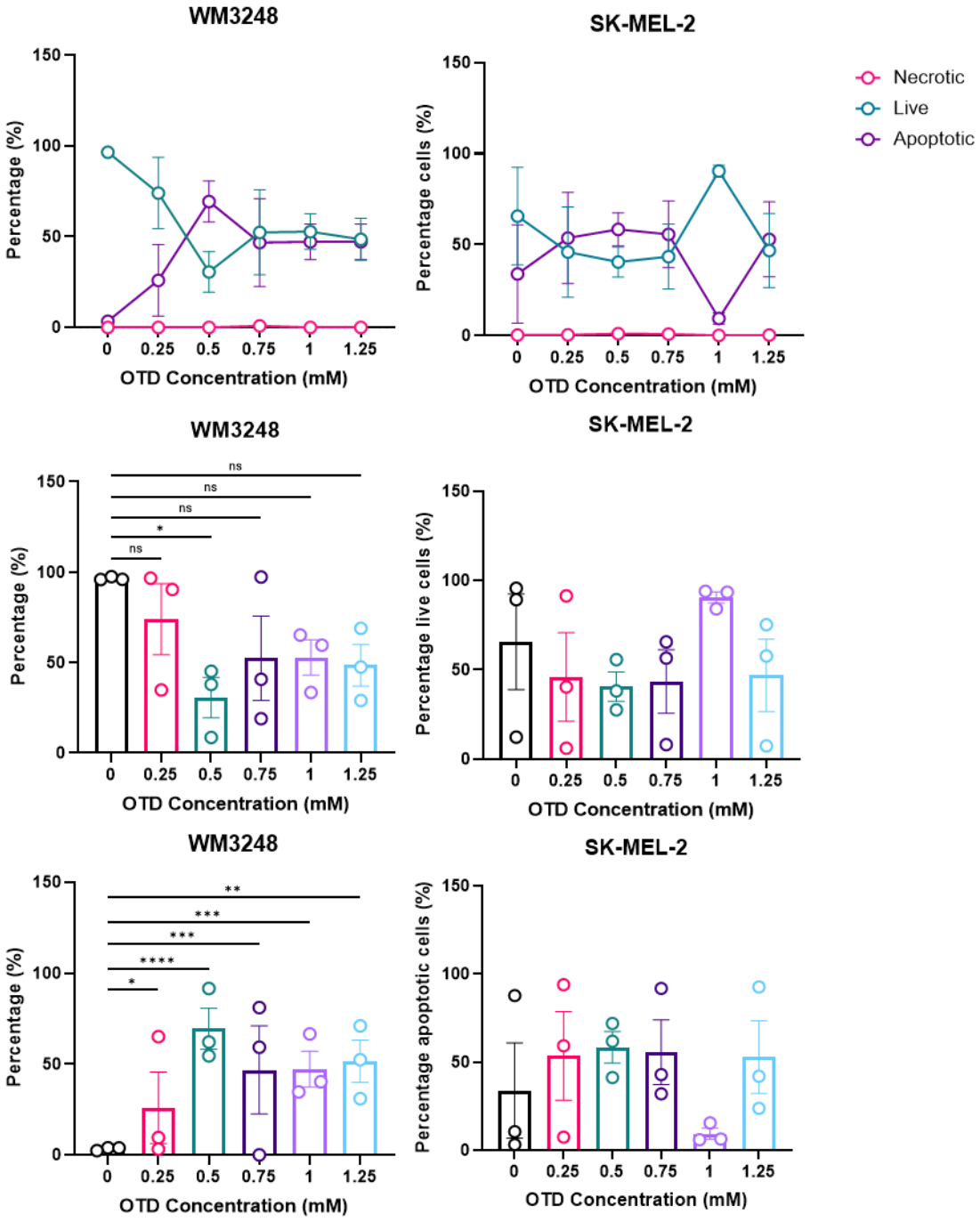

Figure 4.2: *Annexin/PI apoptosis optimisation assay examining the effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) on primary and melanoma cell viability, apoptosis and necrosis. Graphical representation comparing proportions of live, apoptotic and necrotic* ***(A)*** *WM3248 (n=3) and* ***(B)*** *SK-MEL-2 (n=3) melanoma cells at each concentration of OTD. Graphical representation of live cell percentages in* ***(C)*** *WM3248 (n=3) and* ***(D)*** *SK-MEL-2 (n=3) melanoma cells at each concentration of OTD. Graphical representation of apoptotic cell percentages in* ***(E)*** *WM3248 (n=3) and* ***(F)*** *SK-MEL-2 (n=3) melanoma cells at each concentration of OTD. One-way ANOVAs followed by Bonferroni's post hoc test to correct for multiple comparisons were used to calculate the p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), and data are*

presented as mean percentage values ± SEM of three independent experiments in triplicate. Flow cytometry gating strategy is detailed in ***Supplementary Figure 2****.*

4.2.1.3 Optimisation of Mitotracker Assay Method

Initial Mitotracker Green experiments produced unusually high fluorescence intensity across all samples, suggesting oversaturation of the fluorescent signal. To address this, the working concentration of Mitotracker dye was reduced by 25% in subsequent experiments. This adjustment resulted in more defined signal resolution, thereby improving the reliability of mitochondrial mass quantification.

4.2.2 OTD induces cell death in primary melanoma cells

OTD induced cell death was first assessed in primary A375 melanoma cells at 24, 48 and 72 hours using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay whereby cell viability was inferred from relative OD values.Treatment with OTD for 24 hours resulted in significant cell death at all concentrations, with 0.25, 0.5, 0.75, 1 and 1.25mM OTD inducing 64.6%, 75.5%, 77.1%, 76.7% and 77.7% reductions in relative OD value, respectively, compared with culture-medium treated control ($p<0.001$, $p<0.0001$) ***(Figure 4.3A)***. Further incubation for 48 and 72 hours demonstrated no further time dependent reduction in cell viability ***(Figure 4.3C)***.

We next treated primary WM3248 melanoma cells with OTD for 24 hours. Treatment resulted in a similar reduction in cell viability at all concentrations, with 0.25, 0.5, 0.75, 1 and 1.25mM inducing 40.6%, 66.1%, 70%, 64.1% and 58% reduction in relative OD value, respectively, compared with culture-medium treated WM3248 melanoma cells ($p<0.05$, $p< 0.001$) ***(Figure 4.3B)***.

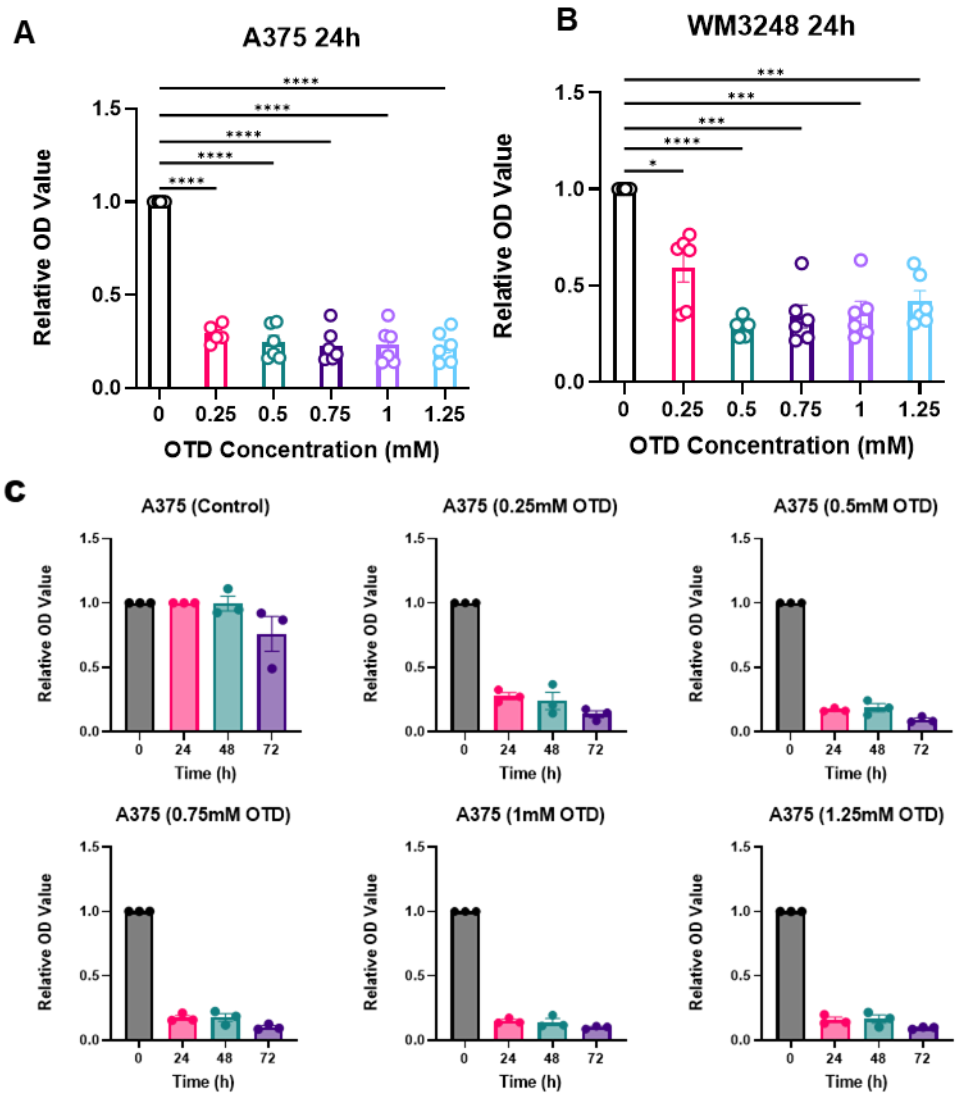

Figure 4.3: *Effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) treatment on cell viability in primary melanoma cells. Primary melanoma cell viability for each concentration of OTD was compared to control at 24 hours in* ***(A)*** *A-375 melanoma cells (n=6) and* ***(B)*** *WM3248 melanoma cells (n=6).* ***(C)*** *Cell viability for each concentration of OTD was compared over time in A-375 melanoma cells (n-3) up to 72 hours. One-way and Two-way ANOVAs followed by Bonferroni's post hoc test to correct for multiple comparisons were used to calculate the p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), following removal of significant outliers, using a Grubbs Test* ***(Supplementary Table 3).*** *Data is presented as mean optical density values ± SEM of at least three independent experiments in triplicate.*

4.2.3 OTD induces cell death in metastatic SK-MEL-2 cells but not WM-164 cells

We next assessed the effect of OTD on cell viability in metastatic WM164 melanoma cells. Following 24 hours of OTD treatment, cell viability was similarly assessed using MTT assay. No significant reduction in viability was observed at all concentrations within 24 hours compared with culture-medium treated control following statistical analysis using one-way ANOVA ***(Figure 4.4A)***. Similarly, when treatment incubation was increased from 24h to 48h and 72h, no significant change in viability was observed ***(Figure 4.4C)***.

