

Investigating Nrf2 and Nrf3 as therapeutic targets in cancer

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This thesis is dedicated to my mum Helen, for her unwavering support and kindness, and in memory of my dad and brother, Peter, and Christopher, who continue to inspire me to this day.

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II) Abstract

The transcription factor Nrf2 acts as a master regulator of oxidative stress by influencing the expression of genes involved in cytoprotection, redox regulation, and the anti-inflammatory response. While Nrf2 provides a protective effect against carcinogenesis in non-malignant cells, Nrf2 overexpression and aberrant activation in patient tumours contributes to increased tumour growth, metastasis, and chemoresistance. Hence, Nrf2 inhibitors are emerging as potential anti-cancer agents.

In this study, seven Nrf2 inhibitors were compared for the first time for in non-small cell lung cancer (NSCLC) and melanoma cell lines. Among these inhibitors, brusatol and triptolide proved the most efficacious at inhibiting cell proliferation and Nrf2 signalling in both NSCLC and melanoma. Additionally, brusatol, triptolide, and luteolin sensitised cells to the BRAF inhibitor vemurafenib. Triptolide also induced apoptosis and sensitised A549 NSCLC cells to the chemotherapeutic agent, gemcitabine, suggesting that Nrf2 inhibition may be potentially beneficial in Nrf2-overexpressing or chemoresistant NSCLC or melanoma tumours.

Two novel mechanisms of inhibiting Nrf2 were also explored. While triptolide is a potent inhibitor of Nrf2, it possesses several off-target effects. To combat this, we present the first triptolide-peptide conjugate designed to target cancer cells with high Nrf2 and EGFR expression. This study also describes the first peptide-based mimic of the MafG bZIP domain, designed to interrupt the Nrf2/MafG protein-protein interaction. This mimic not only repressed Nrf2-ARE binding, and Nrf2 target gene expression, but also sensitised A549 NSCLC cells to gemcitabine.

The transcription factor Nrf3, which has been implicated in several cancer types, was also explored as a potential therapeutic target in cancer. Herein, we show that Nrf3 is upregulated in patient-derived colorectal tumouroids and is highly expressed in MCF-7 breast, HCT116 colorectal, and MIAPACA2 pancreatic cancer cell lines. Moreover, Nrf3 knockdown significantly repressed migration of MIAPACA2 cells, suggesting that Nrf3 may be a useful therapeutic target in pancreatic cancer.

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IV) Publications and presentations

Published Abstracts and Poster Presentations

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Hobson, E., Saffer, D., Inglis, E., Antonios, C., Adekola, G., Prabhu, S., O'Connell, M. (2020) 'Evaluation of effects of small molecules targeting Nrf2 in human cancer cells', *Norwich Cancer Research Network Symposium*, 7th September 2020 (Norwich, UK).

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VII) Abbreviations

ADC	Antibody-drug conjugate
Ac	Acetyl
ANOVA	Analysis of variance
AKT	Protein kinase B
ARE	Antioxidant response element
ATP	Adenosine triphosphate
Bach1/2	BTB and CNC homology 1/2
Bax	BCL2 associated X protein
Bcl-2	B-cell lymphoma 2
BCP	3-bromo-3-chloropropane
BSA	Bovine serum albumin
BTB	Broad-Complex, tramtrack and bric à brac
bZIP	Basic leucine zipper
CDK1	Cyclin-dependent kinase 1
cDNA	Complimentary DNA
CNC	Cap 'N' Collar
CPEB3	Cytoplasmic polyadenylation element binding protein 3
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTR	C-terminal region
CUL1/3	Cullin 1/3
DCM	Dichloromethane
DDI2	DNA damage inducible 1 homolog 2
DIPEA	<i>N,N</i> -Diisopropylethylamine
DJ-1	Protein deglycase 1
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethyl fumarate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
DTT	Dithiothreitol
DUX4	Double homeobox factor 4
ECH	Erythroid cell-derived protein with CNC homology
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide disodium salt hydrate
FBS	Foetal bovine serum
FBW7	F-box and WD repeat domain-containing 7
FITC	Fluorescein isothiocyanate
Fmoc	Fluorenylmethyloxycarbonyl
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEO	Gene Expression Omnibus
GLUT1	Glucose transporter 1
GSH	Glutathione
GSK-3 β	Glycogen synthase kinase 3 beta
GST	Glutathione-S-Transferase
GPX	Glutathione peroxidase
HBTU	Hexafluorophosphate Benzotriazole Tetramethyl Uronium
HO-1	Heme oxygenase-1
HOBt	Hydroxybenzotriazole

HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IC50	Half maximal inhibitory concentration
IVR	Intervening region
Keap1	Kelch-like ECH associated protein 1
mAb	Monoclonal antibody
MALDI-TOF	Matrix-assisted laser desorption/ionization-Time of Flight
MARE	Maf recognition element
MC1R	Melanocortin 1 receptor
MEM	Minimum essential medium
MMP	Matrix metalloprotease
MTT	Thiazolyl Blue Tetrazolium Blue
mRNA	Messenger ribonucleic acid
MRP1-9	Multidrug resistance associated protein 1-9
NADPH	Nicotinamide adenine dinucleotide phosphate
NES	Nuclear export signal
Neh 1-7	Nrf2-ECH homology 1-7
NF-E2	Nuclear factor erythroid 2
NF-κB	Nuclear factor kappa B
NGLY1	N-glycanase 1
NHB1/2	N-terminal homology box 1/2
NLS	Nuclear localisation signal
NMR	Nuclear magnetic resonance
<i>N,N</i> -DMF	<i>N,N</i> -dimethyl formamide
Nrf1/2/3	Nuclear factor erythroid 2-related factor 1/2/3
NSCLC	Non-small cell lung cancer
NST	Asparagine/ serine/ threonine
NTR	N-terminal region
NQO1	NAD(P)H dehydrogenase [quinone] 1
PARK7	Parkinson disease protein 7
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PDC	Peptide-drug conjugate
Pgp	P-glycoprotein
PI3K	Phosphoinositide 3-kinase
POMP	Proteasome maturation protein
PPI	Protein-protein interaction
Prdx	Peroxiredoxin
PVDF	Polyvinylidene difluoride
p53	Tumour protein 53
Rb	Retinoblastoma protein
Rbx1	Ring box protein 1
RNA	Ribonucleic acid
RNASeq	RNA sequencing
RT-qPCR	Reverse transcription- quantitative polymerase chain reaction
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
Skp1	S-phase kinase associated protein 1
sMaf	Small musculoaponeurotic fibrosarcoma
SOD	Superoxide dismutase
SPPS	Solid phase peptide synthesis
SQSTM1	Sequestosome 1
SU	Succinate
TBST	Tris-buffered saline with Tween 20
TCGA	The Cancer Genome Atlas
TFA	Trifluoroacetic acid

TIPS	Triisopropylsilane
TNF- α	Tumour necrosis factor alpha
TP	Triptolide
TXN	Thioredoxin
UHMK1	U2AF homology motif kinase 1
UV	Ultraviolet
VEGFA	Vascular endothelial growth factor alpha
5-FU	5-Fluorouracil
β -TrCP	Beta-Transducin repeat containing protein

Chapter 1: Introduction

1.1 Cancer as an ongoing healthcare issue

Cancer is a condition that incorporates hundreds of diseases that can arise in a variety of tissue types within the body. Cancer typically arises due to abnormal cell growth that can invade surrounding tissues, although symptoms and survival rates vary depending on each cancer type. At present, cancer remains the second leading cause of death worldwide, with an estimated 9.8 million cancer-associated deaths occurring in 2020¹. It is estimated that approximately 50% of people in the UK (born after 1960) will be diagnosed with cancer at some point throughout their life². Early diagnosis and advancements in treatments have fuelled greater chances of survival in a variety of cancer types, with survival rates in the UK doubling in the past 40 years³.

Approximately 375,000 people are diagnosed with cancer each year in the UK, with breast, prostate, lung, bowel, and melanoma cancers comprising the 5 most common types². In the UK, the highest mortality rate is reported in lung cancer (21% of all cancer deaths), followed by bowel (10%), prostate (7%), breast (7%), and pancreatic cancers (6%)⁴. Furthermore, it is predicted that there will be a 55% increase in cancer incidence rates overall between 2020 and 2040 worldwide¹. Understanding the mechanisms of cancer development, and developing innovative treatments therefore remains at the forefront of pharmacological research.

1.1.1 Mechanisms of carcinogenesis

The mechanisms through which cancer develops and progresses are complex and show wide patient to patient variability. Carcinogenesis is a multi-step process, which involves an accumulation of mutations that drive abnormal cell growth, resistance to cell death mechanisms, and metastasis⁵. Such mutations are typically caused by epigenetic alterations or DNA damage that can occur due to a variety of exogenous and endogenous factors⁶. Exogenous factors of DNA damage include chronic exposure to carcinogens (*e.g.*, cigarette smoke), and ultraviolet (UV) radiation. On the other hand, DNA damage can also arise through the generation of by-products of normal cellular processes such as the formation of reactive oxygen species (ROS), produced during energy metabolism^{7,8}. Other factors including genetics, diet, exposure to certain viruses, and advanced age also have roles in promoting carcinogenesis⁶.

Cancer typically arises due to mutations that have accumulated over a number of years, with initial neoplastic transformation often triggered by a mutation in a key tumour suppressor or tumour promoter gene, such as TP53 or BRAF, respectively. For example,

mutations in BRAF, which are present in 50% of melanoma patient tumours, promotes the initial switch between benign (normal) and dysplastic (abnormal) skin naevi (moles), in the first step of melanoma tumorigenesis⁹ (**Figure 1.1**). Such mutations contribute to rapid cell proliferation, allowing mutated cells to expand at a faster rate than normal cells. As the population of mutated cells increases, additional mutations can occur, which further promote the malignant phenotype. To potentiate growth, cancer cells can also begin to invade into the surrounding tissue, with increased invasion correlating with advanced disease stages and poorer clinical outcomes. Following on from local invasion, cells can also undergo epithelial to mesenchymal transition (EMT) and extracellular matrix remodelling, allowing cells to detach away from the primary tumour site and invade into distal organs via the lymphatic and circulatory systems (**Figure 1.1**).

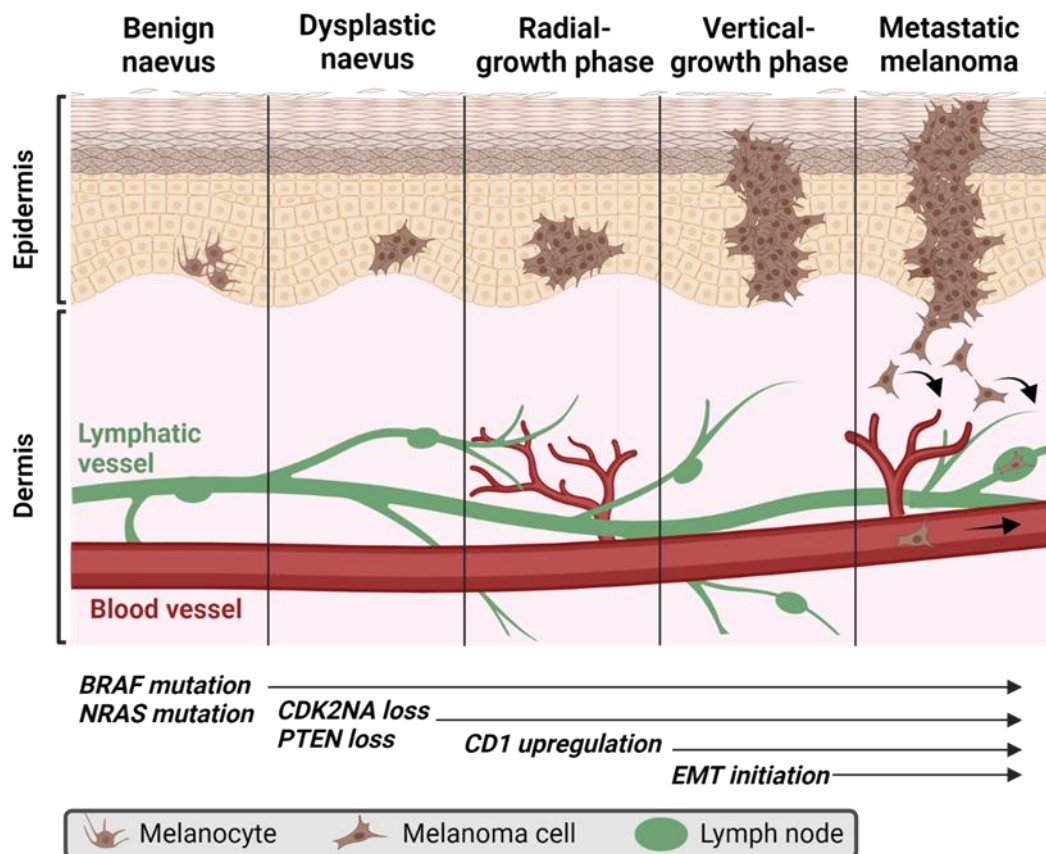


Figure 1.1 The progression of melanoma from benign naevi to metastatic melanoma. Melanoma initiates from the neoplastic transformation of melanocytes. Following on from the formation of dysplastic cells, melanoma penetrates further into the epidermis and dermis layer, before eventually undergoing epidermal to mesenchymal (EMT)-initiated metastasis via the lymphatic and circulatory system. (Adapted from Rahmati *et al.*⁹). Abbreviations: EMT; epithelial to mesenchymal transition.