In contrast, OTD treatment in metastatic SK-MEL-2 melanoma cells revealed a significant reduction in cell viability at all concentrations following 24h exposure to OTD treatment. OTD concentrations of 0.25, 0.5, 0.75, 1 and 1.25mM induced a reduction in relative OD value of 59.2%, 79.1%, 54.9%, 55.4% and 63.3% respectively ($p<0.001$, $p<0.0001$) ***(Figure 4.4B)***

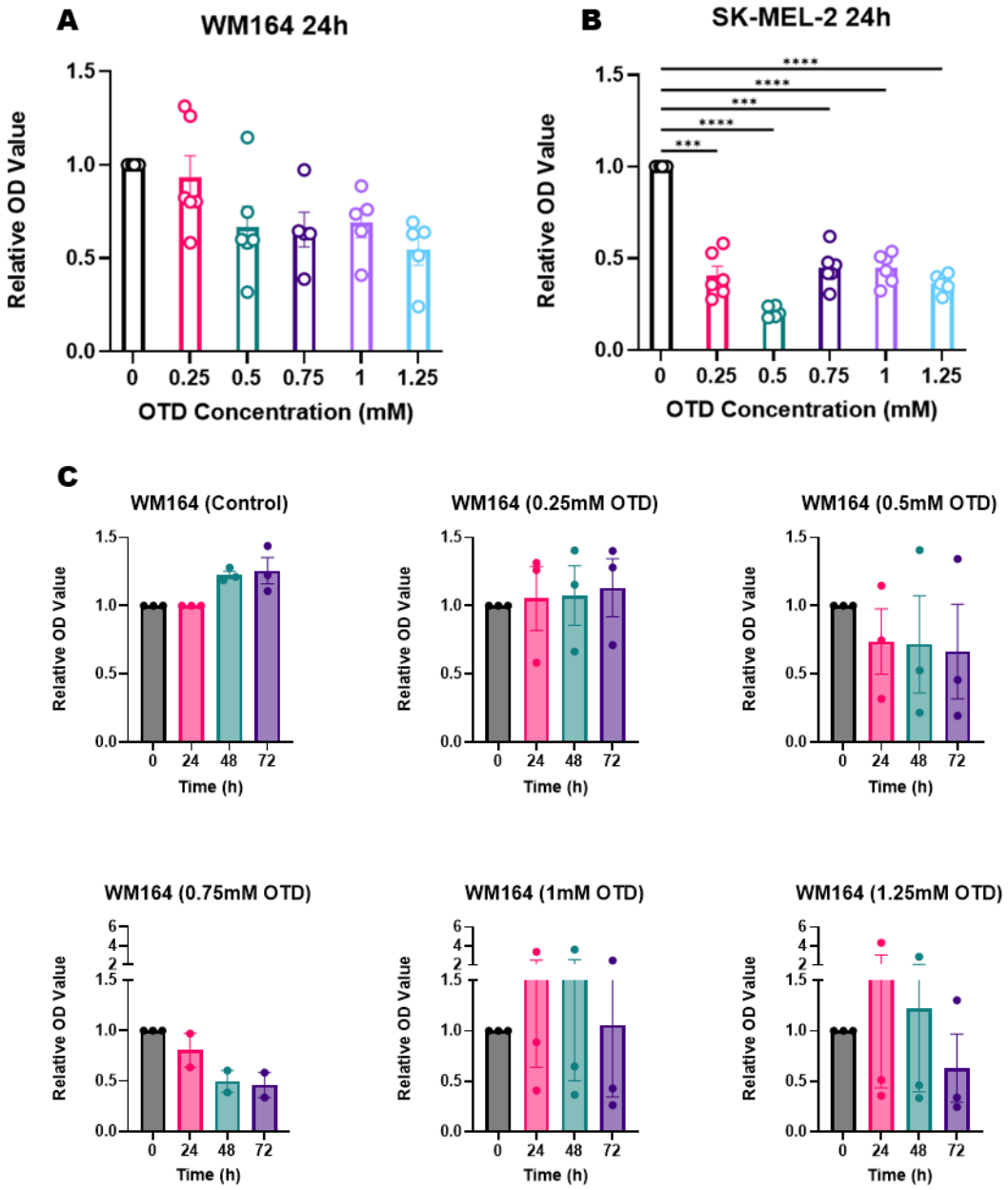

Figure 4.4: *Effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) treatment on cell viability in metastatic melanoma cells. Metastatic melanoma cell viability for each concentration of OTD was compared to control at 24 hours in* ***(A)*** *WM-164 melanoma cells (n=6) and* ***(B)*** *SK-MEL-2 melanoma cells (n=6).* ***(C)*** *Cell viability for each concentration of OTD was compared over time in WM3248 melanoma cells (n-3) up to 72 hours. One-way and Two-way ANOVAs followed by Dunnett/Bonferroni post hoc test to correct for multiple comparisons were used to calculate the p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), following removal of significant outliers, using a Grubbs Test* ***(Supplementary Table 4).*** *Data are presented as mean optical density values ± SEM of at least three independent experiments in triplicate.*

4.2.4 OTD induces cell death via an apoptotic pathway in both primary and metastatic melanoma cells

To ascertain whether OTD induces cell death via apoptosis and/or necrosis in melanoma cells OTD treatment was applied to primary WM3248 and metastatic SK-MEL-2 melanoma cells for 24 hours, following which, cell necrosis and apoptosis were measured using Annexin V/FITC and propidium iodide (PI) assay ***(Figure 4.5).***

Following OTD treatment in WM3248 cells, a significant reduction was observed in the viable cell proportion in parallel with an increase in apoptotic cells with 0.25, 0.5, 0.75, 1 and 1.25mM OTD, inducing a reduction in viability of 64.2%, 98.34%, 89.5%, 87.4% and 67.59% respectively. No significant change was observed in necrosis at all concentrations. Notably, the greatest effect on cell viability was observed at 0.5mM with a 98.34% reduction in viable cells corresponding to an increase of 43.8% and 49.2% in early and late apoptotic/necrotic cells, respectively.

In contrast, OTD treatment in metastatic SK-MEL-2 melanoma cells demonstrated a significant reduction in live cells at a concentration of 0.5mM alone with a reduction in live cells of 55.2% as well as an increase in early and late apoptotic/necrotic cells of 24.1% and 31.1% respectively. At all other concentrations, cell viability remained above 50%.

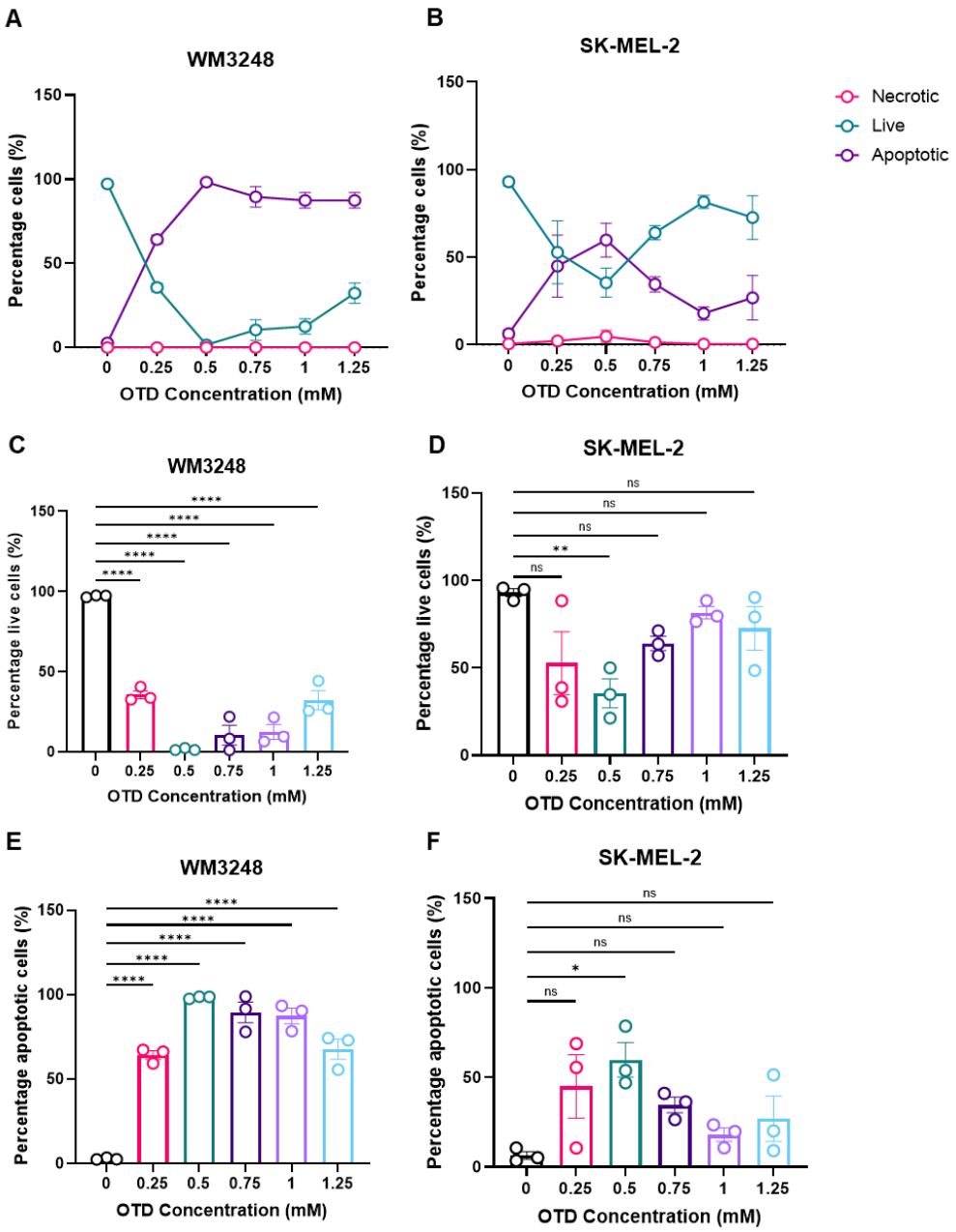

Figure 4.5: *Annexin/PI apoptosis assay demonstrating the effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) on primary and melanoma cell viability, apoptosis and necrosis. Graphical representation comparing proportions of live, apoptotic and necrotic* ***(A)*** *WM3248 (n=3) and* ***(B)*** *SK-MEL-2 (n=3) melanoma cells at each concentration of OTD. Graphical representation of live cell percentages in* ***(C)*** *WM3248 (n=3) and* ***(D)*** *SK-MEL-2 (n=3) melanoma cells at each concentration of OTD. Graphical representation of apoptotic cell percentages in* ***(E)*** *WM3248 (n=3) and* ***(F)*** *SK-MEL-2 (n=3_ melanoma cells at each concentration of OTD. One-way ANOVAs followed by Bonferroni's post hoc test to correct for multiple comparisons were used to calculate the p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), and data are presented as mean percentage values ± SEM of three independent experiments in triplicate. Flow cytometry gating strategy is detailed in* ***Supplementary Figure 2****.*

4.2.5 OTD does not affect mitochondrial mass in primary or metastatic melanoma cells

Finally, the effect of OTD on mitochondrial mass in primary (WM3248) and metastatic (WM164) melanoma cells was assessed using Mitotracker Green (FITC), a fluorescent probe which binds with free thiol groups of cysteine residues associated with mitochondrial proteins to assess mitochondrial mass (Presley *et al.*, 2003). Following 24 hours of OTD treatment and subsequent flow cytometry analysis, mitochondrial mass was assessed by calculating median fluorescent intensity (MFI) in OTD treated samples compared with culture-media treated control. OTD demonstrated no significant effect on MFI and therefore mitochondrial mass, in either cell line when results were assessed using one-way ANOVA ***(Figure 4.6)***.

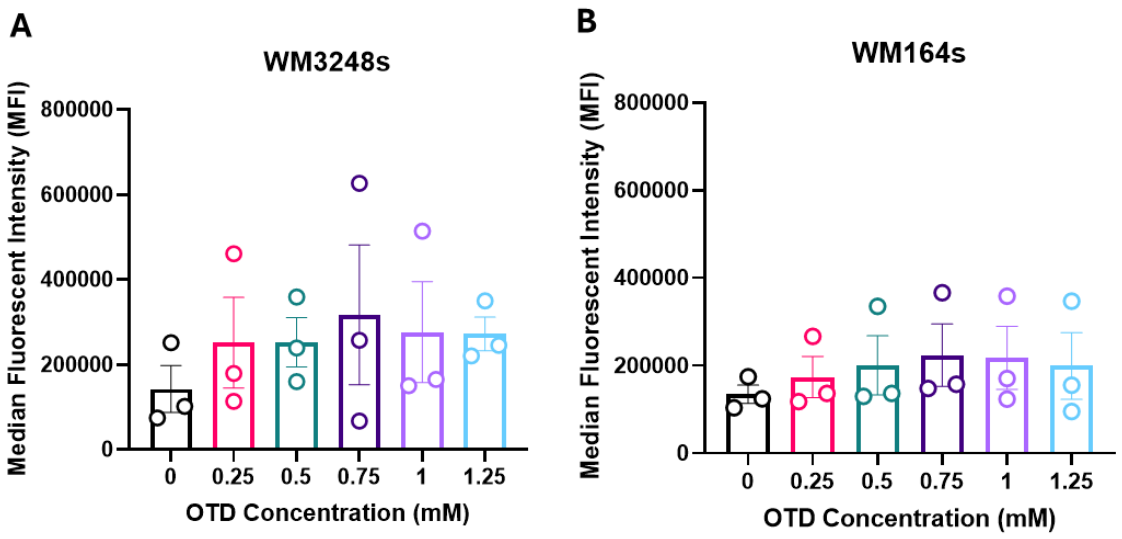

Figure 4.6: *Effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) on mitochondrial biomass in primary and metastatic melanoma cell lines. Graphical representation of mitochondrial biomass for each concentration of OTD compared to control in* ***(A)*** *primary WM3248 and* ***(B)*** *metastatic WM164 melanoma cell lines. One-way ANOVAs followed by Dunnett's post hoc test to correct for multiple comparisons were used to calculate the p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001),*

and data are presented as mean median fluorescent intensity (MFI) values ± SEM of three independent experiments in triplicate. Flow cytometry gating strategy is detailed in ***Supplementary Figure 3****.*

4.3 Discussion

Melanoma is one of the most aggressive cutaneous tumours with an increasing incidence and high propensity for invasion and metastasis (Long *et al.*, 2023). The metastatic process can occur early in the progression of the disease, with an estimated recurrence rate of 20-40% worldwide within 5 years (Peirano *et al.*, 2023), often despite complete surgical excision of the primary tumour. Adjuvant therapies are frequently used to enhance primary treatment modalities, which, in the case of melanoma, is typically surgery. Systemic immune therapy, such as immune checkpoint inhibitors, for example, pembrolizumab and targeted therapies such as nivolumab, are frequently used to treat melanoma and enhance recurrence-free survival by targeting residual micro-metastatic disease (Eljilany *et al.*, 2023). This study evaluated the use of an antineoplastic agent, OTD, as a surgical adjuvant in melanoma.

Our study first assessed the cytotoxicity of OTD on both primary and metastatic melanoma cell lines using an MTT assay. This demonstrated a significant reduction in cell viability at all concentrations in primary A375 and W3248 cells and metastatic SK-MEL-2 cells. In contrast, no significant reduction was observed in metastatic WM164 cells. The MTT assay is commonly employed to assess cell viability; however, it measures metabolic activity, from which viability is inferred. Consequently, the "non-viable" cell population may include quiescent, cytostatic cells (Ghasemi *et al.*, 2021). To provide a more precise evaluation of cell viability, an Annexin V/PI apoptosis assay was utilized.

The Annexin V/PI apoptosis assay was carried out on primary WM3248 and metastatic SK-MEL-2 cell lines. A consistent effect was observed in WM-3248 cells, with a significant reduction in viability observed at all concentrations in both viability studies, however, this was not the case for the SK-MEL-2 group. While a consistent trend was observed in both studies in

the metastatic SK-MEL-2 cell line, the MTT assay demonstrated a significant reduction at all concentrations, whereas the Annexin V/PI study showed significance for a single concentration (0.5mM OTD). As MTT assays infer viability from the degree of metabolic activity, cells in the "non-viable" population may include cytostatic cells (Ghasemi *et al.*, 2021). In contrast, the Annexin/PI assay relies on markers expressed exclusively on dead cells. Therefore, our findings suggest impairment in metabolic activity in lieu of cytotoxicity in SK-MEL-2 melanoma cells at higher concentrations of OTD.

While OTD demonstrates a prominent cytotoxic effect on a variety of cancer cell lines, it has previously been shown that some melanoma cell lines may not respond to OTD treatment. Using the OncoPanel multiplexed cytotoxicity assay, Sofia *et al.* assessed the effect of OTD on over 300 human cancer cell lines, 25 of which were melanoma. Melanoma was the only category with cell line(s) that did not respond to OTD treatment, exhibiting a maximum effective concentration (EC50) above the highest concentration assessed (2 mM OTD). Specific information regarding the resistant cell line(s) was not provided. In contrast, the EC50 for all other cell types was below 0.15 mM (Sofia *et al.*, 2024).

Surprisingly, in our apoptosis assay conducted on WM3248 and SK-MEL-2 cells, the 0.5mM concentration emerged as the most effective concentration in reducing cell viability, with higher concentrations showing recovery of cell viability. This was similarly reflected in SK-MEL-2 cells in the MTT assay. Baron *et al.* also observed this effect in both JL-1 and MSTO-211H malignant mesothelioma cells, with a greater reduction in cell viability observed at 0.5mM compared with higher concentrations (Baron *et al.*, 2022). In melanoma, Sofia *et al.* demonstrated using *in vivo* murine models, that OTD was more effective at attenuating tumour growth at lower doses of 250mg/kg compared with 500 mg/kg and 1000mg/kg in HS-695T metastatic melanoma (Sofia *et al.*, 2024). The effect of OTD on other cell lines has largely been shown to be dose dependent (Buchholz *et al.*, 2017; Jinih *et al.*, 2021).

A possible reason for this effect is the induction of metabolic switching. Majchrzak-Stiller *et al.* suggest that cytotoxic effects of OTD are mediated by inhibition of glycolysis and downregulation of NF-kB transcriptional activity (Majchrzak-Stiller *et al.*, 2023). While most cancer cells rely on aerobic glycolysis to produce energy, aggressive melanoma demonstrates metabolic plasticity, adapting its metabolism in response to environmental changes to promote survival and tumourigenic potential (Avagliano *et al.*, 2020). Inhibitors of glycolysis, similar to OTD, can induce this effect (Lu *et al.*, 2015; Shiratori *et al.*, 2019), which may account for reduced cytotoxicity at higher concentrations of OTD. Concentrations below 0.5mM may be capable of sufficient glycolytic inhibition to cause cell death without triggering changes in metabolism to pathways unaffected by OTD. Meanwhile, the observed recovery of cell viability at 0.75–1.25mM OTD may be attributed to the induction of metabolic switching to facilitate tumour survival.

Our results demonstrated cell death via an apoptotic pathway for both WM3248 cells and SK-MEL-2 melanoma cells. Although the Annexin V/PI assay accurately measures live and early apoptotic cell populations, cells in the "late apoptotic bracket" may contain a subset of necrotic cells, leading to a falsely high apoptotic rate. Annexin-V/PI flow cytometry markers are unable to definitively distinguish between late apoptotic and necrotic cells, as loss of membrane integrity in both late apoptotic and necrotic cells may lead to concurrent Annexin V/PI staining due to exposure of PS on both the outer cell membrane (apoptotic) and the disrupted inner membrane (necrotic). Physiologically, apoptosis results in programmed cell death followed by subsequent phagocytosis of degradation products, however, due to the absence of immune cells in the *in vitro* culture and thus phagocytosis, secondary necrosis may occur (Berghe *et al.*, 2010).

Our final experiment assessed the effect of OTD on mitochondrial biomass in primary and metastatic cell lines. Fontana *et al.* showed that mitochondrial mass correlates with "stemness"

or metastatic potential in melanoma, assessing mitochondrial content in melanospheres, containing cancer stem cells, compared with adherent melanocytes. In addition to increased mitochondrial mass, melanospheres also demonstrated increased oxidative phosphorylation (OXPHOS) complex expression (Fontana *et al.*, 2024). We intended to compare the effect of OTD on metastatic potential (mitochondrial biomass) between a primary cell line (WM3248) to which OTD was cytotoxic and a metastatic cell line (WM164) resistant to OTDs cytotoxic effects, hypothesising that though OTD may not kill WM164s, it may limit their metastatic potential. However, no significant effect was observed in either cell line.

In conclusion, this study chapter demonstrates the antineoplastic functions of OTD in both primary and metastatic cell lines with potential applications as a surgical adjuvant therapy.

Chapter 5: Investigation of the effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) on CD8+ Lymphocytes *in vitro*

5.1 Introduction

Optimal timing for administration of adjuvant therapy in surgical oncology is a vital consideration and must account for various factors, including tumour characteristics, patient factors and drug features (Gaß *et al.*, 2016; Llovet *et al.*, 2024). Neoadjuvant and adjuvant therapy refer to therapies administered before and after the primary treatment, respectively and can include chemotherapy, hormone therapy, molecular targeted therapy and radiation therapy (West and Jin, 2015). In Ireland, the treatment of advanced or metastatic melanoma may include three cycles of neo-adjuvant pembrolizumab followed by 15 cycles postoperatively administered over a maximum of one year (NCCP, 2018; National Centre for Pharmacoeconomics, 2020).

The administration of adjuvant therapies in the early postoperative period may offer enhanced direct and indirect anti-neoplastic action via cytotoxic effects and inhibition of pro-tumourigenic processes such as postoperative inflammation and immunosuppression (Tang *et al.*, 2020). The initiation of surgery induces a sterile proinflammatory immune response followed by a compensatory immunosuppressive effect, which, in instances of oncological surgery, may promote tumour growth (Tohme et al., 2017b). To combat this, there has been increasing research into the administration of anti-inflammatory agents postoperatively to reduce inflammation and thus prevent progression to immunosuppression (Cheng *et al.*, 2019; Xu *et al.*, 2015; Yeh *et al.*, 2015). However, there is a narrow therapeutic window in which to reduce inflammation without inadvertently promoting immunosuppression. In the immediate

postoperative period several proinflammatory factors are released in response to tissue injury including interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-α (TNF-α) (Grimstad *et al.*, 2011). However, cells and factors involved in the adaptive immune response typically arrive later in the wound repair process, such as T lymphocytes, which recognise intracellular antigens presented by antigen presenting cells (APCs). CD8+ T cells are a cytotoxic subset of αβT lymphocytes, which are important effector cells in anti-microbial and anti-tumour immunity. In mice, they typically show peak levels in wounds at days 7-10 postoperatively (Chen *et al.*, 2014) however, studies in humans are limited. Cytotoxic effects of CD8+ T Lymphocytes are mediated by several factors, including interferon-γ (IFNγ), which enhances anti-tumoural cytotoxic effects as well as potentiating CD8+ T lymphocyte function via autocrine mechanisms (Bhat *et al.*, 2017).

Surgical adjuvant, Taurolidine, has been shown to significantly attenuate postoperative inflammation when administered intraoperatively and early postoperatively in colon cancer. The first dose of the therapy was administered at induction of anaesthesia and continued four times per day for four days (Redmond *et al.*, 2018). OTD is a drug analogue of Taurolidine's active component, Taurultam and shows prominent direct anti-neoplastic effects on a variety of cancers (Baron *et al.*, 2022; Barras *et al.*, 2023; Jinih *et al.*, 2021). While Taurolidine has previously shown a suppression in TNFα and IFNγ release from Peripheral Blood Mononuclear Cells (PBMCs) (Wouters *et al.*, 2022), the effect of OTD on specific adaptive immune cells has yet to be investigated.

Therefore, this chapter aims to characterise the effect of OTD on cytokine release from CD8+ T lymphocytes and thus assess whether therapy with OTD is optimal long term or whether a shorter course would be favourable.

5.2 Results

5.2.1 Optimisation of Experimental Conditions

Preliminary optimisation experiments were conducted to refine procedures related to cell recovery following thawing and to optimise immune cell stimulation protocols. These refinements aimed to ensure sufficient viable cell numbers for downstream analysis and to improve the reliability and sensitivity of immune functional assays.

5.2.1.1 Optimisation of Cell Recovery Post-Thaw

Early experiments revealed a higher than anticipated loss of viable PBMCs during the freeze-thaw process, necessitating the use of greater cell numbers than originally planned. This reduction in cell recovery was likely attributable to cell death occurring during thawing and handling, resulting in suboptimal initial cell concentrations for functional assays. To address this, the initial number of cells allocated for thawing was doubled to compensate for post-thaw losses. This modification ensured that sufficient viable cells were consistently available for stimulation and subsequent analysis for all downstream assays.