A key backbone of cancer treatment is therefore early diagnosis and treatment, to limit the potential of the tumour to metastasise and invade other tissues. Regular monitoring of high-risk patients, for example those with certain viral infections, such as the human

papillomavirus, which is linked with cervical cancer, or with a genetic predisposition to certain cancers (e.g., BRCA1/2 mutations in breast and ovarian cancers), is vital in preventing advanced tumour progression.

1.1.2 The Hallmarks of Cancer

During neoplastic transformation, cells become increasingly abnormal and begin to demonstrate several key characteristics, termed the “hallmarks of cancer”, that separate them from non-malignant cells¹⁰. While the initial hallmarks were proposed in 2000¹⁰, two follow up reports by Hanahan and Weinberg, have now described a total of 14 cancer hallmarks^{11,12}, as described in **Figure 1.2**.

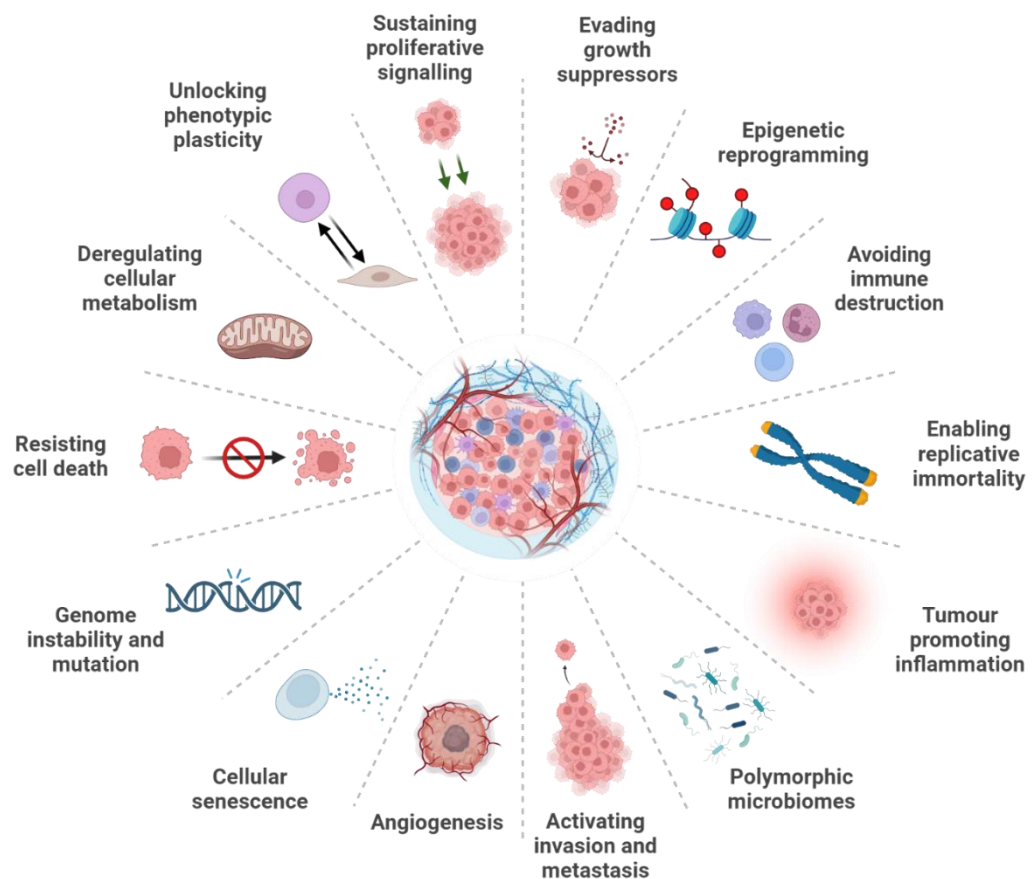


Figure 1.2 The hallmarks of cancer. (Adapted from Hanahan 2022¹¹).

One of the most well researched hallmarks that cancer demonstrates is the ability to evade growth suppressors, which is key in initiating tumorigenesis¹⁰. This occurs through the loss of several tumour suppressor proteins which would normally prevent progression of abnormal cells through the cell cycle¹². While DNA damage can often be repaired by

DNA repair mechanisms, severe DNA damage can be mediated by tumour suppressor proteins, by inducing cell cycle arrest and programmed cell death (apoptosis). A key mediator in this response is p53 (tumour protein p53), which is induced in the presence of DNA damaging agents, such as UV radiation, and works to initiate cell cycle arrest at the G1/S phase, induce the expression of proteins involved in DNA damage repair, and trigger apoptosis of abnormal cells¹³. Loss of p53 function is a common driver of tumorigenesis, with mutations identified in >50% of all cancers¹⁴.

Asides from loss of function mutations in p53, cancer cells can also become resistant to apoptosis via the dysregulation of anti- and pro-apoptotic proteins, such as Bcl-2 and Bax, respectively^{10,15}. Typically, these proteins remain in a tightly regulated balance, with initiation of apoptosis being a quick and transient response to irreparable DNA damage. In cancer however, this balance can be shifted, with upregulations of anti-apoptotic proteins and downregulation of pro-apoptotic proteins allowing for resistance to cell death¹⁶. Resistance to apoptosis therefore further potentiates the malignant phenotype as it prevents the destruction of cells with a high mutational burden and allows them to continue to proliferate and accumulate additional oncogenic mutations¹⁰. Furthermore, this also provides a protective response against treatment methods that induce DNA damage, such as radio- and chemotherapies¹⁷.

Not only can cancer cells evade growth suppressors, but they can also initiate uncontrolled proliferative signalling (**Figure 1.2**). While normal cells can only proliferate in the presence of mitogenic growth signals, such as growth factors, cancer cells are able to promote their own growth signals leading to uncontrolled proliferation¹⁰. These signals are typically propagated via the overactivation of cell signalling pathways, often initiated by increased receptor expression or activation of the pathway in the absence of typical receptor-ligand interactions¹⁸. An example of this is epidermal growth factor (EGF), which binds to the epidermal growth factor receptor (EGFR) to promote proliferation through a number of pathways, including the mitogen activated protein kinase (MAPK) and PI3K/Akt (Phosphoinositide 3-kinase/ Protein Kinase B) pathways¹⁹ (**Figure 1.3**). By overexpressing growth factor receptors such as EGFR, cancer cells can remain responsive to growth factor levels that would not normally elicit a response^{20,21}, leading to enhanced tumour growth.

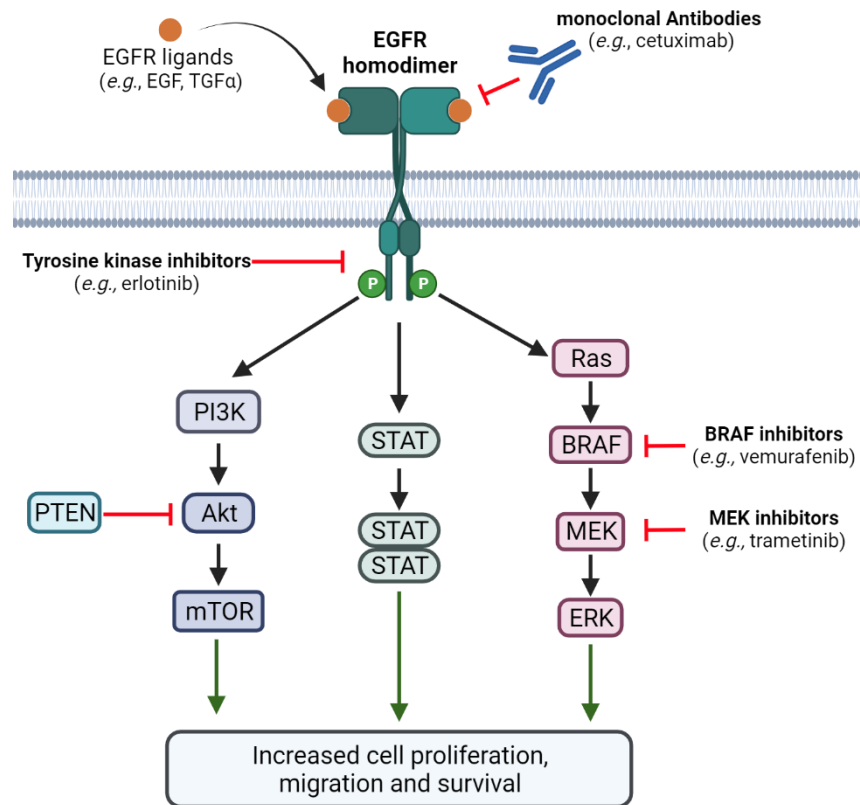


Figure 1.3 The EGFR signalling pathway and current clinical therapeutic interventions that inhibit its overactivation in cancer. EGFR contributes to increased cancer cell proliferation, migration, and survival via its induction of the PI3K/Akt, STAT, and MAPK/ERK pathways. To prevent such signalling, targeted therapies aimed to inhibit mediators of these pathways have now emerged, including anti-EGFR monoclonal antibodies, tyrosine kinase inhibitors, BRAF inhibitors and MEK inhibitors. *Abbreviations: EGF; epidermal growth factor, EGFR; epidermal growth factor receptor, TGFα; transforming growth factor alpha.*

In order to fuel their excessive growth, cancer cells also reprogram metabolic pathways. Although the underlying mechanisms behind this are not always clear and vary among different cancer types, a common observation is that cancer cells upregulate glucose transporters to increase glucose uptake (such as GLUT1), which is often driven by oncogenic signalling pathways, such as PI3K/Akt²². Many cancers also increase expression of enzymes involved in the pentose-phosphate pathway, such as transaldolase 1 (TALDO1) and transketolase (TKT) which assist in the production of ribose and NADPH for nucleotide synthesis²³. Previously, it was thought that cancer cells undergo a ‘glycolytic switch’; a phenomenon wherein aerobic glycolysis is favoured as opposed to oxidative metabolism, despite the former being less efficient in producing ATP¹²; however, this observation is not well understood and remains under contention^{24,25}.

Metabolic reprogramming can also assist in allowing cancer cells to grow under hypoxic conditions in tumours with limited vasculature²⁶. Tumours can also combat hypoxic conditions via the rewiring and growth of blood vessels, in a process called

angiogenesis¹⁰. This is mediated through the upregulation of key angiogenic factors, such as vascular endothelial growth factor-A (VEGF-A)^{27,28}, allowing cancer cells to induce new blood vessel formation localised to the tumour site to promote more efficient nutrient exchange. The formation of new blood vessels in the tumour area also feeds into another key cancer hallmark, wherein tumours can invade other tissues and metastasise¹², which occurs through extracellular matrix remodelling and epithelial to mesenchymal transition (EMT). This is the process in which cancer cells can escape from their primary site of growth and enter blood and lymphatic vessels, eventually extravasating into secondary tissues²⁹ (**Figure 1.1**). This can occur through alterations in cell-cell adhesion molecules, such as E-cadherin, which is downregulated in numerous cancers³⁰.

While such hallmarks are well-established as key potentiators of tumour growth and metastasis, novel characteristics are constantly under investigation. Recent inclusions to the hallmarks of cancer incorporate the ability of tumours to unlock phenotypic plasticity, wherein cancer cells can differentiate and de-differentiate according to their current needs¹¹. There is also emerging evidence that the microbiota plays an influential role in modulating the tumour microenvironment to either inhibit or drive the hallmarks of cancer, including genome instability, uncontrolled cell proliferation and evasion of immune regulation^{11,31}. While mutations are often viewed as the main driving force for pro-tumorigenic phenotype changes, increasing evidence also suggests that non-mutational epigenetic reprogramming is also a hallmark of tumorigenesis in cancer cells^{11,32}. While the hallmarks of cancer are ever evolving, they provide better understanding of potential molecular mechanisms and targets that could be utilised to improve cancer therapy outcomes.

1.1.3 Current chemotherapies

Cancer treatments include chemotherapy, radiotherapy, surgery, and hormone therapy; however, treatment options for patients varies depending on the type and stage of cancer with which they are diagnosed. Patients may be also treated with a combination of these therapies, for example in the case of breast cancer, where chemotherapy can be administered either before surgical resection (termed neoadjuvant therapy) to shrink the tumour or can be administered after surgery (adjuvant therapy) to prevent cancer recurrence. Over 100 drugs are approved by the FDA for chemotherapy, including the “classical” cytotoxic drugs and the more recently developed targeted therapies and biologics (**Table 1.1**).

Table 1.1 Chemotherapies currently used in the clinic. (Adapted from Rang and Dale 2012³³).