5.2.1.2 Optimisation of Immune Cell Stimulation

Initial immune cell stimulation experiments aimed to determine the effect of OTD on CD8+ T lymphocyte function. OTD treatment was applied to PBMCs for a period of 24 hours before stimulating IFNγ production using either 5ug/ml anti-CD3 or 20/1000ng/ml phorbol-12-myristate-13-acetate (PMA)/Ionomycin. Cells were then stained and analysed using flow cytometry to determine the proportions of live, CD8+ and/or IFNγ+ cells. Anti-CD3 stimulation coupled with OTD treatment resulted in an apparent cytotoxic effect in comparison to unstimulated controls ***(Figure 5.1A).*** This effect is most apparent at 0.25mM OTD, demonstrating the greatest variation in viability between the unstimulated and stimulated samples, 58.2% and 29.2% respectively. However, treatment with 0.5mM OTD followed by

anti-CD3 stimulation resulted in the largest reduction in viability, with 21.7% live cells remaining.

In contrast, PMA/Ionomycin stimulation showed a minimal difference in viable cell proportion compared to unstimulated controls ***(Figure 5.1B)***. However, a similar trend in cytotoxicity due to OTD treatment was observed, with the greatest reduction also seen at 0.5mM OTD, 33.7% and 33.6% viability in unstimulated and stimulated samples, respectively.

Minimal production of IFNγ was observed in both the anti-CD3 and PMA/Ionomycin stimulated samples, with the maximum proportion produced being 2.5% in the PMA/Ionomycin stimulated control ***(Figure 5.1C/D)***. On review of the experimental design, we hypothesised that Brefeldin-A (BFA) stock solution, which functions to prevent intracellular cytokine release, may not have been adequately thawed. BFA is formulated in dimethylsufoxide (DMSO), which freezes below 18ºC (stored at 4ºC) thus, if partially frozen, an adequate concentration may not have been formulated in the working solutions.

It should be noted that optimisation testing was carried out on one biological replicate and thus is not sufficiently powered for statistical analysis.

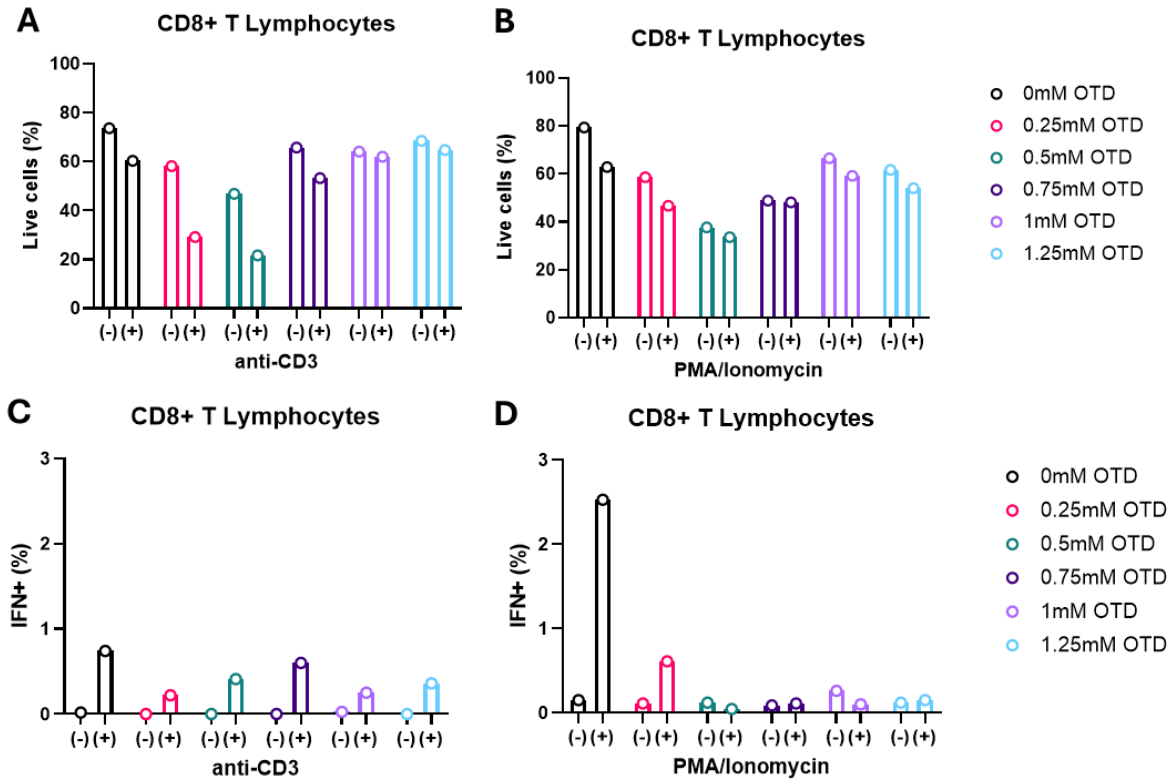

Figure 5.1: *Preliminary experiments assessing the effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) on cell viability and IFNγ release in anti-CD3 and PMA/Ionomycin stimulated CD8+ T lymphocytes. Percentage cell viability for* ***(A)*** *anti-CD3 and* ***(B)*** *PMA/Ionomycin stimulated cells at each concentration of OTD was compared to respective unstimulated controls. Percentage IFNγ production in CD8+ T lymphocytes for* ***(C)*** *anti-CD3 and* ***(D)*** *PMA/Ionomycin stimulated cells at each concentration of OTD was compared to respective unstimulated controls. Values are expressed as single percentage values from one experiment and thus are not powered for statistical analysis. Flow cytometry gating strategy is detailed in* ***Supplementary Figure 4****.*

5.2.2 OTD suppresses cytokine release in PMA/Ionomycin stimulated CD8+ T cells

Taking findings from our optimisation experiments into account, PBMCs from four healthy donors were treated with OTD and stimulated with 20/1000ng/ml PMA/Ionomycin prior to staining and flow cytometry analysis. Suppression of IFNγ production to baseline was observed at all concentrations of OTD ***(Figure 5.2A)***. IFNγ levels in the stimulated control without OTD treatment showed a significant increase of 9.6% compared to the unstimulated control (p**** <0.0001). However, no significant difference was observed between stimulated samples treated with OTD compared with their respective controls.

To assess the effect of PMA/Ionomycin stimulation and/or OTD treatment on cell viability, the CD8+ live cell proportion was determined. Mean live cell proportion remained above 60% at all concentrations of OTD with or without stimulation. No significant difference was observed with regard to OTD concentration or PMA/Ionomycin stimulation ***(Figure 5.2B)***.

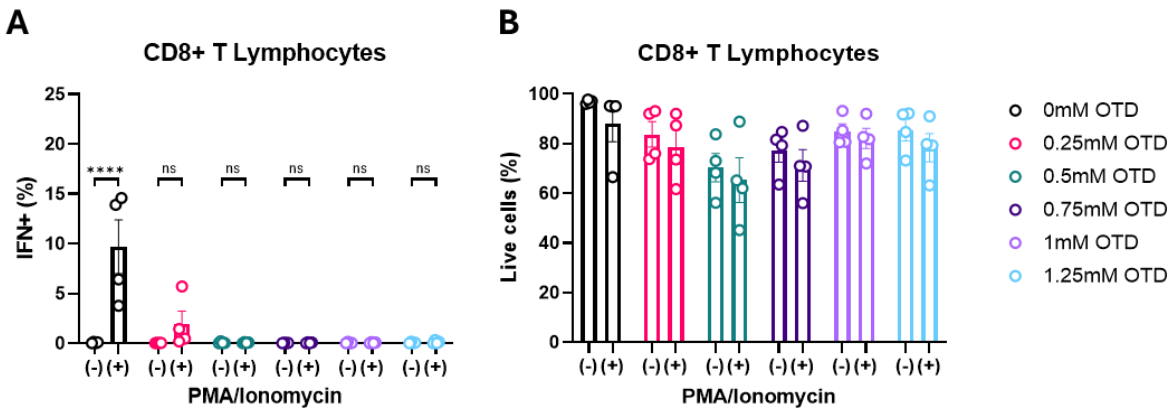

Figure 5.2**: Effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) on cell viability and IFNγ release in PMA/Ionomycin stimulated CD8+ T lymphocytes. **(A)** Percentage IFNγ production in CD8+ T lymphocytes for PMA/Ionomycin stimulated cells at each concentration of OTD was compared to respective unstimulated controls (n=4). **(B)** Percentage cell viability PMA/Ionomycin stimulated cells at each concentration of OTD was compared to respective unstimulated control (n-4). Results were analysed using one-way ANOVA compared with respective controls. p* < 0.0001. One-way ANOVAs followed by Bonferroni's post hoc test to correct for multiple comparisons were used to calculate the p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), following removal of significant outliers, using a Grubbs Test **(Supplementary Table 4).** and data are presented as mean percentage values ± SEM of four independent donors. Flow cytometry gating strategy is detailed in **Supplementary Figure 4**.*

5.2.3 Reduction in anti-CD3 concentration reduces cytotoxic effect and preserves IFN-γ production in CD8+ T Cells

A similar experiment was carried out using anti-CD3 stimulation at a reduced concentration of 2ug/ml following results of optimisation. PBMCs from the same four donors were treated with OTD and stimulated with 2ug/ml anti-CD3 prior to staining and flow cytometry analysis. Improved cell viability was observed with the mean live cell proportion remaining above 60% at all concentrations of OTD with or without stimulation ***(Figure 5.3B)***. Lowering the anti-CD3 concentration preserved IFNγ production, with levels in the stimulated control without OTD treatment showing a significant increase of 2.2% compared to the unstimulated control

(p**<0.001). At all test concentrations of OTD, suppression of IFNγ production was observed with no significant difference between stimulated samples treated with OTD compared with their respective controls ***(Figure 5.3A)***.

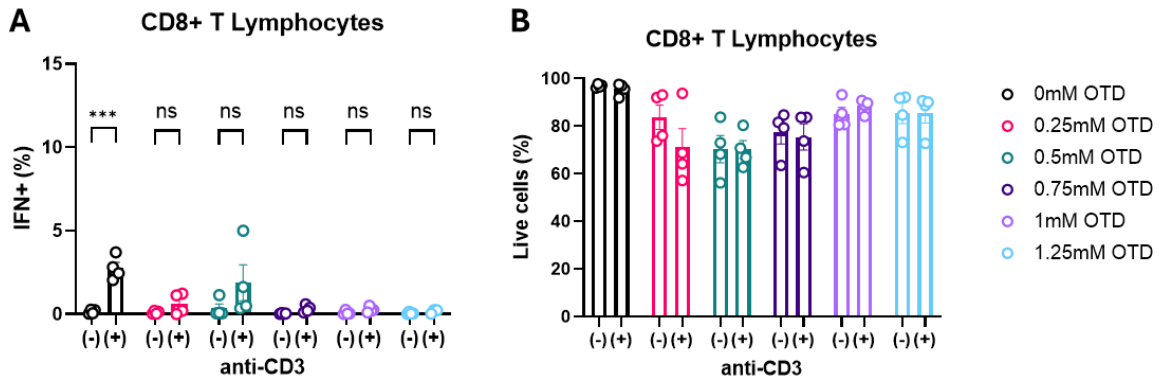

Figure 5.3: *Effect of 1,4,5-Oxathiazinane-4,4-dioxide (OTD) on cell viability and IFNγ release in anti-CD3 stimulated CD8+ T lymphocytes.* ***(A)*** *Percentage IFNγ production in CD8+ T lymphocytes for anti-CD3 stimulated cells at each concentration of OTD was compared to respective unstimulated controls (n=4).* ***(B)*** *Percentage cell viability anti-CD3 stimulated cells at each concentration of OTD was compared to respective unstimulated control (n-4). Results were analysed using one-way ANOVA compared with respective controls. p**** < 0.0001. One-way ANOVAs followed by Bonferronit's post hoc test to correct for multiple comparisons were used to calculate the p-values (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001), following removal of significant outliers, using a Grubbs Test* ***(Supplementary Table 5).*** *Data is presented as mean percentage values ± SEM of four independent donors. Flow cytometry gating strategy is detailed in* ***Supplementary Figure 4****.*

5.3 Discussion

Tissue trauma following surgery in oncology can lead to an increased risk of recurrence due to excessive inflammation and compensatory, pro-tumourigenic immunosuppression (Tohme et al., 2017b). A narrow therapeutic window exists in which inflammation may be reduced in order to prevent progression to immunosuppression and recurrence. This thesis aimed to evaluate OTD as a potential surgical adjuvant in melanoma for administration in the early postoperative period. Having previously discussed the effects of OTD on human dermal fibroblast and melanoma cell lines, this study examined the effect of OTD on cytotoxic CD8+ T lymphocytes involved in adaptive immune responses.

Our results demonstrated that, while OTD is not cytotoxic to CD8+ T lymphocytes, IFNγ release is significantly impaired at all concentrations following stimulation with PMA/Ionomycin or anti-CD3. Preservation of adaptive immune responses is of vital importance in the prevention of disease progression following surgery. Immunostimulatory cytokines such as IFNγ exert anti-tumoural activity via both direct growth inhibition and indirect enhancement of immune cell function (Zaidi, 2019). In chronic wounds, functional impairment in cytokine secretion is observed in both αβ+ and Vδ1+ T lymphocytes compared to acute wounds in an ex-vivo study conducted on human tissue. Notably, there were no significant differences in the population levels of these T lymphocyte subsets between the two groups. (Toulon *et al.*, 2009). Animal models estimate peak levels of CD8+ T lymphocytes in the wound at 7-10 days post wounding (Chen *et al.*, 2014). Ananth *et al.* suggest impairment of adaptive immunity begins in the early postoperative period with surgical stress inducing a reduction in CD8+ T lymphocyte IFNγ production beginning 24 hours postoperatively and continuing for 7-10 days in mouse models (Ananth *et al.*, 2016). While there are limited studies available in humans, Martin *et al.* demonstrated that T lymphocyte levels typically peak in

human wounds between days 8 and 14 postoperatively, however, subset analysis was not reported in this study (Martin and Muir, 1990).

Our initial optimisation experiments indicated a high proportion of CD8+ T lymphocyte death associated with anti-CD3 stimulation at a concentration of 5ug/ml. The concentration of anti-CD3 for adequate stimulation of cytokine release is highly variable in the literature, with concentrations ranging from 1ug/ml to 20ug/ml and stimulation times varying from 6 hours to 4 days (Uhl *et al.*, 2023; Biller *et al.*, 2001; Gupta and Maecker, 2015; Machicote *et al.*, 2018). At a concentration of 2ug/ml anti-CD3, cell viability was preserved with live cell proportion remaining above 60% across all concentrations of OTD with or without anti-CD3 stimulation. Although significant IFNγ release was observed in stimulated controls, levels were much lower than that of PMA/Ionomycin stimulation at 2.2% and 9.6% respectively. Further optimisations could be justified, including a prolonged anti-CD3 incubation period of 24 hours, with BFA added in the final 5 hours.

Initial optimisations revealed minimal IFNγ production with a maximum proportion of 3% in the PMA/Ionomycin stimulated control. We hypothesised that BFA may not have been adequately thawed due to the high freezing point of DMSO in which stock solution if formulated (18ºC). BFA prevents the release of intracellular cytokines from CD8+ T lymphocytes following stimulation, so that they may be measured during flow cytometry analysis. Thus, an optimal concentration may not have been achieved in subsequent working solutions. In the absence of BFA, IFNγ would likely be released extracellularly and lost during the staining process. For subsequent experiments, BFA was allowed to thaw at room temperature for a minimum of 15 minutes, resulting in enhanced detection of IFNγ intracellularly (maximum of 9.6% in PMA/Ionomycin stimulated control).

While not significant, it should be noted that a trend was visible in optimisations and to a lesser extent in replicated experiments whereby reduction in CD8+ T lymphocyte viability was most apparent at 0.5mM OTD. This was similarly observed in chapter 4 in melanoma cell lines, in which we hypothesised that the effect may be due to induced metabolic switching as a result of glycolytic inhibition by OTD. T lymphocytes also demonstrate metabolic plasticity depending on environment and state of activation, with active phenotypes relying on glycolysis and inactive, memory phenotypes relying on fatty acid oxidation or oxidative phosphorylation (Slack *et al.*, 2015). Inhibitors of glycolysis, similar to OTD, can induce metabolic switching in CD8+ T lymphocytes to oxidative phosphorylation and a memory phenotype (Sukumar *et al.*, 2013). This may clarify our findings regarding the observed trend in CD8+ T lymphocyte viability and suppression of cytokine release. The potential induction of metabolic switching may promote a shift in cellular state towards an inactive memory phenotype, suppressing IFNγ release as well as enhancing survival at higher concentrations of OTD. In order to investigate this hypothesis comprehensively, further studies would be required into the effects of OTD on cell metabolism.

Therefore, this study demonstrates that OTD suppresses cytokine release in CD8+ lymphocytes, leading to implications in the timing of its potential use as a surgical adjuvant. We suggest that OTD should be administered early postoperatively, however, this study is limited by the in-vitro experimental design. The SURGUVANT trial previously examined the use of Taurolidine in colorectal cancer surgery, exhibiting an excellent safety profile, reduction of systemic inflammatory markers and decreased incidence of wound complications. The first treatment dose was administered at induction of anaesthesia and treatment continued for four days (Redmond *et al.*, 2018). In contrast, the first trial examining the use of OTD in humans, currently at stage 1/2, involves a considerably longer duration of therapy. Therapy is to be administered for up to five weeks postoperatively in patients with pancreatic cancer, though

the outcomes of this trial have yet to be published (Kasi and Iglesias, 2022). Definitive conclusions regarding the optimal duration of OTD therapy in melanoma require further studies using *in vivo* animal models followed by studies in human subjects.

Chapter 6: Discussion and Future Perspectives

Surgery is often an essential component of cancer treatment, with approximately 58% of patients with cancer requiring at least one surgical procedure globally (Perera *et al.*, 2021). However, it is well established that surgical intervention is associated with an increased risk of recurrence and metastasis (Kim, 2018; O'Connor et al., 2020; Tohme et al., 2017b). This pattern relates to a variety of factors, including tumour cell dissemination (Kameyama *et al.*, 2023), choice of anaesthetic (Brogi and Forfori, 2022) and excessive surgical inflammation (Yasui *et al.*, 2021).

Surgery causes an initial proinflammatory immune response, or systemic inflammatory response syndrome (SIRS), followed by a compensatory anti-inflammatory or immunosuppressive response, predisposing the host to septic complications and providing an optimal environment for tumour growth and metastasis (Verdonk *et al.*, 2021). Given the critical role of surgery in cancer treatment, the development of adjuvant therapies is imperative to mitigate the adverse effects of surgical procedures and enhance the overall efficacy of anti-cancer interventions.

In this regard, this thesis sought to evaluate the effectiveness of OTD as an adjuvant therapy following melanoma excision to reduce postoperative inflammation and eradicate potential residual melanoma cells without impacting physiological processes such as wound healing.

6.1 The future of adjuvant therapy in Melanoma

In Ireland, medical treatment for melanoma, either as a monotherapy or as a surgical adjuvant, is typically reserved for late stage tumours (HSE, 2023). However, there is emerging evidence suggesting that adjuvant therapy may also be beneficial for patients with early-stage melanoma. Notably, Wan *et al.* identified a subset of individuals diagnosed with stage II melanoma who may be at an elevated risk of recurrence based on specific histological features (Wan *et al.*, 2022). Furthermore, phase III of the Keynote-716 trial demonstrated significant improvement in recurrence free survival (RFS) rates following administration of Pembrolizumab in stage IIB and stage IIC melanoma (Luke *et al.*, 2024).

Previous studies demonstrate a significant association between inflammation and melanoma progression and metastasis (Kainulainen *et al.*, 2022; Landsberg *et al.*, 2016; Ohno *et al.*, 2019). Moreover, preclinical studies have demonstrated the potential use of anti-inflammatory agents as adjuvants in melanoma treatment, such as inhibitors of cyclooxygenase (COX-2) (Tudor *et al.*, 2020) and interleukin-6 (IL-6) (Hailemichael *et al.*, 2022). Although clinical studies analysing the impact of postoperative inflammation on melanoma progression are limited, several *in vivo* animal models have established a correlation between surgical-induced inflammation and melanoma progression (Antonio *et al.*, 2015; Krall *et al.*, 2018; Mintz and Silvers, 1993) indicating a potential use for anti-inflammatory therapies in the postoperative period.

Our findings indicate that OTD effectively reduces levels of pro-inflammatory cytokines, IL-6 and IL-8 in human dermal fibroblast (HDF) cells, while also demonstrating cytotoxic effects against melanoma cells. Elevated levels of IL-6 and IL-8 have emerged as prognostic markers in melanoma, as shown in recent studies (Laino *et al.*, 2020; Levati *et al.*, 2024; Tobin *et al.*, 2019). Notably, Tobin *et al.* demonstrated that IL-6 and IL-8 participate in the recruitment of immunosuppressive myeloid-derived suppressor cells (MDSCs), with higher concentrations of

these cytokines being associated with poorer median overall survival outcomes (Tobin *et al.*, 2019). Furthermore, patients with higher levels of IL-6 have shown reduced responses to immunotherapy, including ipilimumab, nivolumab and pembrolizumab (Y. Wang *et al.*, 2022). Hailemichael *et al.* describe that inhibition of IL-6 can improve immune checkpoint blockade by reducing toxicity and enhancing anti-tumour immunity (Hailemichael *et al.*, 2022), leading to a stage II clinical trial (NCT04940299) evaluating the combination of IL-6 inhibitor, Tocilizumab, with ipilimumab and nivolumab in the treatment of metastatic melanoma.

Additional anti-inflammatory effects of OTD have been demonstrated by Majchrzak-Stiller *et al.,* showing impairment of the nuclear factor-κB (NF-kB) pathway in pancreatic cancer cell lines via inhibition of p65/DNA binding (Majchrzak-Stiller *et al.*, 2023). The NF-kB pathway is a significant promoter of tumour progression(Janostiak *et al.*, 2017) and immunotherapy resistance (Li et al., 2024) in melanoma, however, at present there are no clinically approved NF-kB inhibitors for this disease. Targeting this pathway using OTD may therefore represent a novel approach to overcoming treatment resistance and enhancing responsiveness to existing adjuvant therapies.

From a clinical translation perspective, OTD's pharmacological profile lends itself to multiple delivery strategies that could complement current treatment paradigms. Topical application represents a particularly attractive option, allowing high local drug concentrations while limiting systemic exposure (Pfirrmann et al., 2024), which may be especially relevant for superficial primary melanomas. Intraoperative lavage or topical application to the surgical bed could provide locoregional control in the immediate postoperative period, targeting residual microscopic disease during a window of heightened inflammatory signalling (Bezu et al., 2024). Systemic administration could also be explored in advanced or disseminated disease given the oral tolerability of OTD, however, our results indicate that further studies may be required for use of OTD in metastatic melanoma.

Combination strategies may further enhance clinical efficacy. Given its NF-κB inhibitory activity, OTD could be integrated with immune checkpoint inhibitors or targeted therapies to reduce immune evasion and resistance mechanisms (Li et al., 2024; Yu et al., 2020). Reduction of proinflammatory cytokine release may also counteract pro-tumour inflammation in the perioperative setting, potentially improving the efficacy of adjuvant immunotherapies.

These considerations suggest OTD could be strategically positioned within the evolving adjuvant therapy landscape for melanoma, potentially expanding treatment options beyond conventional systemic therapy. Further preclinical and clinical studies will be required to define optimal dosing, timing, safety and identification of patient subsets most likely to benefit from its integration into perioperative and adjuvant treatment regimens

6.2 Wound healing considerations of surgical adjuvant therapy

Postoperative wound complications have a significant impact on recurrence rates and prognosis of several cancers (Beecher *et al.*, 2018). Given the elevated incidence of wound complications associated with anti-cancer therapies, such as chemotherapy (Słonimska *et al.*, 2024) and radiotherapy (Haubner *et al.*, 2012) it is essential to carefully consider the timing of administration and the impact of these therapies on cellular functions involved in wound healing.