	Class of treatment	Method of action	Examples
“Classical” chemotherapy agents	Alkylating agents	Causes damage to DNA and prevents DNA repair mechanisms, either by the addition of an alkyl group onto DNA (at guanine), or by permanently coordinating with DNA.	• Cyclophosphamide • Cisplatin • Dacarbazine • Carmustine
	Antimetabolites	Prevent cell division and growth by disrupting DNA production. This is achieved by either inhibiting purine or pyrimidine synthesis, or by competing for DNA bases in DNA synthesis, thereby preventing correct cell replication.	• 5-Fluorouracil (5-FU) • Methotrexate • Gemcitabine • 6-Mercaptopurine
	Plant alkaloids	Derived from natural products, these treatments have varying mechanisms of action, including preventing microtubule assembly (vincristine), stabilising microtubule polymers (paclitaxel) and inhibiting topoisomerase activity (etoposide).	• Vincristine • Vinblastine • Paclitaxel • Docetaxel • Etoposide
	Topoisomerase inhibitors	Topoisomerase acts to prevent damage caused by DNA supercoiling, by “cutting” DNA at specific points to untangle DNA strands. Topoisomerase inhibitors thereby prevent DNA replication and DNA damage repair mechanisms.	• Doxorubicin • Etoposide • Mitoxantrone • Irinotecan
Targeted therapies and biologics	Tyrosine kinase inhibitors	Inhibit tyrosine kinases from activating signalling cascades leading to protein activation. This reduces the activity of signalling cascades that can assist in cancer growth, for example by targeting the EGF receptors and BCR-ABL kinase.	• Sorafenib • Erlotinib • Gefitinib • Imatinib
	Serine/threonine kinase inhibitors	Inhibit the kinase activity of serine/threonine kinases such as BRAF and MEK to repress proliferative signalling pathways.	• Vemurafenib • Trametinib • Selumetinib
	Monoclonal antibodies	Bind specifically to targets (e.g., receptors) on the surface of cancer cells to disrupt signalling pathways that assist in cancer growth.	• Cetuximab (anti-EGFR) • Trastuzumab (anti-HER2) • Ipilimumab (anti-CTLA-4) • Pembrolizumab (anti-PD-1)
	Antibody and peptide drug conjugates	These therapies utilise a monoclonal antibody or peptide specific for a target on cancer cells, linked to a cytotoxic “warhead”. The antibody/peptide binds to the target and releases the cytotoxic in the vicinity of the cancer cells, thereby reducing systemic side effects.	• Trastuzumab emtansine • Brentuximab vedotin

The “classical” cytotoxic drugs encompass alkylating agents, antimetabolites, plant alkaloids, topoisomerase inhibitors and cytotoxic antibiotics (**Table 1.1**). The majority of these therapies target pathways involved in DNA synthesis and cell division, thereby preventing further cancer cell growth to minimise tumour progression. Although many of these drugs are routinely used in therapy, often alongside other therapy approaches such as surgery and radiotherapy³⁴, they do possess several key drawbacks that limit their clinical use. One of the main issues with the classical cytotoxics is that they target all proliferating cells and therefore do not act preferentially against cancer, meaning that they can induce numerous systemic side effects including anaemia, gastrointestinal symptoms, hair loss, bruising and neuropathy^{34,35}. Not only does this limit the therapeutic window of these treatments, but also places a great physical burden on patients.

Additionally, these chemotherapies do not take into account the heterogeneity of patient tumours, resulting in variable response rates³⁵.

In recent years, research has therefore shifted to therapies that could potentially bypass such side effects. A key approach in this strategy is the use of personalised medicine, wherein treatments are tailored according to the individual biological landscape of a patient's tumour. Personalised medicine relies on a patient's genomic and proteomic data to identify whether certain treatments are likely to be effective depending on the expression and activity of biological targets involved in the drugs mechanism of action³⁶. A leading example of this is the advent of BRAF inhibitors, which specifically target mutations in the BRAF proto-oncogene that lead to uncontrolled cell proliferation via hyperactivation of the MEK/ERK signalling pathway (**Figure 1.3**) in cancers such as melanoma, thyroid, colorectal and non-small cell lung cancers³⁷. While wild-type BRAF in non-neoplastic cells is only activated following binding of growth factors to their respective receptor tyrosine kinases, cells with mutated BRAF are able to induce the MEK/ERK pathway even in the absence of receptor tyrosine kinase-ligand binding, resulting in constitutive pathway activation and sustained proliferative signalling³⁸. BRAF inhibitors, such as vemurafenib, specifically target BRAF overactivation caused by mutations, to repress proliferative signalling^{38,39}.

While many targeted therapies are small molecules, personalised medicine also encompasses the use of biologics such as monoclonal antibodies (mAb). These therapies include the immune checkpoint inhibitors, which work by repressing the "evasion of immune destruction" hallmark of cancer¹² (**Figure 1.2**). This is achieved using mAbs that block receptor-ligand interactions between tumour and immune cells that act as checkpoints to inhibit immune recognition of tumour cells, such as the PD1 (programmed death receptor 1) and CTLA4 (cytotoxic T-lymphocyte-associated protein 4) receptors⁴⁰. By blocking these immune checkpoints, immune cells, such as T killer cells, are reactivated to recognise and destroy tumour cells⁴¹. Several mAb therapies also target receptors that are overexpressed on cancer cells to block binding of their endogenous ligands and therefore repress excessive proliferative signalling (**Table 1.1**). One such example is the anti-EGFR mAb cetuximab, which is currently licensed in colorectal, and head and neck cancers to block the PI3K/Akt and MEK/ERK signalling pathways by blocking EGFR activation⁴². As mAbs possess high affinity binding to their targets, they are often more specific than small molecules and possess lower systemic side effects than traditional chemotherapies, such as cytotoxic agents⁴².

Furthermore, mAbs can also be conjugated to cytotoxic agents, to produce antibody-drug conjugates (ADCs), which aim to minimise the systemic side effects of potent cytotoxic drugs by delivering the cytotoxic agent within the local area of the tumour⁴³. These therapies work by use of an antibody that recognises antigens expressed on cancer cells, linked to a cytotoxic “payload” via a chemical linker. Following binding of the antibody to its target, the ADC is internalised, and the linker is cleaved via lysosomal degradation, resulting in release of the payload within the tumour cell. An example of this is trastuzumab emtansine which targets the tubulin inhibitor mertansine to human epidermal growth factor receptor 2 (HER2)-overexpressing breast cancers via its conjugation to the anti-HER2 mAb trastuzumab⁴⁴. ADCs have shown good clinical success, with 14 currently approved for clinical use, and over 100 in clinical development⁴⁴.

Based upon the success of ADCs, another similar targeted therapy has recently emerged into the clinic, termed peptide drug conjugates (PDCs). While similar in theory to ADCs, PDCs utilise smaller peptide molecules to specifically direct radio- and chemo- therapies to overexpressed molecular targets on cancer cells⁴⁵. These peptide molecules not only possess similar binding affinities to receptors as observed with mAbs, but also demonstrate lower immunogenic responses and can better penetrate the tumour stroma due to their smaller size⁴⁶. Additionally, PDCs are also cheaper to produce than ADCs, due to the high cost involved in producing and purifying antibodies⁴⁶. While many are under clinical investigation, two PDCs have so far made it to the clinic; Lu¹⁷⁷ dotatate, and Meflufen, which target a radiotherapeutic agent to somatostatin-overexpressing neuroendocrine cancers, and induce the release of alkylating agents in myeloma tumours, respectively^{47,48}.

Although targeted therapies and biologics are more specific and in general possess lower systemic side effects than classical cytotoxic drugs, they do still come with some drawbacks. Many of the antibody therapies are associated with inflammatory and autoimmune effects; for example, the checkpoint inhibitors nivolumab (PD1 inhibitor) and ipilimumab (CTLA-4 inhibitor) have been found to induce severe side effects in 16.3% and 27.3% of patients, respectively⁴⁰. Furthermore, while such therapies may induce a good initial response rate, patient tumours often become resistant which can reduce drug efficacy. If this occurs, patients may be given combination therapies, with the inclusion of a drug that acts on a different target, however these do not always solve the issue of chemoresistance and often are associated with further chance of severe side effects³⁵.

1.1.4 Chemoresistance- the need for new therapies

Although the success of chemotherapies depends on many factors, a key challenge facing successful chemotherapy is the emergence of chemoresistance⁴⁹. As demonstrated in several of the hallmarks of cancer¹¹ (**Figure 1.2**), cancer is well known to rapidly adapt to changes in its environment, including the administration of chemotherapy agents. While chemoresistance is often battled by the addition of another chemotherapeutic agent to the treatment regime, many cancers then begin to develop multidrug resistance, which greatly hinders therapeutic options further down the line.

Such resistance mechanisms can either be intrinsic or acquired, meaning that resistance can be present originally in the tumour, or can develop following drug treatment⁵⁰. Cancer cells can achieve resistance through mutations and epigenetic modifications, leading to increased drug efflux, drug inactivation, increased repair of drug-induced DNA damage, inhibition of cell death, decreased drug influx and modification of the drug target^{50,51} (**Figure 1.4**).

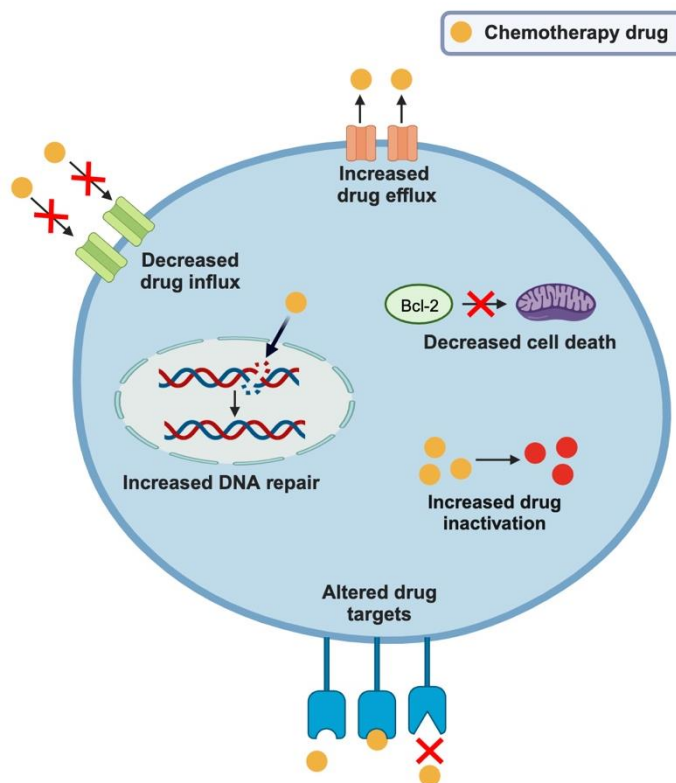


Figure 1.4 The mechanisms through which chemoresistance can develop. Chemoresistance can occur through multiple mechanisms, including decreasing drug influx, increasing drug efflux, increasing DNA repair in response to DNA damaging agents, altering drug targets, and resisting drug-induced cell death.

Increased drug efflux can occur through the upregulation of efflux transporters, such as the ATP-binding cassette (ABC) transporters⁵². These include the multidrug resistance-associated protein 1 (MRP1), breast cancer resistance protein (BCRP) and ABCB1 (multidrug resistance protein 1 (MDR1) or P-glycoprotein (P-gp)). Perhaps the most well studied of these is ABCB1, which has been shown to increase efflux of a number of treatments including methotrexate, doxorubicin⁵³, etoposide⁵⁴, vinblastine⁵⁵ and paclitaxel⁵⁶. Alternatively, the influx of drugs into the cell can also be modified, for example through the downregulation of drug solute carriers, such as solute carrier 19A1 (SLC19A1), which is necessary for the influx of several antifolate therapies, including methotrexate⁵⁷. Cancer cells can also inactivate drugs once they enter the cell, both through increased detoxification enzymes and via downregulation of enzymes required to convert drugs to their active metabolites. One of the key perpetrators of this is the family of glutathione transferases (GSTs), which are Phase II enzymes involved in glutathione conjugation. Upregulation of GSTs, which is observed in a large majority of cancers including colorectal, lung, breast and pancreatic cancers, is known to reduce the activity of several chemotherapeutic drugs such as cisplatin, etoposide and doxorubicin⁵⁸, which has led to the development of GST inhibitors as potential chemotherapies⁵⁹.

Cancer cells can also modify drug targets to render treatments less effective, often by altering their expression level or function. For example, an acquired mutation in the extracellular domain of the EGFR receptor (S492R) confers resistance to the anti-EGFR mAb cetuximab in colorectal cancer⁶⁰. Mutations can also promote elevated expression of a chemotherapy target, rendering the therapy less effective. This is a common mechanism of resistance of imatinib, a tyrosine kinase inhibitor that targets the Bcr-Abl fusion protein, which is the result of a common chromosomal translocation in chronic myeloid leukaemia patients. In this case, point mutations in the BCR-ABL gene have been shown to induce increased Bcr-Abl expression associated with imatinib resistance⁶¹. Increased DNA-damage repair can also induce chemoresistance, with overexpression of DNA-repair enzymes such as PARP1 (poly (ADP-ribose) polymerase 1), an enzyme that repairs single strand breaks in DNA, resulting in reduced sensitivity to DNA-damage inducing treatments⁵⁰. This has led to the development of PARP inhibitors as chemotherapeutic agents⁶², such as olaparib, which are currently licenced in ovarian and breast cancers. Despite this, cancer can still rely on other DNA damage repair mechanisms, including the O(6)-methylguanine DNA methyltransferase (MGMT) enzyme, which repairs guanine O6 alkylation in response to alkylating agents, such as dacarbazine⁶³.

Finally, as mentioned earlier as a hallmark of cancer (**Figure 1.2**), tumours can also become resistant to chemotherapies through inhibition of cell death mechanisms such as apoptosis¹⁰. Often, anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL and Mcl-1) are upregulated, while pro-apoptotic proteins (Bax and Bad) are downregulated, thereby reducing apoptosis¹⁷. Furthermore, given that cancer cell populations are heterogeneous, it is possible that a single tumour may possess more than one mechanism of drug resistance and tumours may also develop multidrug resistance to therapies of similar mechanisms of action (**Table 1.2**).

Table 1.2 Common resistance mechanisms of chemotherapy drugs used in the clinic.

Treatment	Resistance mechanisms	Ref
"Classical" cytotoxic drugs	Increased drug inactivation (e.g., enhanced enzymatic modification via cytochrome P450s). Reduced drug uptake via downregulation or mutation of influx pumps (e.g., SLC19A1). Upregulation of anti-apoptotic proteins (e.g., Bcl-2). Upregulation of drug efflux pumps (e.g., P-gp). Enhanced DNA damage response mechanisms (e.g., elevated PARP1 expression or (6)-methylguanine DNA methyltransferase, MGMT).	49,64
BRAF inhibitors	Gain of function mutations in downstream effects of BRAF (e.g., MEK). Loss of function in PTEN, resulting in hyperactivation of the PI3K/Akt pathway.	38,65
Immune checkpoint inhibitors	Severe T cell exhaustion, resulting in their reduced function. Mutations in PTEN, EGFR and MYC. Overexpression of other immune checkpoint receptors and ligands (e.g., B- and T-lymphocyte attenuator, BTLA).	66,67
Tyrosine kinase inhibitors	Mutations in kinase domains of tyrosine kinases (e.g., BCR-ABL1 and EGFR). Overexpression of drug efflux transports (e.g., MDR1, MRP1). Overexpression of tyrosine kinases. Overactivation of Met/HGF and STAT3 signalling pathways.	68-70
Serine/threonine kinase inhibitors	MEK1/2 or ERK1/2 mutations Hyperactivation of receptor tyrosine kinases (e.g., EGFR, MET). Loss of PTEN function to promote hyperactivation of PI3K/Akt pathway. Upregulation of microRNA's such as miR-514a, miR-125a and miR-34a.	38,65
Monoclonal antibodies	Loss of PTEN function, resulting in PI3K/Akt activation. Mutations in receptors (e.g., HER2) that prevent antibody binding.	71
Antibody drug conjugates	Downregulation of the molecular target (e.g., HER2 for trastuzumab emtansine) Decreased lysosomal degradation of cleavable linkers, resulting in reduced delivery of payload. Increased expression of drug efflux transporters (e.g., MRP1).	72,73

The mechanisms of resistance vary for each chemotherapy and tumours can often use several methods of resistance at one time, as outlined in **Table 1.2**. Therefore, it is essential to explore new targets for chemotherapy to improve treatment options and response rates. Furthermore, additional therapies that could sensitise cancers to current therapies is also of clinical importance to prevent blockages in patient treatment due to chemoresistance. Two proteins that are emerging as potential targets in chemotherapy

are the transcription factors Nrf2 (nuclear factor erythroid-2 related factor 2) and Nrf3 (nuclear factor erythroid-2 related factor 3), which belong to the Cap 'N' Collar family.

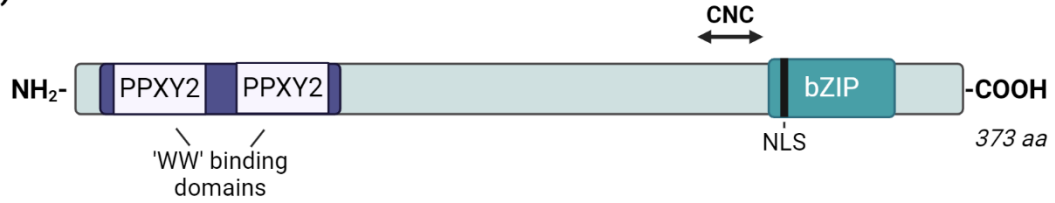
1.2 The Cap 'N' Collar family of transcription factors

The Cap 'N' Collar (CNC) family are a set of transcription factors containing a highly conserved 43-amino acid region, that regulate cellular homeostasis and coordinate the cells response to a range of stress signals, including oxidative stress, inflammation, and infection. The Cap 'N' Collar family was named after the CNC gene that was identified in the *Drosophila melanogaster* invertebrate⁷⁴, following discovery of the family's first member, NF-E2 (nuclear factor erythroid 2). 8 CNC members have been identified thus far, including Skn-1 (Skinhead family 1) in *Caenorhabditis elegans* (*C. elegans*) and the 6 mammalian members; NF-E2, Nrf1 (nuclear factor erythroid-2 related factor 1), Nrf2, Nrf3, Bach1 (BTB and CNC homology 1), and Bach2 (BTB and CNC homology 2).

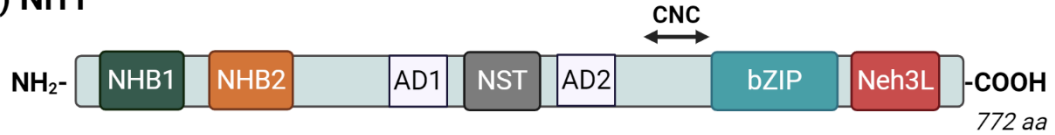
Of the non-mammalian members, the *D.melanogaster* CNC gene was identified in 1991, and is now known to be vital in embryonic development, aging and stress responses⁷⁵. The second non-mammalian family member to be identified was the *C. elegans* ortholog; Skn-1, which was found in 1992⁷⁶. Similarly to the CNC gene in *D.melanogaster*, Skn-1 also plays an important role in embryonic development, homeostasis and the oxidative stress response. Due to their high similarity to the mammalian CNC members, both CNC and Skn-1 have been important models of studying the role of CNC family members in humans⁷⁷.

Although the structure and functions of the different CNC family members vary (as displayed in **Figure 1.5**), they all share a basic region leucine zipper (bZIP) domain in their C-termini, which is essential for their ability to associate with DNA and either induce or repress transcription of their respective target genes.

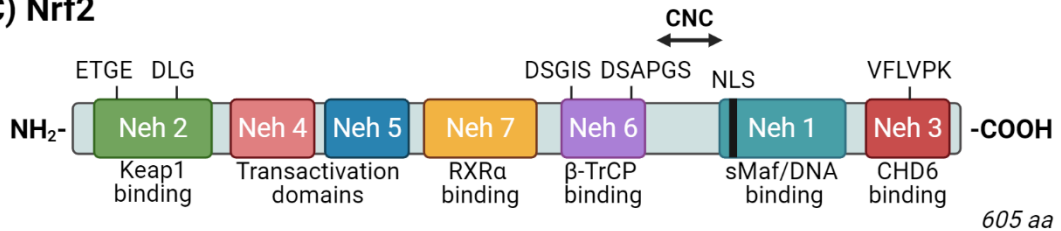
(A) NF-E2



(B) Nrf1



(C) Nrf2



(D) Nrf3

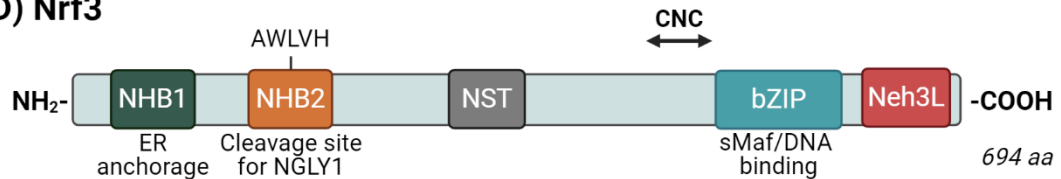


Figure 1.5 The structures of the NF-E2, Nrf1, Nrf2 and Nrf3 CNC transcription factors. Each member of the CNC family contains a highly conserved 43-amino acid CNC region located N-terminally to its bZIP domain. The bZIP domain facilitates interactions with other bZIP proteins, such as small Mafs, which permits binding to antioxidant response elements on CNC target genes. Other structural domains exist, such as the NHB1/2 domains in Nrf1 and Nrf3 and the Neh domains in Nrf2, however each member appears to vary in their structure and function. *Abbreviations: CNC; Cap 'N' Collar, bZIP; basic region leucine zipper, NLS; nuclear localisation signal, NHB1/2; N-terminal homology box 1/2, AD1/2; acidic domain 1/2, NST; asparagine/serine/threonine domain, Neh; Nrf2-ECH homology, Neh3L; Neh3-like, aa; amino acids.*

Through their bZIP domain, the CNC family of transcription factors form heterodimers with other bZIP proteins, such as c-Jun, and sMafs (small musculoaponeurotic fibrosarcoma), such as MafK, MafF and MafG^{78,79}. Heterodimers of sMaf and CNC proteins can then translocate into the nucleus, where they recognise specific sequences located in the promoter region of their target genes. These sequences, termed the antioxidant response element (ARE), all share the same core sequence of 5'-TGACnnnGC-3', where *n* is any base⁸⁰. The ARE was initially identified in the rat glutathione S-transferase Ya subunit gene⁸¹ and was later found as a key element

responsible for the action of phenolic antioxidant compounds in 1991 by Rushmore *et al.*⁸⁰. The ARE is now well studied and has been located in the promoters of hundreds of genes that regulate the antioxidant response to oxidative stress. Interestingly, the ARE shares sequence overlap with other response elements, such as the Maf recognition element (MARE), which facilitates binding of sMaf homodimers and heterodimers with CNC proteins, demonstrating that CNC family members can influence a wide range of genes⁸².

1.2.1 NF-E2

In 1989, the first mammalian CNC family member was identified, revealing the NF-E2 protein (also known as p45 NF-E2). NF-E2 is predominantly expressed in haematopoietic cells, such as erythrocytes, mast cells and granulocytes⁸³, where it induces expression of globin proteins, such as β -globin⁸⁴. The structure of NF-E2 still remains largely under characterised, with only a few key protein domains confirmed to date (**Figure 1.5A**). As is essential for the function of CNC family members, NF-E2 contains a bZIP domain⁸⁵, which is necessary for its interaction with sMaf proteins⁸⁶. Within this bZIP domain is a nuclear localisation sequence (NLS), which facilitates nuclear translocation of NF-E2⁸⁷. Furthermore, NF-E2 also possesses a TAD domain, containing two key motifs; PPXY1 and PPXY2 (**Figure 1.5A**). These domains (denoted PPXY for the presence of proline (P) tyrosine (Y), and 'X' meaning any other amino acid) are capable of binding to 'WW' domains (tryptophan-containing motifs) in numerous other proteins⁸⁸. As mutation of the PPXY1/2 motifs results in loss of transcriptional activity of NF-E2, it is hypothesised that these domains are essential for the binding of NF-E2 to other transactivator proteins^{89,90}.

Due to its expression within haematopoietic cells NF-E2 is predominantly known for its involvement in haematopoiesis⁹¹. This role has been further demonstrated using NF-E2 knockout mice, which display severe aberrations in haematopoiesis, with 90% of litter mates dying from haemorrhage associated with platelet deficiency and thrombocytopenia^{92,93}. NF-E2 also appears to have a role in bone development, with NF-E2 knockout mice showing increased bone mass⁹⁴ and impaired placental vascularisation⁹¹, suggesting a role for NF-E2 in non-haematopoietic cells. More recently, mutations and overexpression of NF-E2 have also been observed in myeloproliferative neoplasms, such as acute myeloid leukaemia (AML)⁹⁵, although more research is required to understand its clinical relevance.

1.2.2 Nrf1

Several years after the discovery of NF-E2, a second mammalian CNC family member was discovered, termed nuclear factor erythroid-2 related factor-1 (NFE2L1 or Nrf1), based upon its similarity to NF-E2⁹⁶. Unlike NF-E2 however, Nrf1 expression appears to be ubiquitous⁹⁶. Furthermore, several isoforms of Nrf1 exist, most notably the TCF11⁹⁷ and LCFR1⁹⁸ isoforms, which correspond to full-length and truncated proteins, of apparent weights of 120 kDa and 65 kDa, respectively. Like other CNC members, Nrf1 also possesses the conserved CNC domain⁹⁶, located N-terminally to its bZIP domain, and can associate with sMaf proteins^{99,100}. Nrf1 also possesses two key transactivation domains called acidic domain 1 and 2 (AD1/2), which are separated by an asparagine/serine/ threonine-rich domain (NST)^{101,102} (**Figure 1.5B**). The NST sequence, also conserved in the CNC and Skn-1 invertebrate orthologs and in human Nrf3 proteins, is required for glycosylation of Nrf1, which appears to be important for its cellular localisation¹⁰².

A further transactivation domain also exists in the Nrf1 C-terminus, termed Neh3-like (Neh3L) for its similarity to the Neh3 domain in Nrf2, however little is known about this domain other than that it appears to act as a repressor of Nrf1 activation¹⁰³. In its glycosylated form, Nrf1 predominantly resides within the endoplasmic reticulum (ER), where it is anchored by its N-terminal domains, NHB1 and NHB2 (N-terminal homology box 1/2)^{101,102,104}. Under normal conditions, Nrf1 is sequestered in the ER, where it is ubiquitinated by Hrd1 (3-Hydroxy-3-methylglutaryl reductase degradation 1) and degraded via the ER-associated degradation pathway. When activated however, for example in times of proteotoxic stress, Nrf1 is released from the ER, where it is deglycosylated by NGLY1 (N-glycanase 1)¹⁰⁵ and cleaved by the aspartyl protease DDI2 (DNA damage inducible 1 homolog 2)^{106,107}, to produce a truncated form of Nrf1 that can translocate to the nucleus and induce ARE-driven gene transcription¹⁰⁶.