In melanoma, Antonio *et al* demonstrate in zebrafish expressing mutant Ras^{G12V} oncogene, that repeated wounding leads to a higher incidence of local tumour formation (Antonio *et al.*, 2015). Likewise, case reports in humans have demonstrated a rare incidence of melanoma in chronic wounds (Kelahmetoglu *et al.*, 2018; Łakomska *et al.*, 2023). Skin malignancy may also manifest in approximately 2% of all burn scars, with melanoma representing 6% of cases (Ma *et al.*, 2024).

This thesis therefore considered whether OTD treatment may adversely affect wound healing function. To this end, *in vitro* assessments were carried out on dermal fibroblasts demonstrating the absence of cytotoxicity and preservation of migratory function at concentrations of 0.75mM OTD and below. These findings are clinically relevant as they indicate that OTD could be safely administered in the perioperative period without impairing fibroblast viability or wound closure dynamics.

An additional advantage of OTD is its potential for topical administration, which may achieve high local drug concentrations at the surgical site while minimising systemic exposure (Pfirrmann et al., 2024). This targeted delivery approach could support local tumour control and reduce the risk of postoperative recurrence while preserving wound integrity and reducing the likelihood of treatment-related complications. Such localised delivery strategies align with the growing emphasis on therapies that minimise systemic toxicity while maintaining or enhancing local efficacy in the postoperative setting.

Therapeutic wound dressings may also be used as a delivery system for adjuvant therapies such as OTD in the postoperative period. Alginate hydrogels are known to enhance wound healing by providing moisture, absorbing exudate and eliciting antimicrobial function (Froelich *et al.*, 2023). These hydrogels also offer a biocompatible platform for local delivery of anti-tumour agents, potentially allowing controlled release of OTD at the wound site. For example, Chen *et al.* demonstrate in mouse models that a sprayable calcium alginate hydrogel, containing the tumour-targeting nanodrug (denoted as HIL@Z), reduces melanoma recurrence and metastasis while promoting postoperative wound healing. The drug's anti-tumour effects stem from the generation of reactive oxygen species (ROS), nitric oxide (NO), and reactive nitrogen species (RNS), causing oxidative damage to residual tumour cells while the enhanced wound healing is linked to increased oxygen levels, which downregulate hypoxia-inducible factor-1α (HIF-

1α) and upregulate vascular endothelial growth factor (VEGF), promoting angiogenesis, re-epithelialization, and tissue regeneration (Chen *et al.*, 2024).

Taken together, these findings suggest that the topical delivery of OTD is a clinically feasible strategy that could be integrated into the postoperative management of melanoma. By combining localised anti-tumoural activity with preservation of wound healing function, OTD may offer an important advantage over conventional systemic therapies in the surgical adjuvant setting.

6.3 Timing of adjuvant therapy in oncological surgery

In general, short-term stress associated with surgery elicits immune cell stimulation and enhanced recovery. However, in the case of oncological surgery, where stress is applied to a chronically stressed environment, we see an exacerbated inflammatory effect triggering compensatory immunosuppressive responses, ultimately leading to increased susceptibility to infections and tumour recurrence (Dhabhar, 2014).

Elevated postoperative neutrophil-to-lymphocyte ratios (NLRs) have shown prognostic value in determining poor disease-free survival (DFS) and overall survival (OS) across a range of cancers. Neutrophils are associated with the promotion of tumourigenesis, while lymphocytes are typically linked to anti-tumoural responses. Interestingly, a meta-analysis by Wu *et al.* showed that while NLRs demonstrate prognostic value, there is no correlation with tumour stage or clinicopathological features (Wu *et al.*, 2021), indicating that NLR may be influenced by the surgical intervention. Excessive surgical inflammation leads to increased levels of neutrophils (Kumagai *et al.*, 2020) and a compensatory reduction in lymphocytes correlating to the degree of surgical stress (Sun *et al.*, 2019). Therefore, it is reasonable to assume that post-operative NLR could also be used to indicate response to anti-inflammatory surgical adjuvants.

Targeting of excessive surgical inflammation to prevent compensatory immunosuppression is confined to a narrow therapeutic window. In the initial post-operative period, cells of the innate immune system dominate, with neutrophils arriving within four hours (Kim *et al.*, 2008) and macrophages accumulating within 24-48 hours of wounding (Rodrigues *et al.*, 2019) initiating pro-inflammatory responses. In contrast, cells of the adaptive immune system such as CD4+ and CD8+ T lymphocytes, show peak levels in wounds at days 5-10 and days 7-10 postoperatively, respectively (Chen *et al.*, 2014). The rapid onset of wound inflammation would suggest that the optimal timing for initiation of surgical adjuvant therapies to reduce excessive inflammation is in the early postoperative period. However, the duration of the therapy is contingent on the preservation of adaptive immune responses.

Our findings demonstrated *in vitro* that OTD is cytotoxic to melanoma cells and reduces proinflammatory cytokine production in HDF cells while preserving their migratory wound healing function. While further *in vivo* studies are required, these findings would suggest that OTD should be both safe and efficacious as a surgical adjuvant in the early postoperative period, when innate immune responses dominate and excessive inflammatory signalling may promote tumour survival and recurrence. By targeting proinflammatory cytokines during this critical window, OTD may help limit the protumoural effects of surgical stress while preserving wound integrity.

However, this therapeutic window is time sensitive. OTD negatively impacted the functionality of CD8+ T lymphocytes by suppressing IFNγ release, indicating potential interference with adaptive immune responses if administered during the late postoperative period. As CD8+ T lymphocytes play a crucial role in anti-tumour immunity (Xu et al., 2015), inappropriate timing could blunt beneficial immune activation. While studies in humans are limited, Martin and Muir demonstrate that T lymphocyte levels peak in wound tissue between days 8 and 14 postoperatively using pan-T cell markers (Martin and Muir, 1990). Subset analysis has been

carried out in animal models, with Chen *et al.* showing peak tissue levels of CD8+ T lymphocytes in mice at 7 to 10 days postoperatively (Chen *et al.*, 2014). This suggests that the administration of OTD should be restricted to the immediate postoperative period, ideally within the first week after surgery, to target early innate inflammatory signalling while avoiding interference with later adaptive T cell mediated anti-tumour responses.

6.4 Phasic dose-responses in adjuvant therapy: tailoring treatment doses for optimal effects

Dose-response curves to cytotoxic anti-cancer therapies *in vitro* are typically represented by linear, dose dependent curves with a proportional increase in therapeutic effect with increasing drug concentrations. However, the introduction of biological complexity in *in vivo* animal models and subsequent species-specific differences in translation to human animal models, variability in dose-responses can be observed (Algharably *et al.*, 2019; Martin and Dimmitt, 2019).

Despite previous studies demonstrating a dose-dependent response to OTD in pancreatic cancer (Buchholz *et al.*, 2017), Merkel cell cancer (Gambichler *et al.*, 2023), breast cancer (Jinih *et al.*, 2021) and ovarian cancer (Kim *et al.*, 2024), our results indicated a non-linear response to OTD treatment in primary (WM-3248) and metastatic (SK-MEL-2) melanoma cell lines. Dose response curves for these cell lines may be described as either hormetic/J shaped or U shaped, with potential recovery of cytotoxic function unclear within the range of concentrations tested. Hormetic/J shaped curves describe a bi-phasic enhanced response at low concentrations, followed by a diminished response at high concentrations. U-shaped curves, on the other hand, are characterised by a triphasic response with enhanced response at low concentrations, followed by treatment resistance at intermediate concentrations and finally, recovery of treatment response at high concentrations (Ramaiah *et al.*, 2016).

Preliminary testing revealed a U-shaped dose response curve or triphasic response in cytotoxicity to anti-CD3 stimulated CD8+ T Lymphocytes, albeit testing was carried out in a single biological replicate and thus not powered for statistical analysis. Subsequent testing using multiple replicates and a reduced concentration of anti-CD3, negating its apparent cytotoxic effects and the initial triphasic response was not observed.

A U-shaped dose response curve or triphasic response was also observed in the treatment of malignant mesothelioma cell lines using OTD. A steep reduction in cell viability was observed at 0.5mM OTD in both JL-1 and MSTO-211H cell lines, followed by a diminished effect at their respective intermediate concentrations, ultimately leading to recovery of cytotoxic activity at higher concentrations (Baron *et al.*, 2022). In melanoma, an *in vitro* study by Gambichler *et al.* demonstrates a dose-dependent response to OTD treatment, although it should be noted that specific concentrations of OTD (0.5mM - 2.5mM) at which this effect was observed were not reported in this study (Gambichler *et al.*, 2023). *In vivo* murine models, however, show more effective attenuation of tumour growth at lower doses of 250mg/kg compared with 500 mg/kg and 1000mg/kg in HS-695T metastatic melanoma (Sofia *et al.*, 2024).

The molecular mechanism underpinning this effect remains unclear. We hypothesise that our findings may be explained by the induction of metabolic switching in melanoma. The cytotoxic effects of OTD are linked with the inhibition of glycolysis via GAPDH and Hexokinase-2 inhibition (Majchrzak-Stiller *et al.*, 2023). The aggressive nature of melanoma is thought to relate to its metabolic plasticity. While metabolism in most cancer cells relies on aerobic glycolysis to rapidly produce energy, melanoma is capable of adapting its metabolic phenotype to promote survival and tumourigenic potential (Avagliano *et al.*, 2020). Inhibitors of glycolysis, similar to OTD, can induce metabolic switching (Lu *et al.*, 2015; Shiratori *et al.*, 2019), which may account for reduced cytotoxicity at higher concentrations of OTD. We

hypothesise that at 0.5mM OTD, cytotoxicity is mediated by glycolytic inhibition as seen in previous cell lines. However, at concentrations above this level, glycolytic inhibition triggers a change in the metabolic phenotype of melanoma, switching to pathways unaffected by OTD to ensure tumour cell survival. Potential recovery of cytotoxic function could be explained by alternative mechanisms of toxicity at higher doses of OTD. The observation of a similar trend in our assessment of OTD cytotoxicity in anti-CD3 stimulated CD8+ T Lymphocytes may also be mediated by GAPDH inhibition, with T lymphocytes also demonstrating metabolic plasticity (Slack *et al.*, 2015).

Phasic dose-response dynamics have important implications for patient selection and clinical dosing strategies. Hormetic or U-shaped response curves imply that excessively high doses may paradoxically reduce therapeutic efficacy, whereas intermediate or lower doses may offer a more favourable balance between anti-tumour activity and preservation of immune function. Identifying patients whose tumour biology may be more responsive to lower OTD concentrations, for example, those with glycolysis-dependent metabolic phenotypes, could help maximise treatment response while minimising toxicity.

Furthermore, the potential for immunomodulatory effects, as demonstrated in our CD8+ T lymphocyte assays, reinforces the need for careful dose titration to avoid impairing adaptive anti-tumour immunity in the postoperative setting.

From a safety perspective, tailoring OTD doses to exploit the lower end of the therapeutic range could minimise potential off-target effects, reduce the risk of systemic toxicity and align with topical or locoregional delivery strategies that concentrate drug exposure at the surgical site. This precision-based dosing model could allow OTD to be selectively applied to patients most likely to benefit based on metabolic phenotype, tumour burden and postoperative inflammatory burden.

Further studies are necessary to validate this theory, however, should it be substantiated, the therapeutic potential of OTD may be significantly augmented through combination therapy with inhibitors targeting alternative metabolic pathways, such as oxidative phosphorylation or fatty acid metabolism.

6.5 Conclusion

In conclusion, this thesis represents a series of preclinical, *in vitro* analyses supporting the use of OTD as a surgical adjuvant in melanoma. Collectively, our findings demonstrate significant cytotoxic activity against both primary and metastatic melanoma cell lines, a reduction of pro-inflammatory cytokine production in HDF cells and preservation of HDF migratory wound healing function and viability. Conversely, OTD negatively impacts adaptive CD8+ T lymphocytes via inhibition of anti-tumoural cytokine release which may restrict the duration of OTD therapy.