Unlike NF-E2 and despite its ability to bind to NF-E2 elements in the β -globin gene locus control region, Chan *et al.*¹⁰⁸ identified that Nrf1 does not appear to have a function in controlling globin gene expression. Despite this, Chan *et al.* also found that constitutive Nrf1 knockout in mice results in embryonic lethality¹⁰⁸. Additionally, in isolated fibroblasts from Nrf1 knockout mouse embryos, expression of genes involved in glutathione synthesis, such as GCLC (Glutamate—cysteine ligase catalytic subunit) and GCLM (Glutamate—cysteine ligase regulatory subunit) was decreased, and cells were more sensitive to pro-oxidant compounds^{109,110}. Nrf1 deletion in human cells has also demonstrated lower levels of antioxidant genes in response to oxidative insults, such as arsenic, including GCLC, GCLM and NQO1 (NAD(P)H:quinone oxidoreductase 1)¹¹¹,

suggesting that Nrf1 plays a role in the oxidative stress response. Nrf1 has also been found to have a role in proteostasis, where it regulates the expression of genes necessary for proteasome assembly, such as the ARE-containing proteasome subunit (PSM) genes^{112,113}. Nrf1 is also induced by proteasome inhibitors, such as MG-132, which promotes Nrf1 accumulation and nuclear translocation¹¹⁴. This therefore also suggests that Nrf1 may play a role in diseases with dysregulated proteasome and antioxidant activity, such as cancer, neurodegenerative and cardiovascular conditions^{113,115-118}.

1.2.3 Nrf2

Nrf2 (nuclear factor erythroid-2 related factor-2) is the most widely studied of the CNC family members and is commonly referred to as a “master regulator” of oxidative stress, due to its ability to regulate genes with cytoprotective, anti-inflammatory and detoxification activities^{119,120}. Following the identification of NF-E2¹²¹ and Nrf1⁹⁶, Nrf2 was originally isolated in 1994 by Moi *et al.*¹²² through a bacterial expression screening of a K562 cDNA library, using an NF-E2 motif as a recognition probe. In their study, Moi *et al.* not only observed the ubiquitous expression of Nrf2, but also identified its bZIP domain, earning it its place among the other CNC family members¹²². Shortly after, in 1996, Nrf2 alongside Nrf1, was found to regulate genes that contained a *cis*-regulatory antioxidant response element (ARE) in their promoter region, which had previously been found to induce expression of antioxidant genes, such as the rat glutathione-S-transferase-Ya⁸¹. Over 250 ARE-containing target genes of Nrf2 have since been identified, and Nrf2 dysfunction has been noted in numerous chronic inflammatory diseases, aging and in cancer¹¹⁹.

1.2.3.1 Structure of Nrf2

The human Nrf2 protein is encoded by the NFE2L2 gene located on chromosome 2q31.2, and consists of 7 domains, termed Neh 1-7 (Nrf2-ECH homology 1)¹²³, as displayed in **Figure 1.5C**. Containing the bZIP domain, Neh1 is responsible for heterodimerisation of Nrf2 with sMaf proteins (e.g., MafG, MafF and MafK) necessary for Nrf2's interaction with ARE sequences^{79,124}. Within the Neh1 also lies a nuclear localisation signal (NLS)¹²⁵, responsible for nuclear import of Nrf2, and a nuclear export signal (NES), which promotes Nrf2 nuclear export when not masked by other bound proteins such as sMafs¹²⁶. The second domain, Neh2, is a lysine-rich domain that contains two key motifs, ETGE and DLG, located in the N-termini of Nrf2^{127,128}. Through these motifs, Neh2 interacts with Keap1 (Kelch-like ECH-associated protein 1), a negative regulator of Nrf2 and substrate adapter protein for the Cullin-3 E3 ligase

complex that facilitates Nrf2 turnover^{129,130}. Here, the lysine residues of Nrf2 can become poly-ubiquitinated, allowing for proteasomal degradation via the ubiquitin-proteasome system (UPS)¹²⁸.

The Neh3 domain is necessary for transactivation, protein stability and contains a VFLVPK motif¹²³, which is essential for Nrf2's ability to bind CHD6 (Chromodomain-helicase DNA-binding protein 6)¹³¹. Interestingly, the Neh3 domain appears to be conserved across other members of the CNC family, including Nrf1, where it also acts as a transactivation domain¹⁰³. Furthermore, the Neh4 and Neh5 domains located in the N-terminus of Nrf2 are also involved in Nrf2's transactivation activity and facilitate interactions with transcriptional activators such as CREB (cAMP Responsive Element binding protein)-binding protein to enhance transcriptional activity¹³². The sixth domain, Neh6, is another major domain involved in Nrf2 regulation via a Keap1-independent degradation mechanism involving β -TrCP (β -transducin repeat-containing protein)¹³³. Neh6 contains DSGIS and DSAPGS binding motifs, which can become phosphorylated by the action of GSK-3 β (glycogen synthase kinase 3- β)¹³³. This phosphorylated form of Nrf2 is then recognised by the SCP/ β -TrCP E3 ligase complex¹³⁴, causing Nrf2 to be marked for degradation^{134,135}. The Neh7 domain was the last to be identified and is responsible for the binding of RXR α (retinoic X receptor α), which, when bound, can inhibit the interaction between Nrf2 and AREs¹³⁶.

1.2.3.2 Keap1-dependent regulation of Nrf2

Nrf2 is a relatively unstable protein, with a short half-life of approximately 13-30 minutes, depending on cell type¹³⁷⁻¹³⁹. Keap1, the negative regulator of Nrf2, was originally identified by a yeast two-hybrid screen¹²⁸, and has since been recognised as a substrate adapter for the E3 ligase complex^{129,134,139}. It is through its association with a Cullin3 (CUL3)-E3 ligase complex that Keap1 mediates the proteasomal degradation of Nrf2¹³⁰. Keap1 consists of five domains (**Figure 1.6**)- the N-terminal region (NTR), Broad complex, Tramtrack and Bric-a-Brac (BTB) domain, the linker intervening region (IVR), the Kelch repeat domain, and the C-terminal region (CTR)¹⁴⁰.

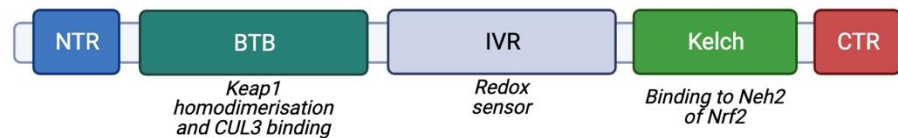


Figure 1.6 The structural domains of Keap1. The E3 ligase substrate adaptor Keap1 acts as a negative regulator of Nrf2 by sequestering it in the cytosol. This is achieved via a protein-protein interaction between the Keap1 Kelch domain and the Neh2 domain of Nrf2. Keap1 also possesses a BTB domain that is responsible for its homodimerisation and subsequent recruitment of the CUL3 E3 ligase complex, which polyubiquitinates Nrf2 to mark it for proteasomal degradation. Keap1 also contains a IVR domain, which senses oxidative stress via its cysteine residues, which can become modified in the presence of oxidative stress, causing activation of the Nrf2 signalling pathway. *Abbreviations: NTR; N-terminal region, BTB; Broad complex, Tramtrack and Bric-a-Brac, IVR; intervening region, CTR; C-terminal region, CUL3; Cullin 3.*

The BTB domain is responsible for Keap1 homodimerisation and binding of CUL3 to form the CUL3- E3 ligase complex^{141,142}. Keap1 contains 27 cysteine residues that can be modified in the presence of electrophilic activators, such as sulforaphane, which modify the Keap1/Nrf2 interaction to induce Nrf2 activation¹³⁸. Among these cysteine residues, Cys151, Cys273, Cys288 are the most important for the Nrf2/Keap1 interaction, which are located within the IVR and BTB domains of Keap1, suggesting that these domains act as redox sensors¹⁴³. Finally, Keap1 also contains 6 β -propeller Kelch repeats which make up the Kelch domain in the C-terminus of Keap1¹⁴⁴, responsible for association with the Neh2 domain of Nrf2¹²⁸ (**Figure 1.7**).

During non-stressed conditions, Nrf2 is maintained at low levels within the cytosol and is prevented from entering the nucleus via binding to a Keap1 homodimer¹⁴². As displayed in **Figure 1.7**, Nrf2/Keap1 binding has been described previously as a “hinge-and-latch” model, in which one molecule of Keap1 binds first to the higher affinity ETGE motif of Neh2 via its Kelch domain (the hinge), followed by binding of the lower affinity DLG motif to a second Keap1 molecule (the latch)¹⁴⁵. This binding configuration allows for easy ubiquitination of the lysine-rich area of Neh2, which is required for proteasomal degradation of Nrf2 via the UPS¹²⁹. Once bound to Nrf2, Keap1 recruits CUL3 and ring box protein 1 (Rbx1) to form the CUL3-E3 ligase complex¹³⁰. Nrf2 is then polyubiquitinated at its lysine rich region and is degraded by the 26S proteasome¹³⁷ (**Figure 1.7A**).

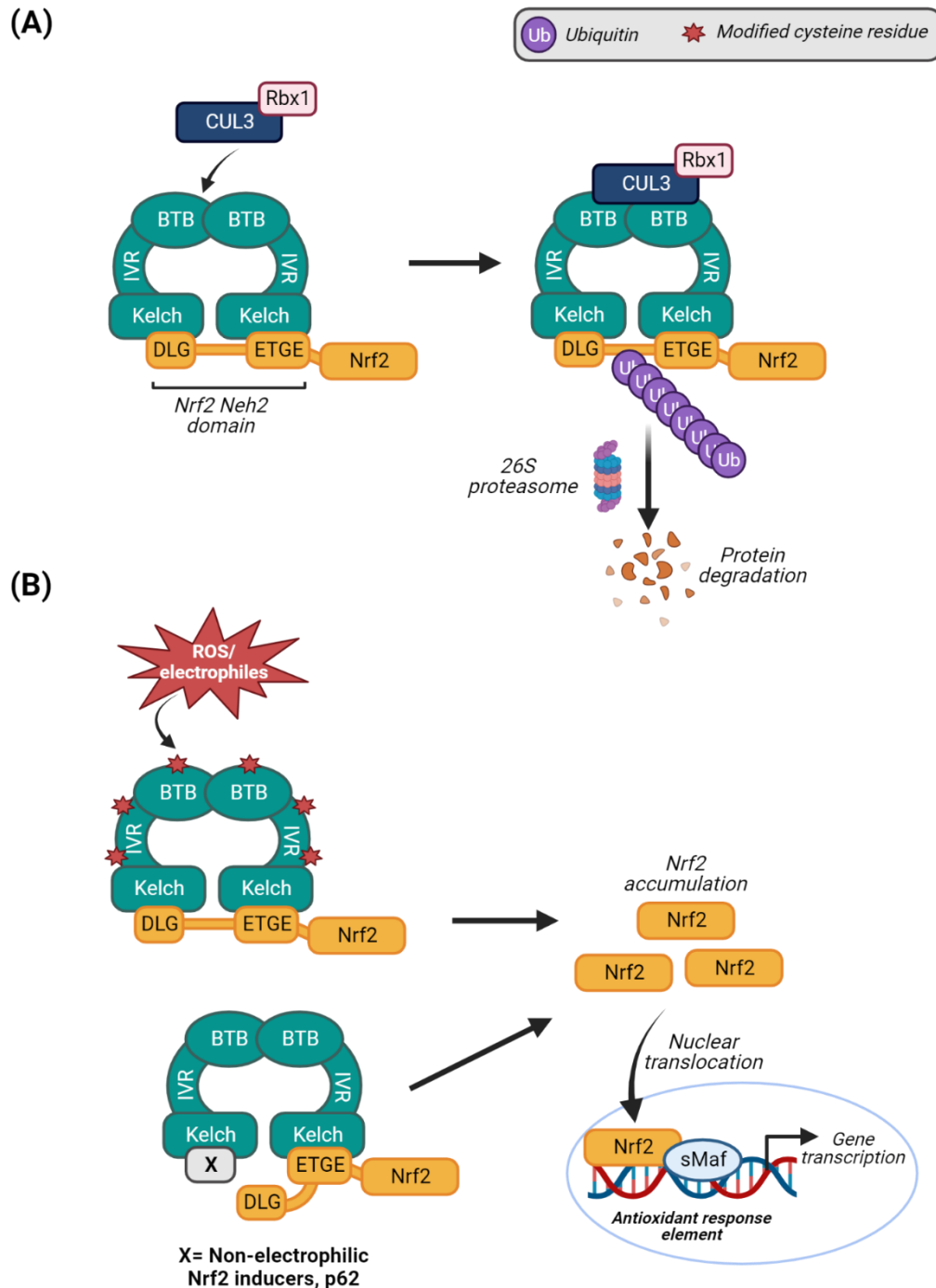


Figure 1.7 Keap1-mediated regulation of Nrf2 in non-stressed and stressed conditions. (A) In non-stressed conditions, Nrf2 is sequestered by Keap1, which binds as a homodimer via interaction between its Kelch domain and the DLG and ETGE motifs of the Nrf2 Neh2 domain. Keap1 can then recruit the CUL3/Rbx1 E3 ligase complex via its BTB domain, resulting in ubiquitination of Nrf2 at the lysine rich region in Neh2, and subsequent degradation by the 26S proteasome. (B) In stressed conditions, such as in the presence of high ROS levels or electrophilic Nrf2 inducers, cysteine residues in Keap1 become modified, preventing Nrf2 ubiquitination. In the presence of non-electrophilic Nrf2 inducers or other Keap1-binding proteins such as p62, the lower affinity DLG motif of Nrf2 dissociates from Keap1, hence Keap1 cannot target Nrf2 for degradation. If not degraded, Nrf2 accumulates in the cytosol, following which it can form heterodimers with sMaf proteins. Nrf2/sMaf heterodimers can translocate to the nucleus and bind to antioxidant response elements on a number of cytoprotective genes, inducing their transcription.

During stressed conditions, for example in the presence of ROS, UV radiation, and xenobiotics, or following exposure to electrophilic and non-electrophilic Nrf2 inducers, the ubiquitination of Nrf2 is prevented, leading to Nrf2 accumulation and subsequent nuclear translocation (**Figure 1.7B**). For example, high levels of ROS or electrophilic compounds (e.g., sulforaphane), can modify cysteine residues in Keap1 that are essential for Keap1-mediated proteasomal degradation of Nrf2, such as Cys151, Cys273 and Cys288¹⁴⁶. While these modifications were initially believed to act via the 'hinge and latch' model, to 'release' the DLG 'latch' of Nrf2 from Keap1^{145,147}, a recent study by Horie *et al.* determined that such modifications do not result in the release of the DLG motif but instead may induce conformational changes in Keap1 that prevent Nrf2 ubiquitination¹⁴⁸. Although many Nrf2 activators/inducers, including bardoxolone methyl, sulforaphane, and curcumin, act to modify Keap1 cysteine residues, at present the exact mechanism of how this prevents Nrf2 ubiquitination is not fully understood.

On the other hand, non-electrophilic Nrf2 inducers such as the Keap1-binding protein p62/SQSTM1 (ubiquitin binding protein p62/ sequestosome 1) and pharmacological protein-protein interaction (PPI) inhibitors such as the peptides PRL295 and NG262, do appear to act by dissociating the DLG motif of Nrf2 from the Kelch domain of one Keap1 monomer¹⁴⁸ (**Figure 1.7B**). Without binding of both the ETGE and DLG motifs of Nrf2 to the Kelch domains of the Keap1 homodimer, Keap1 cannot induce Nrf2 ubiquitination, thereby preventing Keap1 from targeting Nrf2 for degradation^{127,129,145,149}. This allows Nrf2 to accumulate in the cytosol, where it can form heterodimers with sMaf proteins. The Nrf2/sMaf heterodimer can then translocate into the cell nucleus, recognise ARE sequences on target genes and induce their transcription¹⁵⁰ (**Figure 1.7B**).

1.2.3.3 Keap1-independent regulation of Nrf2

Although Keap1 is a key regulator of Nrf2, other regulation mechanisms also exist, as described in **Figure 1.8**. One such Keap1-independent mechanism involves the β -TrCP/Skp1/CUL1/Rbx1 E3 ubiquitin ligase complex. In this mechanism, GSK-3 β phosphorylates Nrf2 via a DSGIS motif found in Neh6, allowing binding of β -TrCP to both the DSGIG and DSAPGS located in this domain¹³³. β -TrCP can then associate with a Skp1/CUL1/Rbx1 E3 ubiquitin ligase complex, thus marking Nrf2 for ubiquitination and proteasomal degradation^{134,135} (**Figure 1.8**).

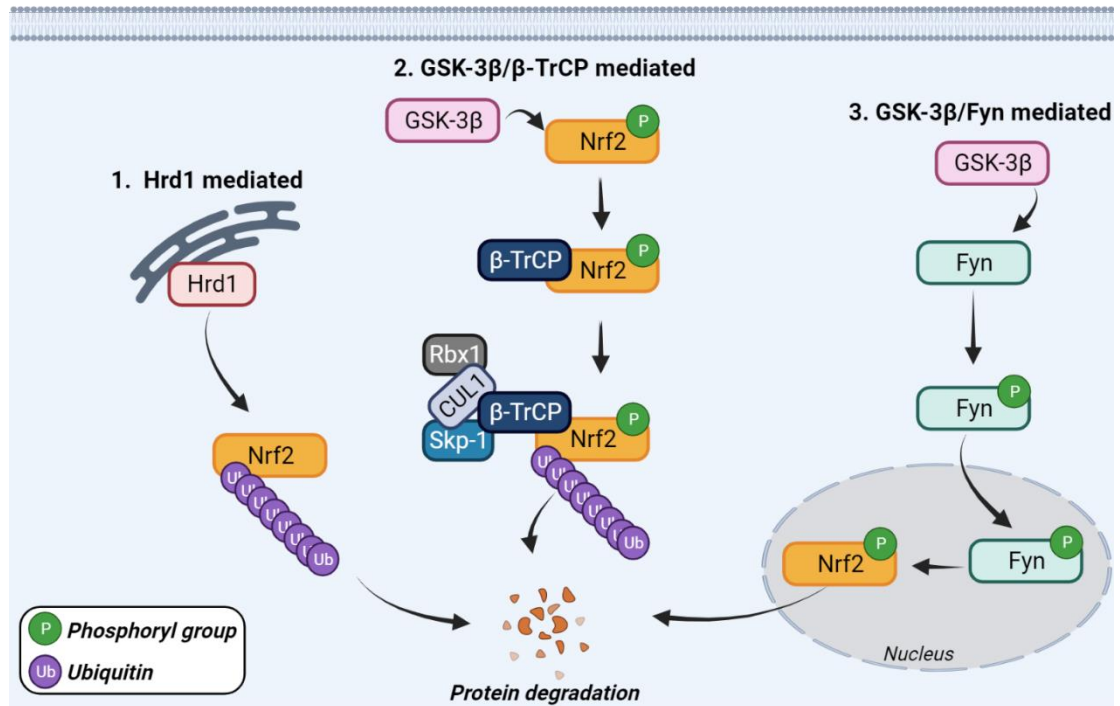


Figure 1.8 Keap1-independent mechanisms of Nrf2 degradation. Nrf2 is degraded by multiple mechanisms aside from Keap1 including via Hrd1, β -TrCP and Fyn. **(1)** In times of ER-stress, Nrf2 is ubiquitinated by the ER-associated Hrd1 E3 ligase complex, inducing Nrf2 proteasomal degradation. **(2)** Nrf2 is phosphorylated by GSK-3 β , allowing β -TrCP to associate with Nrf2 via its Neh6 domain. β -TrCP then recruits the CUL1/Skp-1/Rbx1 ligase complex, which ubiquitinates Nrf2, thereby promoting its proteasomal degradation. **(3)** Fyn is phosphorylated by GSK-3 β , leading to its nuclear accumulation. Fyn can then phosphorylate tyrosine⁵⁶⁸ on Nrf2, resulting in Nrf2 nuclear export and degradation.

Furthermore, Nrf2 is also regulated by a GSK-3 β phosphorylation cascade which restricts nuclear accumulation of Nrf2. Activation of GSK-3 β (e.g., by hydrogen peroxide) can phosphorylate threonine residues in Fyn (a tyrosine kinase), which accumulates in the nucleus. Phosphorylated Fyn can then in turn phosphorylate Nrf2 at Tyr⁵⁶⁸, leading to Nrf2 nuclear export and degradation via the ubiquitin system¹⁵¹ (**Figure 1.8**). Additionally, the ER-associated E3 ubiquitin ligase Hrd1 (HMG-CoA reductase degradation protein 1) has also been shown to induce Nrf2 degradation in times of ER stress, via binding to the Neh4 and Neh5 domains during liver cirrhosis¹⁵² (**Figure 1.8**).

Epigenetic modification or mutations of Nrf2 or of its negative regulators, such as hypermethylation or point mutations in Keap1, can also induce Nrf2 protein expression¹⁵³. Other proteins can also compete with Nrf2 for binding to Keap1, such as p62, which activates Nrf2 by preventing its association with the Kelch domain of Keap1¹⁵⁴. Furthermore, two other members of the CNC family, Bach1 and Bach2, which mainly act as transcriptional repressors¹⁵⁵, have also been shown to play a role in

restricting Nrf2 activity. Like Nrf2, Bach1 and Bach2 associate with sMaf proteins and bind to the ARE, hence, Bach1 and Bach2 can compete with Nrf2 for ARE-binding and can therefore act to negatively regulate Nrf2 target gene expression^{156,157}.

Nrf2 also cross-talks with numerous other signalling pathways, such as the NOTCH, NF- κ B (nuclear factor κ -B), PI3K/Akt and AhR (aryl hydrocarbon receptor) pathways, with many of these relationships being bidirectional, where the two pathways feedback on each other. For example, Nrf2 regulates Notch1 expression, due to a functional ARE sequence in the NOTCH1 gene, while Nrf2 is also regulated by NOTCH¹⁵⁸. In this case, Nrf2 knockout not only abrogates NOTCH signalling in murine fibroblasts, but also impairs liver regrowth following hepatectomy, suggesting a key role of the Nrf2/NOTCH pathway in liver disease.

Furthermore, Nrf2 also cross-talks with NF- κ B, which is a key mediator in the response to pro-inflammatory stimuli¹⁵⁹. Not only do Nrf2 target genes (such as NQO1 and HO-1) assist in repressing NF- κ B transcriptional activity, but NF- κ B also represses Nrf2 via competing for binding to the CBP (CREB-binding protein)-p300 transcriptional coactivator complex¹⁶⁰. As both Nrf2 and NF- κ B require CBP-p300 to induce transcription, binding of NF- κ B to CBP-p300 reduces the ability of Nrf2 to mediate target gene transcription¹⁶⁰.

1.2.3.4 Nrf2 downstream target genes

Once in the nucleus, Nrf2 can induce the transcription of hundreds of genes, such as Phase II detoxification enzymes, drug efflux pumps, enzymes regulating glutathione or haem metabolism, and drug conjugation enzymes¹¹⁹, as summarised in **Table 1.3**. Currently over 250 Nrf2 target genes have been validated¹⁶¹, however transcriptomic profiling in tandem with proteomic studies of transient overexpression of Nrf2, conducted by Liu *et al.*¹¹⁷ and Ibrahim *et al.*¹¹⁶, identified hundreds of other potential Nrf2 target genes involved in homeostasis regulation. Importantly, Nrf2 target genes all contain one or more ARE sequences located in their promoter region^{80,162}, which allows for recognition by Nrf2/sMaf heterodimers. Induction of Nrf2 via stress signals or by exposure to phenolic antioxidants, results in rapid transcription of Nrf2 target genes, which act to regulate homeostasis, cytoprotection, and anti-inflammatory activity. For example, previous research by our lab demonstrates that exposure to the pro-inflammatory stimulant, lipopolysaccharide, induces transcription of Nrf2 target genes such as NQO1 and HO-1¹⁶³.

Table 1.3 Examples of validated Nrf2 target genes and their function.

Acronym	Name	Function	Ref.
<i>Xenobiotic detoxification</i>			
NQO1	NAD(P)H dehydrogenase quinone 1	Reduction of reactive quinones	79,164-166
ADH7	Alcohol dehydrogenase 7	Alcohol metabolism	161,167-169
AKR	Aldo-keto reductases (e.g., AKR1B10, AKR1C1, AKR1C2)	Aldehyde and ketone reduction	166,170-172
ALDH	Aldehyde dehydrogenase (e.g., ALDH1A1, ALDH3A1, ALDH7A1)	Catalyses aldehyde oxidation	161,166,168,169
CYPs	Cytochrome P450 (CYP1B1, CYP2A5, CYP2B9)	Catalyses xenobiotic oxidation and reduction	173-175
CES	Carboxylesterases (e.g., CES1H, CES1G)	Catalyses ester and amide bonds	169
SOD	Superoxide dismutase (e.g., SOD3)	Catalyses the dismutation of superoxide radicals	165,167
<i>Xenobiotic transport</i>			
ABC	ATP-binding cassette transporters (e.g., ABCB6, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCG2)	Transport and efflux of numerous xenobiotics	156,157,176-180
OATP2B1	Organic anion-transporting polypeptide 2B1	Organic ion uptake transporter	169
Pgp	P-glycoprotein (ABCB1)	Major drug efflux pump	173,179,181
<i>Glutathione metabolism</i>			
GCLC	Glutamate-Cysteine Ligase Catalytic Subunit	Catalyses the rate limiting step in glutathione synthesis	164,166,168,182,183
GCLM	Glutamate-Cysteine Ligase Modifier Subunit	Catalyses the rate limiting step in glutathione synthesis	164,165,182
GSR	Glutathione Reductases (e.g., GSR1)	Reduces glutathione disulfide to glutathione	164,168
GPX	Glutathione Peroxidase (e.g., GPX1, GPX2, GPX4)	Scavenges ROS and oxidises glutathione to glutathione disulfide	166,168,184
<i>Conjugation</i>			
GST	Glutathione- S- transferases (e.g., GSTA1, GSTA2, GSTA3, GSTA5, GSTM1, GSTM2, GSTM3)	Conjugates reduced glutathione to xenobiotics for detoxification	81,165,168,169,180
UGT	UDP glucuronosyltransferases (e.g., UGT1A1, UGT2B7)	Conjugates glucuronic acid to xenobiotics for detoxification (glucuronidation)	166,185
SULT	Sulfotransferases (e.g., SULT1A1, SULT1A2)	Conjugates sulfo groups with xenobiotics for detoxification	166,186
<i>Thioredoxin enzyme system</i>			
TXN1	Thioredoxin-1	Reduces oxidised proteins	166,168,187,188
TXNRD1	Thioredoxin reductase 1	Catalyses the reduction of thioredoxin	161,164,166,189
PRDX	Peroxiredoxins (e.g., PRDX1, PRXD6)	Catalyses the reduction of peroxides	161,166,168,187
SRXN1	Sulfiredoxin-1	Reactivates peroxiredoxins	161,164,166
<i>Haem metabolism</i>			
HO-1	Haem oxygenase-1	Converts haem to biliverdin	155,164,166,168,190,191
BLVR	Biliverdin reductases (e.g. BLVRA, BLVRB)	Catalyses the conversion of biliverdin to bilirubin	166,168,191
FTL	Ferritin light chain (e.g., FTL1)	Iron storage	164,191,192
FTH	Ferritin heavy chain (e.g., FTH1, FTHL12, FTHL17)	Iron storage	164,191,192
FECH	Ferrochelatase	Catalyses final stage of haem biosynthesis	164,173,191
<i>NADPH regeneration and PPP pathway</i>			
G6PD	Glucose-6-phosphate 1-dehydrogenase	NADPH generation in PPP pathway	164,166,168,193
PGD	6-phosphogluconate dehydrogenase	NADPH generation in PPP pathway	164,169,193
ME1	Malic enzyme 1	NADPH generation via conversion of malate in TCA cycle	164,193
TALDO1	Transaldolase 1	Role in nonoxidative stage of PPP	169,193
TKT	Transketolase isoform 1	Role in nonoxidative stage of PPP	193
IDH1	Isocitrate dehydrogenase 1	NADPH generation in PPP pathway	168,193
<i>Transcription factors</i>			
AHR	Aryl hydrocarbon receptor	Regulation of xenobiotics and CYP450s	175
PPAR γ	Peroxisome proliferator-activated receptor gamma	Regulates adipogenesis and lipid uptake	194
RXR α	Retinoid X receptor alpha	Inhibits the Nrf2/Keap1 pathway	136
CEBP α	CCAAR/enhancer binding protein alpha	Regulation of adipogenesis	169