Importantly, these findings support a therapeutic model in which OTD could be administered during the immediate postoperative period, targeting early innate inflammatory responses and minimal residual disease while avoiding interference with adaptive immune activation. This short course, perioperative strategy may align well with topical or locoregional delivery approaches, minimising systemic exposure while enhancing local control.

The observation of non-linear, hormetic dose-response curves underscores the need for precision dosing strategies to maximise tumour cytotoxicity while preserving immune function. Future patient selection may therefore benefit from metabolic profiling to identify those most likely to respond to lower, well-tolerated doses.

This thesis highlights several promising avenues for future research. The most obvious next step is the evaluation of OTD using *in vivo* animal models, ultimately progressing to human

clinical trials. However, several further *in vitro* studies have also been highlighted that warrant consideration and may take precedence.

Our results revealed variability in dose-response patterns across different melanoma cell lines, leading to the hypothesis that this dose-response variability may be linked to metabolic phenotype switching triggered by inhibition of glycolysis. Further studies are required to clarify the impact of OTD on metabolic signalling pathways as well as the potential synergistic effects of OTD given in combination with alternative metabolic inhibitors. Such combinations could enhance efficacy while allowing lower, safer OTD dosing in the clinical setting,

Likewise, the effect of OTD on IL-6 and IL-8 release may support its use in combination with immunotherapy to combat resistance. Taken together, these findings suggest that OTD has potential not only as a standalone therapy, but as a targeted perioperative adjuvant integrated into multimodal treatment strategies.

While our findings support the use of OTD following melanoma excision, further exploration into its effects on other cell types, such as neutrophils, macrophages and CD4+ T lymphocytes, could provide further insights into the role of OTD in modulating innate and adaptive immune responses in the postoperative period. Ultimately, the clinical translation of OTD will depend on further preclinical characterisation, carefully designed dose-finding studies, optimal timing of administration and integration with existing standard of care treatments to maximise antitumour and anti-inflammatory activity while preserving immune responses.

Limitations

Despite the strengths of this thesis, several important limitations must be acknowledged. These limitations reflect the constraints of preclinical, in vitro experimental design and highlight areas that require further investigation prior to clinical translation. Understanding these limitations not only contextualises the findings presented but also helps to shape the direction of future studies, particularly in bridging the gap between bench research and perioperative clinical application of OTD.

7.1 In Vitro Monocultures and Lack of Tumour Microenvironment Modeling

All experiments were conducted in 2D monocultures, which do not recapitulate key features of the melanoma tumour microenvironment (TME), including stromal architecture and crosstalk with immune and endothelial cells. These factors can substantially alter drug signalling and bioavailability compared with 2D systems. Advanced 3D cultures, spheroids, organoids and xenograft models capture diffusion barriers and cell-to-cell interactions more comprehensively, often yielding different pharmacological responses (Antoni et al., 2015; Edmondson et al., 2014).

In addition, findings have not yet been validated in animal models. Preclinical to clinical translation is frequently constrained by the biological complexity of in vivo systems, where variability in dose-response relationships often emerges. As a result many promising in vitro effects failing to reproduce in vivo (Algharably et al., 2019; Martin and Dimmitt, 2019).

7.2 Restricted Cell-line diversity, Omission of Normal Melanocyte Controls and Single Timepoint Analysis

The inclusion of a broader range of cell lines, representing diverse genetic melanoma subtypes (e.g. BRAF, NRAS and wild-type) as well as broader non-malignant cell populations, such as

melanocytes and keratinocytes, could have contextualised the current findings, providing a more comprehensive understanding of OTD's pharmacological profile. Melanocytes and keratinocytes represent a clinically relevant, non-malignant subset of cells, likely to be directly exposed to OTD in a topical or locoregional delivery setting. Their inclusion would have enabled a more thorough assessment of OTD's selectivity, potential off-target effects in normal tissue and, particularly in the case of keratinocytes, a more comprehensive analysis of its impact on wound healing dynamics.

Furthermore, most assays were conducted as a single post-treatment time point. Incorporating a dynamic time-course analysis could have provided enhanced insight into the kinetics of cytotoxicity, cytokine release and immune modulation, which may have distinguished transient versus sustained treatment effects (Van De Vyver et al., 2021; Wellach and Riemer, 2025).

7.3 Focus on a Single Immune Subset

Functional immune readouts in this study focused on CD8+ T lymphocytes. However, other immune populations, such as CD4+ T lymphocytes, macrophages and neutrophils, play critical roles in melanoma progression, immune evasion and therapeutic response (Knoedler et al., 2023; Wendlinger et al., 2024; Q. Zhou et al., 2022). Their omission constrains the ability to fully characterise the immunomodulatory effects of OTD, as its impact on the broader immune landscape remains unexamined.

7.4 Limited Mechanistic Insight into Phasic Dose-Response and Immune Suppression

Non-linear (hormetic/U-shaped) responses were observed in this study, but detailed mechanistic dissection was beyond its scope. Hormesis is a well-recognised phenomenon in toxicology and pharmacology, often reflecting adaptive cellular responses to stress. Interpreting such patterns requires tailored modelling and mechanistic validation to guide

appropriate dose selection (Ramaiah et al., 2016; Wan et al., 2024). Likewise, the observed suppression of IFNγ production in CD8+ T lymphocytes suggests immunomodulatory effects that warrant deeper pathway analysis of underlying signalling pathways and metabolic dependencies.

7.5 Absence of Logarithmic Scaling

Log scaling of concentrations was not applied to dose-response visualisation or analysis in this study. Logarithmic transformation of the x-axis can aid in interpretation for wide concentration ranges (Motulsky et al., 2004; Ritz and Streibig, 2005). Given the relatively narrow concentration range tested here and the presence of non-monotonic (hormetic) patterns, the omission of log scaling is defensible. However, applying log axes or transformations in sensitivity analyses could improve comparability with other studies and resolution of low-dose effects, which may be otherwise obscured on a linear axis.

7.6 Annexin/PI Interpretation

Annexin/PI staining distinguishes viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+) and necrotic (Annexin V^-/PI^+). A major limitation of this method is its inability to clearly differentiate late apoptotic from necrotic cells, as both exhibit loss of membrane integrity and stain for Annexin V and PI. This can lead to overestimation of late apoptotic fractions, particularly in in vitro systems where the absence of immune cell mediated phagocytosis allows apoptotic cells to progress to secondary necrosis (Berghe et al., 2010). To increase interpretive accuracy, additional validation measures, such as caspase activation or mitochondrial potential assays, should be used to confirm cell death pathways.

7.7 Conclusion

Taken together, these limitations highlight the preclinical and exploratory nature of this work and the need for more physiologically relevant and mechanistically detailed studies to support

clinical translation of OTD. Future research should prioritise incorporation of more complex co-culture systems, normal skin cell types and in vivo models to better replicate the tumour microenvironment and the perioperative wound landscape. Broader cell-line panels, extended time-course analysis and expansion of the immune cell subset would provide a more comprehensive understanding of OTD's effects on both tumour biology and host immunity. Likewise, mechanistic studies exploring the basis of the phasic dose-response pattern, as well as dose optimisation, will be crucial in translating OTD into a clinically viable perioperative adjuvant. Acknowledging these limitations strengthens the interpretation of the current findings and defines a clear and actionable roadmap for the next phase of investigation.

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Appendix

Appendix A: Original Study Design and Rationale

9.1.1 Background and Study Objectives

The original research project plan included the investigation of perioperative circulating cell-free DNA (cfDNA) dynamics in melanoma patients undergoing surgical excision. Levels of cfDNA were to be quantified at three key perioperative time-points: preoperatively, early postoperatively and late postoperatively. Next-generation sequencing (NGS) was planned to assess tumour-derived genetic alterations across these phases. In addition, photographic documentation of the lesion and subsequent surgical site was intended to evaluate potential correlations between wound healing outcomes and cfDNA levels or mutational burden.

This study aimed to explore the utility of cf-DNA as a perioperative biomarker of tumour burden, residual disease and potential wound healing complications in melanoma patients.

9.1.2 Study Design and Ethical Approval

Ethical approval was obtained from the Clinical Research Ethics Committee (CREC) of the Cork Teaching Hospitals [CREC Review Reference Number: ECM 4 (p) 09/08/2022 & ECM 5 (10) 09/08/2022 & ECM 3 (aa) 24/10/2023]. A comprehensive study protocol was developed, outlining inclusion and exclusion criteria as well as standardised procedures for blood collection, plasma processing and cfDNA storage. Two patient samples were collected and processed in accordance with this protocol, as described in the Methods chapter.

9.1.3 Limitations and Discontinuation

The study encountered significant logistical challenges, including low participation rates due to the requirement for additional hospital visits for sample collection. Despite collaboration efforts with additional clinical departments in Plastic Surgery and Dermatology to enhance recruitment, the pace of enrolment was insufficient to generate analysable data within the

project timeframe. Consequently, the clinical study was withdrawn from the scope of this thesis, though it has continued under the supervision of the original research team.

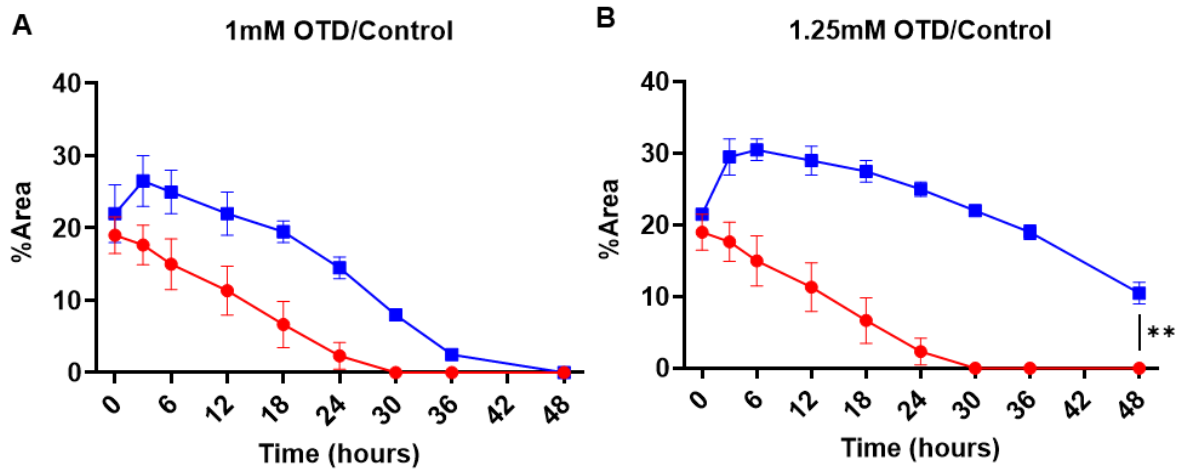
9.1.4 Shift in Research Focus

While the clinical cfDNA study was originally intended to constitute a major component of this thesis, in vitro experiments examining the effects of 1,4,5- Oxathiazine-4,4-dioxide (OTD) were also included in the initial study design as a complementary line of investigation. We hypothesized that the clinical cfDNA study would provide translational insight into the immunomodulatory effect of surgical stress, while in vitro analyses were designed to assess cellular responses to OTD therapy across cell types relevant to the perioperative tumour microenvironment.

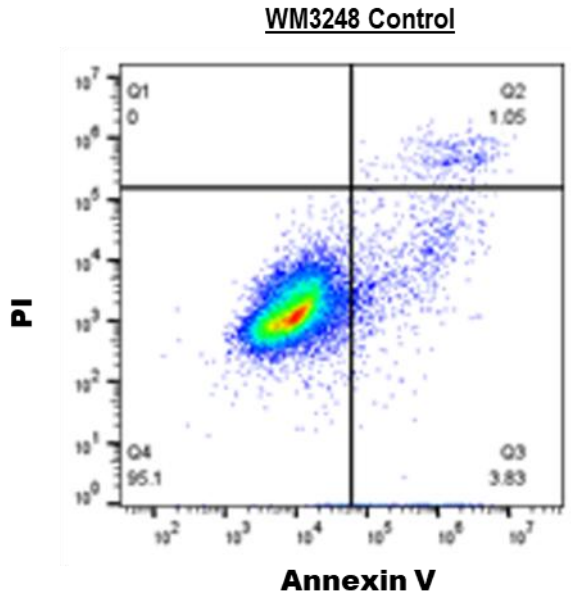
Following the discontinuation of the cfDNA study, the in vitro studies were expanded upon to form the primary focus of the thesis. The selected cell models included human dermal fibroblast (HDF) cells, primary and metastatic melanoma cell lines and $CD8^+$ T lymphocytes. These cell lines were chosen to represent key components of the tumour and post-operative microenvironment. HDF cells were included to explore the effect of OTD on wound healing (HDF migration), inflammatory cytokine release and viability. Melanoma cell lines provided insight into the direct tumour responses of OTD comparing primary and metastatic cell lines due to the inherent biological differences between them. CD8+ T lymphocytes were used to assess immune activation (IFN-γ expression) as a marker of cytotoxic function present later in the postoperative period.