1.2.4 Nrf3

Nrf3 was first isolated in 1999 by Kobayashi *et al.*¹⁹⁵, where it was identified as a member of the CNC family and was co-expressed with MafG. Although Nrf3 is expressed in many tissue types, its highest expression is within the placenta¹⁹⁵. Nrf3 predominantly exists as a membrane-bound glycoprotein that is localised to the ER via its N-terminus¹⁰¹. However, under stressed conditions, Nrf3 can become cleaved, producing a smaller form that can translocate to the nucleus and associate with sMaf proteins¹⁹⁶. Currently, the exact physiological role of Nrf3 has not been characterised, although it appears to have a potential role in ER and oxidative stress responses. While Nrf3 has previously remained in the shadow of its sister-protein, Nrf2, it has recently gained interest due to its potential involvement in a number of human cancers¹⁹⁷.

1.2.4.1 Structure of Nrf3

Although Nrf3 possesses the canonical CNC domain adjacent to its bZIP domain^{195,196} (**Figure 1.5D**), the structure of Nrf3 appears to be most similar to that of Nrf1. Like other bZIP CNC proteins, Nrf3 forms heterodimers with sMaf proteins^{196,198}, which in turn bind to ARE sequences on target gene promoters, to either induce or repress transcription¹⁹⁸. Like Nrf1, Nrf3 contains a NST domain located N-terminally to the CNC region, which facilitates its glycosylation and subsequent localisation within the cytosol^{101,102}. Nrf3 also possesses similar NHB1 and NHB2 domains in its N-terminus to Nrf1^{101,199}, which are responsible for its ER-anchorage and enzymatic cleavage, respectively (**Figure 1.5D**).

The NHB1 domain is responsible for the anchorage of Nrf3 to the ER, keeping it sequestered in the cytosol. The NHB2 domain contains a AWLVH motif, which acts as a cleavage site for DDI2, which mediates the proteolytic cleavage of Nrf3 that is necessary for its nuclear translocation. Additionally, Nrf3 also appears to possess a similar Neh3-like domain (Neh3L) in its C-terminus, although its function is not yet characterised¹⁰¹.

1.2.4.2 Regulation of Nrf3

Although the exact mechanisms through which Nrf3 signalling becomes activated are yet to be fully elucidated, treatment with the ER-stress inducers thapsigargin and tunicamycin induces Nrf3 expression in smooth muscle²⁰⁰ and choriocarcinoma cells¹⁹⁹, suggesting that Nrf3 is a mediator of ER stress. Additionally, Chenais *et al.*¹⁹⁶ identified that treatment of choriocarcinoma cells with the pro-inflammatory cytokine TNF α induces Nrf3, once again suggesting that Nrf3 is a stress-inducible protein. Additionally,

incubation of HEK293 cells (an embryonic kidney cell line) with the proteasome inhibitor MG-132 also appeared to stabilise Nrf3 expression²⁰¹, suggesting that its turnover is likely mediated via proteasomal degradation, potentially in a similar mechanism to its sister proteins NF-E2, Nrf1 and Nrf2. In fact, similarly to the other CNC family members, Nrf3 expression is tightly regulated within the cell (**Figure 1.9**).

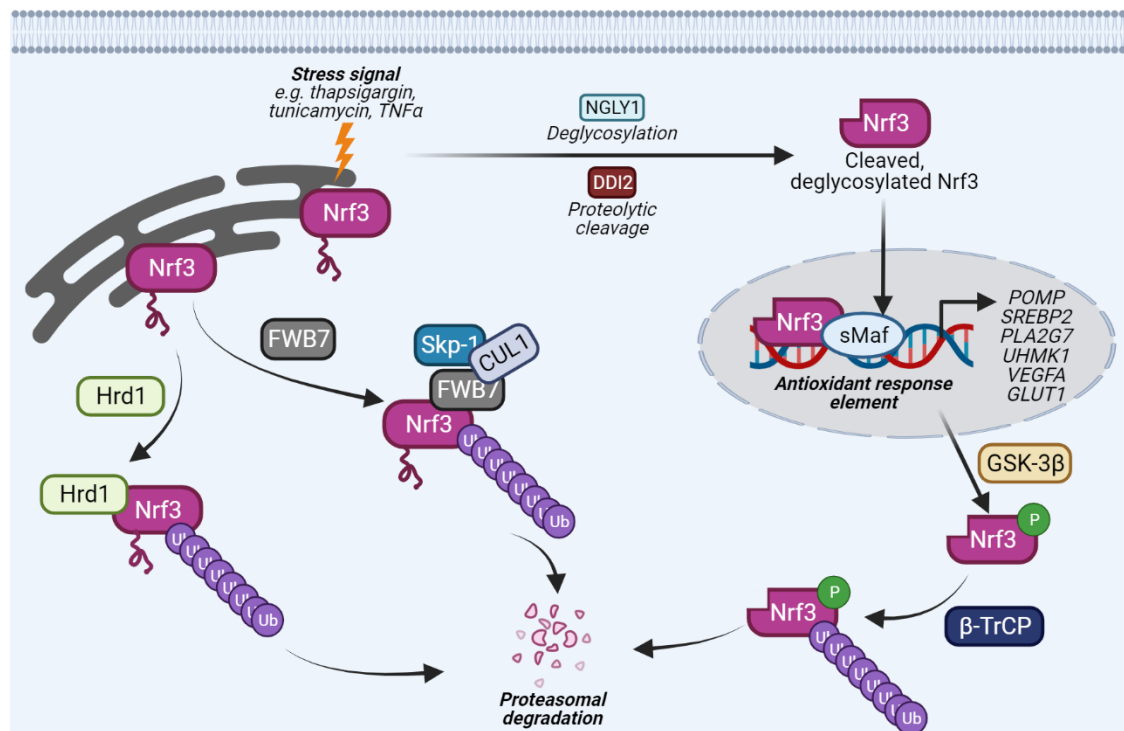


Figure 1.9 Mechanisms of Nrf3 signalling in stressed and non-stressed conditions. Under normal (non-stressed) conditions, Nrf3 is glycosylated and anchored in the endoplasmic reticulum (ER) via its NHB1 domain. In the ER, Nrf3 is degraded via ubiquitination by the E3 ligase Hrd1. Alternatively, Nrf3 can also be degraded by the FWB7/CUL1/Skp-1 E3 ligase complex, which ubiquitinates Nrf3 at its Lys⁷⁷ residue to mark it for proteasomal degradation. In stressed conditions, for example in the presence of ER-stressors such as tunicamycin and thapsigargin, Nrf3 is cleaved by the DDI2 aspartyl protease at its NHB2 domain and is deglycosylated via NGLY1. Cleaved, deglycosylated Nrf3 can then translocate into the nucleus to induce, or repress, gene transcription. Nrf3 can also be exported from the nucleus via β-TrCP-mediated ubiquitination.

Three main forms of the Nrf3 protein have been identified thus far, which include a glycosylated full-length form (~120 kDa), deglycosylated full-length form (~100 kDa), and a deglycosylated, cleaved form (~76 kDa), which possess half-lives of approximately 40, 15 and 20 minutes, respectively¹⁹⁹. Both the 120 kDa and 100 kDa forms appear to be the main forms of Nrf3 expressed within the cell, and are mainly ER-localised, suggesting that full length Nrf3 is typically ER-bound¹⁹⁹. The 76 kDa form on the other hand, appears to be induced mainly during times of cellular stress, and is the main form detected in the nucleus, suggesting that Nrf3 must first be cleaved in order to induce its transcriptional activity¹⁰¹.

Under normal, non-stressed conditions, full-length Nrf3 is glycosylated at its NST domain and is sequestered in the cytosol, where it remains bound to the ER via its NHB1 N-terminal domain^{101,202} (**Figure 1.9**). Nrf3 is then ubiquitinated at its Lys⁷⁷ residue, by the E3 ubiquitin ligase substrate FBW7 (F-box and WD repeat domain-containing 7), which recruits the FBW7/SCF E3 ligase complex, to mark Nrf3 for proteasomal degradation²⁰¹. Nrf3 can also be ubiquitinated by the Hrd1 E3 ligase in the endoplasmic reticulum. Additionally, phosphorylation of Nrf3 by GSK-3 β also appears to be necessary for Nrf3 degradation, and may promote β -TrCP-mediated proteasomal degradation of Nrf3²⁰¹ (**Figure 1.9**) Upon induction of stress however, Nrf3 is released from the ER via cleavage of the NHB2 domain by the DDI2 aspartate protease²⁰². Following this, Nrf3 is then deglycosylated by the action of NGLY1, resulting in the 76 kDa form that can associate with sMaf proteins and translocate to the nucleus to induce or repress transcription of certain ARE-containing genes¹⁹⁹ (**Figure 1.9**).

1.2.4.3 Nrf3 function and transcriptional role

To date, very little research has been conducted on the transcriptional role and physiological function of Nrf3. Initially Nrf3 was believed to have a similar function to Nrf1 due to their structural similarity (**Figure 1.5**), however unlike Nrf1, Nrf3 knockout mice develop normally and show no phenotypic differences compared to wild-type mice²⁰³. Upon challenge by pro-inflammatory and carcinogenic stimuli, such as butylated hydroxytoluene and benzo[a]pyrene, Nrf3 knockout mice are more susceptible to respiratory distress²⁰⁴ and tumour growth²⁰⁵, respectively²⁰⁴. This suggests that Nrf3 may have a protective role in inflammatory disease and carcinogenesis, perhaps similar to its sister protein Nrf2.

Although Nrf3 is able to form heterodimers with sMaf proteins and can recognise ARE sequences on potential target genes^{195,196,198}, it appears to have a weaker transcriptional role than that of other CNC family members and also seems to act more often as a transcriptional repressor. For example, in transcriptomic profiling studies of CNC family members conducted by Liu *et al.*¹¹⁷ and Ibrahim *et al.*¹¹⁶ found that when overexpressed, Nrf3 only affected 1.5% of the active transcriptome, compared to that of 7.2% and 9.3% observed with Nrf1 and Nrf2, respectively^{116,117}. Furthermore, Ibrahim *et al.* also observed that of the transcripts that were affected by Nrf3 overexpression, only 176 were upregulated and 404 transcripts were downregulated¹¹⁶. Although Nrf3 is therefore believed to be involved in the regulation of ARE-containing genes, only 8 Nrf3 target genes containing ARE sequences have been validated thus far (**Table 1.4**)

Table 1.4 Validated Nrf3 target genes.

	Acronym	Name	Function	Ref.
Transcriptional activator	POMP	Proteasome maturation protein	Chaperone protein for formation of the 20S proteasome.	²⁰⁶
	SREBP2	Sterol Regulatory Element Binding Transcription Factor 2	Transcription factor that mediates sterol-regulated genes for cholesterol homeostasis.	²⁰⁷
	PLA2G7	Phospholipase A2, group 7	Phospholipase involved in phospholipid catabolism during inflammatory and oxidative stress.	²⁰⁸
	UHMK1	U2AF Homology Motif (UHM) Kinase 1	Serine/threonine kinase that promotes cell cycle progression.	²⁰²
	VEGFA	Vascular endothelial growth factor alpha	Growth factor that induces vascular endothelial cell proliferation and migration.	²⁰⁹
	GLUT1	Glucose transporter 1	Glucose transport	²¹⁰
Transcriptional repressor	PRDX6	Peroxiredoxin 6	Involved in redox regulation of H ₂ O ₂ and phospholipid hydroperoxides.	²¹¹
	DUX4	Double homeobox factor 4	Represses CDK1 (Cyclin Dependent Kinase 1) to prevent cell cycle progression.	²¹²
Unclear role	NQO1	NAD(P)H Quinone Dehydrogenase 1	Redox protein involved in reduction of reactive quinones.	^{101,198}

As displayed in **Table 1.4**, Nrf3 acts as a transcriptional activator of POMP, SREBP2, PLA2G7, UHMK1 and VEGFA, suggesting that it may be involved in proteostasis, cholesterol homeostasis, endothelial cell growth and cell cycle progression^{202,206,207,209}. Nrf3 also represses the expression of DUX4, which inhibits the cell cycle inhibitor CDK1, to thereby induce cell cycle progression²¹². Finally, Sankaranarayanan and Jaiswal²¹³ found that Nrf3 negatively regulates the NQO1 detoxification enzyme in HEPG2 hepatocellular carcinoma cells, however this is contradicted by Zhang *et al.*¹⁰¹ who observed that Nrf3 may also induce NQO1, suggesting that Nrf3's role in redox regulation is not as clear-cut as Nrf2.