The in vitro approach provided a controlled environment to study the effects of OTD on individual cell types, which may inform future work using more robust co-culture, in vivo or clinical investigations. Although distinct from the cfDNA study, this work remained aligned with the broader context of this thesis.

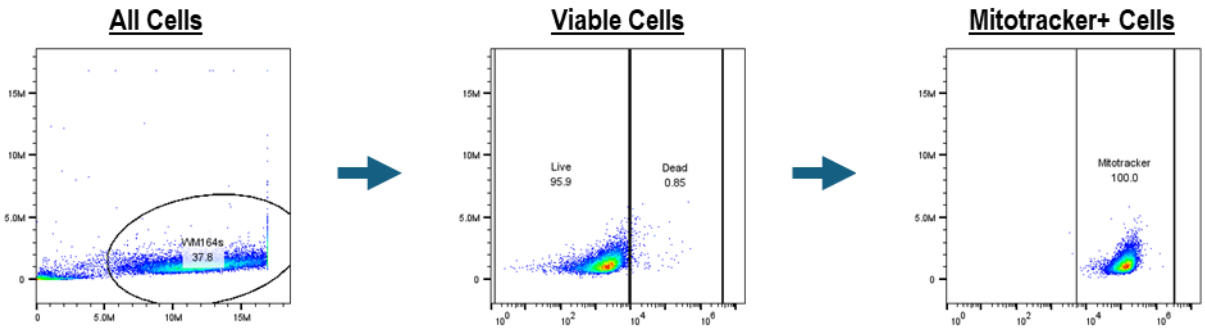
Appendix B: Supplementary Figures



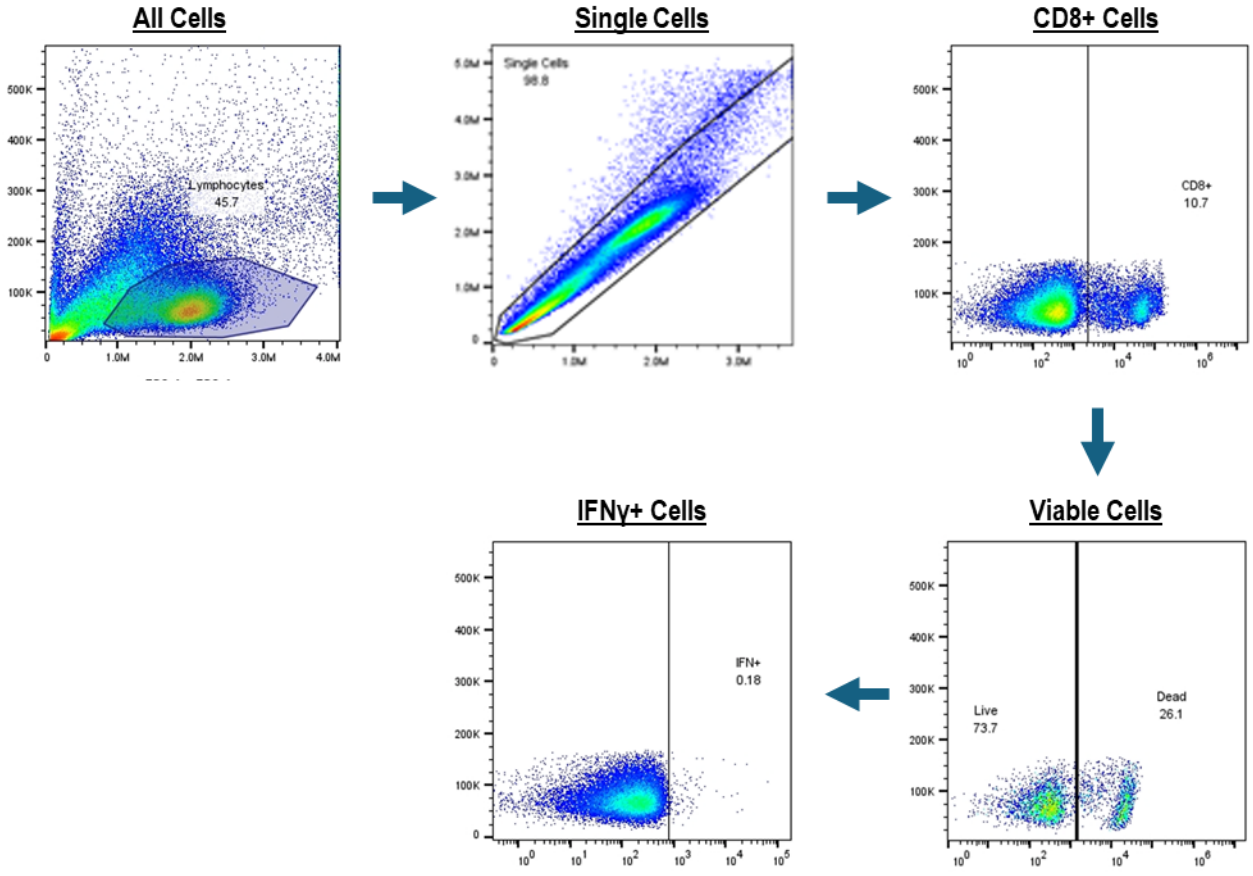
Supplementary Figure 1: *Effect of 1,,4,5-Oxathiazinane-4,4-dioxide (OTD) treatment on Human Dermal Fibroblast (HDF) cell migration measured by in vitro wound healing assay up to 48 hours. Percentage wound area was quantitatively analysed (n=3) over time for OTD at* ***(A)*** *1mM and* ***(B)*** *1.25mM compared to control. Wound area was evaluated using Image J version 1.53t and a wound area calculator Two-way ANOVAs followed by Bonferroni's post hoc test to correct for multiple comparisons were used to calculate the p-values (**p < 0.01), and data are presented as mean values ± SEM of three independent experiments in triplicate.*



Supplementary Figure 2: *Representative flow cytometry gating plot for intracellular Annexin V/PI staining of WM3248 melanoma cells. Cells were treated with OTD prior to staining. The representative image depicts the gating strategy in the control group, whereby Q1 (Annexin V^-/PI^+), Q2 (Annexin V^+/PI^+) Q3 (Annexin V^+/PI^-) and Q4(Annexin V^-/PI^-)cells were considered necrotic, late apoptotic, early apoptotic and viable respectively. The same gating strategy was also used for the SK-MEL-2 cell line.*



Supplementary Figure 3: *Representative images of flow cytometry gating strategy for quantification of mitochondrial mass using Mitotracker Green staining. WM164 cells were treated with OTD prior to staining. Representative images depict the gating strategy in the control group, which was applied to all other treatment groups.*



Supplementary Figure 4: *Flow cytometry gating strategy for intracellular IFNγ production by CD8+ T lymphocytes. PBMCs were stimulated with anti-CD3 and treated with OTD. Representative images depict the gating strategy in the control group, which was applied to all other treatment groups. The same gating strategy was also used for PMA/Ionomycin stimulations.*

Appendix C: Supplementary Tables

Grubbs' outlier test was used to detect outlier data as presented below. Testing was carried out using Graphpad prism statistical software at a 95% confidence interval ($p<0.05$). Values detected as significant ($p<0.05$) were subsequently removed.

***Supplementary Table 1**: Grubb's outlier test performed on cytokine quantification data in Human Dermal Fibroblast (HDF) cells, presented in Figure 3.3*

Cytokine	OTD Concentration (mM)	Unstimulated (IL-1α-)				Stimulated (IL-1α+)			
		Total Biological Replicates (n)	Within LOD* (n)	Outliers (n)	Sample Size (n)	Total Biological Replicates (n)	Within LOD* (n)	Outliers (n)	Sample Size (n)
IL-6	**0**	6	6	0	6	6	6	0	6
	0.25	6	6	0	6	6	6	0	6
	0.5	6	6	0	6	6	6	0	6
	0.75	6	6	0	6	6	6	0	5
	1	6	5	1	4	6	6	0	6
	1.25	6	6	1	5	6	5	0	4
IL-8	**0**	6	6	0	6	6	6	0	6
	0.25	6	6	0	6	6	6	0	6
	0.5	6	6	0	6	6	6	1	5
	0.75	6	6	0	6	6	5	0	6
	1	6	5	0	5	6	6	0	6
	1.25	6	4	0	4	6	6	1	5

*The number of biological replicates with cytokine levels outside the limits of detection (LOD) were removed from the analysis prior to outlier testing

Supplementary Table 2: *Grubb's outlier test performed on percentage cell viability data in Human Dermal Fibroblast (HDF) cells, presented in Figure 3.4*

OTD Concentration (mM)	**Human Dermal Fibroblast (HDF) cells**		
	Total Biological Replicates (n)	Outliers (n)	Sample Size (n)
0	6	0	3
0.25	6	0	3
0.5	6	0	3
0.75	6	1	2
1	6	0	3
1.25	6	1	2

Supplementary Table 3: *Grubb's outlier test performed on percentage cell viability data in primary melanoma (A-375 and WM3248) cell lines, presented in Figure 4.2*

OTD Concentration (mM)	**A-375 cells**			**WM3248 cells**		
	Total (n)	Outliers (n)	Sample Size (n)	Total (n)	Outliers (n)	Sample Size (n)
0	6	0	6	6	0	6
0.25	6	1	5	6	0	6
0.5	6	0	6	6	1	5
0.75	6	0	6	6	0	6
1	6	0	6	6	0	6
1.25	6	0	6	6	0	6

Supplementary Table 4: *Grubb's outlier test performed on percentage cell viability data in metastatic melanoma (WM164 and SK-MEL-2) cell lines, presented in Figure 4.3*

OTD Concentration (mM)	WM164 cells			SK-MEL-2 cells		
	Total (n)	Outliers (n)	Sample Size (n)	Total (n)	Outliers (n)	Sample Size (n)
0	6	0	6	6	0	6
0.25	6	0	6	6	0	6
0.5	6	0	6	6	0	6
0.75	6	1	5	6	0	6
1	6	1	5	6	0	6
1.25	6	1	5	6	0	6

Supplementary Table 5: *Grubb's outlier test performed on percentage IFNγ data in PMA/Ionomycin stimulated CD8+ T lymphocytes, presented in Figure 5.2*

OTD Concentration (mM)	Unstimulated (PMA/Ionomycin-)			Stimulated (PMA/Ionomycin+)		
	Total Donors (n)	Outliers (n)	Sample Size (n)	Total Donors (n)	Outliers (n)	Sample Size (n)
0	4	0	4	4	1	3
0.25	4	0	4	4	0	4
0.5	4	0	4	4	0	4
0.75	4	0	4	4	0	4
1	4	0	4	4	0	4
1.25	4	0	4	4	0	4

Supplementary Table 6: *Grubb's outlier test performed on percentage IFNγ data in PMA/Ionomycin stimulated CD8+ T lymphocytes, presented in Figure 5.3*

OTD Concentration (mM)	Unstimulated (Anti-CD3-)			Stimulated (Anti-CD3+)		
	Total Donors (n)	Outliers (n)	Sample Size (n)	Total Donors (n)	Outliers (n)	Sample Size (n)
0	4	0	4	4	0	4
0.25	4	0	4	4	0	4
0.5	4	0	4	4	0	4
0.75	4	0	4	4	0	4
1	4	0	4	4	0	4
1.25	4	0	4	4	1	3