1.2.5 Bach1 and Bach2

Two other human CNC transcription factors exist; Bach1 and Bach2. Although Bach1 and Bach2 possess a bZIP domain which allows them to heterodimerise with MafG, MafF and MafK proteins²¹⁴, they differ in structure to other CNC members. In their N-termini they contain a BTB/POZ (broad complex, tram track, bric-a-brac/pox virus and zinc finger) domain, which facilitates interactions with other co-repressor proteins, such as histone deacetylase 3 (HDAC3)²¹⁵. Bach1 and Bach2 also possess a cytoplasmic localisation signal (CLS) in their C-termini, which is responsible for their localisation in

the cytosol¹⁵⁷. Additionally, both Bach proteins also contain several cysteine-proline motifs, which bind haem to repress their transcriptional activity^{216,217}.

Bach1 is expressed in a variety of tissues, but Bach2 is mainly expressed in B cells, T cells, neural cells and macrophages²¹⁸. Unlike the other CNC members, the Bach proteins predominantly act as transcriptional repressors, where they regulate the expression of genes involved in redox homeostasis, B cell development, inflammation, haem metabolism and haematopoiesis, as reviewed by Zhang *et al.*²¹⁹ and Zhou *et al.*²²⁰ 2016^{220,221}. Acting as transcriptional repressors, Bach1 and Bach2 selectively bind some ARE and MARE (Maf recognition elements) sequences, thereby preventing binding of other bZIP transcription factors such as Nrf2²¹⁴. By competing for binding, the Bach proteins can therefore repress Nrf2 gene transcription, such as HO-1, to induce ROS and inflammation. Interestingly, Bach1 is also a target gene of Nrf2, wherein it is induced by Nrf2 overexpression, which therefore acts as an inhibitory feedback mechanism for Nrf2 target gene expression²²².

1.3 Nrf2 as a therapeutic target

Oxidative stress and inflammation are involved in the pathogenesis of a majority of chronic conditions, including neurodegenerative, cardiovascular, renal, metabolic diseases and cancer. Nrf2, as the master regulator of oxidative stress, has therefore been researched in several different diseases for its potential role in disease onset and progression (**Figure 1.10**).

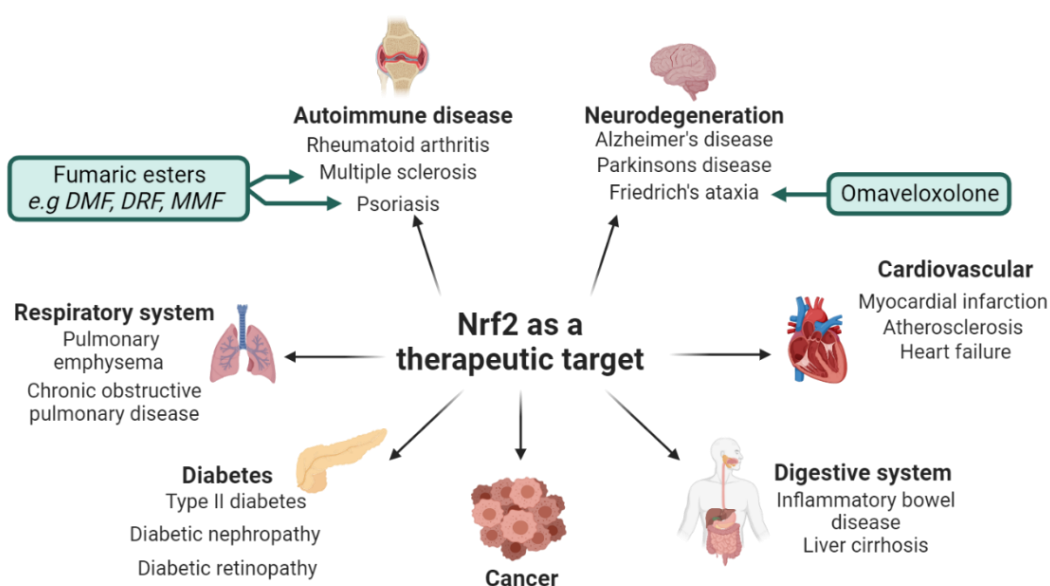


Figure 1.10 Schematic depicting the diseases in which Nrf2 has been investigated or is currently used as a therapeutic target. Nrf2 has been implicated in a multitude of autoimmune, inflammatory, and metabolic diseases. Currently, only 4 Nrf2 modulators are approved for clinical use; dimethyl fumarate (DMF), diroximel fumarate (DRF), monomethyl fumarate (MMF) and omaveloxolone (as displayed in cyan boxes).

In non-neoplastic disease, Nrf2 generally plays a protective role in preventing ROS accumulation and sustained inflammation, as extensively reviewed by Cuadrado *et al.*²²³. For example, in cardiovascular disease, Nrf2 has been shown to induce cardioprotective effects, such as in murine models of ischemia-reperfusion injury²²⁴ and heart failure²²⁵. Loss of Nrf2 function via Nrf2 knockout has also been shown to promote oxidative-stress induced fibrosis, apoptosis and loss of contractility of cardiomyocytes in diabetic mice²²⁶. On the other hand, overactivation of Nrf2 within the heart also appears to have detrimental effects and may promote pressure-overload induced cardiomyopathy²²⁷.

As oxidative stress contributes to disease progression and pathogenesis in both type I and II diabetes, Nrf2 function has been studied in numerous murine models of diabetes, however its exact clinical role continues to be unclear. One study by Uruno *et al.*²²⁸, using Keap1 knockdown mice, crossed with db/db mice (a type II diabetes mouse model) showed that Nrf2 induction delayed onset of diabetes and enhanced insulin sensitivity in db/db mice. Furthermore, using a murine model of streptozotocin-induced diabetic neuropathy, administration of the Nrf2 activators sulforaphane and cinnamic aldehyde resulted in improved renal function²²⁹. However, in contrast, Keap1 knockout mice fed a high-fat diet demonstrated more frequent diabetic phenotypes²³⁰, suggesting a more complex role of Nrf2 in diabetes which warrants further investigation.

Nrf2 function has been shown to be impaired in several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Friedrich's ataxia, multiple sclerosis and Huntington's disease, which is often associated with increased ROS levels and dysregulated proteostasis²³¹. For example, in a murine model of multiple sclerosis (MS), Nrf2 knockout also exacerbates MS symptoms and results in a more rapid onset of disease²³². Certainly, Nrf2 also appears to play a protective role in proteinopathy in animal models of tauopathy and amyloidopathy in Alzheimer's disease (AD)²³³, and α -synucleinopathy in Parkinson's disease (PD)²³⁴. This protective role is often observed to be diminished in AD patients, for example a meta-study of gene expression in AD patients conducted by Wang *et al.*²³⁵ found that Nrf2 activity was significantly reduced in AD patient brains, compared to healthy tissue. On the contrary however, increases in the levels of the Nrf2 downstream target genes NQO1, HO-1, glutathione reductase and glutathione peroxidase in AD patient brain tissue²³⁶⁻²³⁸ have also been observed in brains of AD patients, suggesting that Nrf2's role in neurodegenerative disease progression is not clear-cut and may be dependent on the type and stage of disease²³⁹.

Given the potential involvement of Nrf2 in many diseases, much research has gone into exploring methods of activating Nrf2 in chronic diseases, to combat ROS, inflammation

and cellular damage. Currently, the main method in which this has been explored is using natural product and synthetic derived activators of the Nrf2 pathway, such as organosulfur compounds (e.g., oltipraz and sulfurophane), triterpenoids (e.g., omaveloxolone). Many of these compounds activate Nrf2 by disrupting the Keap1/Nrf2 interaction via modification of Keap1 cysteine residues such as Cys¹⁵¹, thereby preventing Keap1-mediated Nrf2 degradation²⁴⁰, as displayed in **Figure 1.7B**. Protein-protein interaction (PPI) inhibitors are also being explored as Nrf2 activators. In fact, previous research from our lab has identified a cell penetrating peptide that disrupts the interaction between Nrf2/Keap1 to promote HO-1 mRNA expression and repress secretion of the pro-inflammatory cytokine TNF- α ²⁴¹.

Currently, to date, only one Nrf2 activator has been approved for clinical use in the UK; dimethyl fumarate (DMF), which is licensed for relapsing remitting MS in the UK and US, and for psoriasis in the UK and Germany²⁴². DMF is a fumaric ester sold under the brand name Tecfidera that acts by both activating Nrf2 and by inhibiting NF- κ B. In clinical trials, DMF was shown to not only reduce relapse rates of MS patients, but also markedly decreased the frequency of MS lesions compared to previous standard of care therapies for MS²⁴³. In the US, two other fumaric ester derivatives are also licensed for use in relapsing remitting MS: diroximel fumarate (Bafiertam) and monomethyl fumarate (Vemurity)²⁴⁴ (**Figure 1.10**). Furthermore, in pre-clinical studies, fumarate derivatives can also induce cardioprotective effects in ischemia-reperfusion injury²²⁴, and improve neuronal preservation and dyskinesia symptoms of Huntington's disease²⁴⁵, suggesting their potential use in other diseases.

Although currently only licensed in the US, in 2023 another Nrf2 activator, omaveloxolone, was approved for Friedreich's ataxia (**Figure 1.10**), which is a rare neurodegenerative disorder associated with rapid disease onset and reduced life-expectancy²⁴⁶. Alongside showing a strong safety profile in clinical trials²⁴⁷, patients administered omaveloxolone saw improvements in their modified Friedreich's Ataxia Rating Scale (mFARS) scores, which is the main marker of disease progression in Friedreich's ataxia²⁴⁶. Additionally, omaveloxolone administration was also associated with increases in serum levels of ferritin, which is a transcriptional target of Nrf2²⁴⁶. Currently, omaveloxolone is the only disease-modifying therapy to be licenced for Friedreich's ataxia, however further *in vivo* studies have also identified that omaveloxolone may also be a useful therapeutic intervention in other oxidative-stress related diseases such as osteoarthritis²⁴⁸, intracerebral haemorrhage²⁴⁹, and sepsis-induced cardiomyopathy²⁵⁰.

Many other Nrf2 activators are also in clinical development including another oleanane triterpenoid Nrf2 activator, bardoxolone methyl. Initially investigated for its potential use in solid tumours, such as melanoma, bardoxolone methyl gained interest due to its ability to significantly improve glomerular filtration rates²⁵¹ and has since been explored for use in chronic kidney disease²⁵². Although hampered by a Phase 3 trial that was prematurely terminated in 2013 due to occurrence of several severe cardiovascular-related adverse effects^{253,254}, clinical trials of bardoxolone methyl have since restarted with improved patient eligibility criteria²⁵⁵. Several other Nrf2 activators also continue to be investigated for clinical use, including curcumin, resveratrol, oltipraz and sulfurophane^{223,256}. Although these therapies may be useful in some chronic conditions, it is important to understand that the role of Nrf2 in certain cell types and stages of disease may differ, hence Nrf2 activation may not always be a useful therapeutic strategy, as reviewed by Dodson *et al.*²⁵⁷. This has been especially apparent when exploring Nrf2 in the context of cancer, wherein Nrf2 appears to play context-dependent dual roles in both preventing and furthering cancer development.

1.3.1 The dual role of Nrf2 in cancer

The biological mechanisms underpinning cancer development and progression are often complex (as outlined in **Chapter 1.1**), and Nrf2's involvement in cancer is no exception to this. Although originally identified to have a role in chemoprevention, Nrf2 upregulation has been observed to promote tumorigenesis and promotes chemoresistance in a wide variety of Nrf2 “addicted” cancers^{149,258} (**Figure 1.11**). The influence of Nrf2 in chemoprevention is largely due to the role that it plays in protecting non-malignant cells against oxidative stress, such as ROS, UV radiation and carcinogens, which could result in oncogenic mutations²⁵⁹ (**Figure 1.11A**).

Nrf2 activation also promotes anti-inflammatory activity, hence can block cellular damage induced by periods of chronic inflammation, and oxidative stress generated by normal cellular processes²⁶⁰. This has been evidenced through Nrf2 knockout studies in a variety of *in vivo* cancer models; for example, when exposed to carcinogens such as benzo[a]pyrene, Nrf2 deficient mice developed higher gastric tumour burdens than that observed in wild-type mice²⁶¹. Nrf2 knockout mice also showed increased incidence of 7,12-dimethylbenz(a)anthracene- induced skin cancer²⁶² and azoxymethane/DSS–induced colorectal cancer²⁶³. While Nrf2 is typically induced by cellular stressors, it can also be stabilised by the tumour suppressor genes BRCA1 and p21^{264,265}, suggesting that Nrf2 target genes are key mediators of chemoprevention. With several chemopreventive compounds, such as sulforaphane, curcumin and resveratrol known to

induce their protective effects via Nrf2-dependent pathways, Nrf2 has therefore long been explored as a potential therapeutic target for chemoprevention^{266,267}.

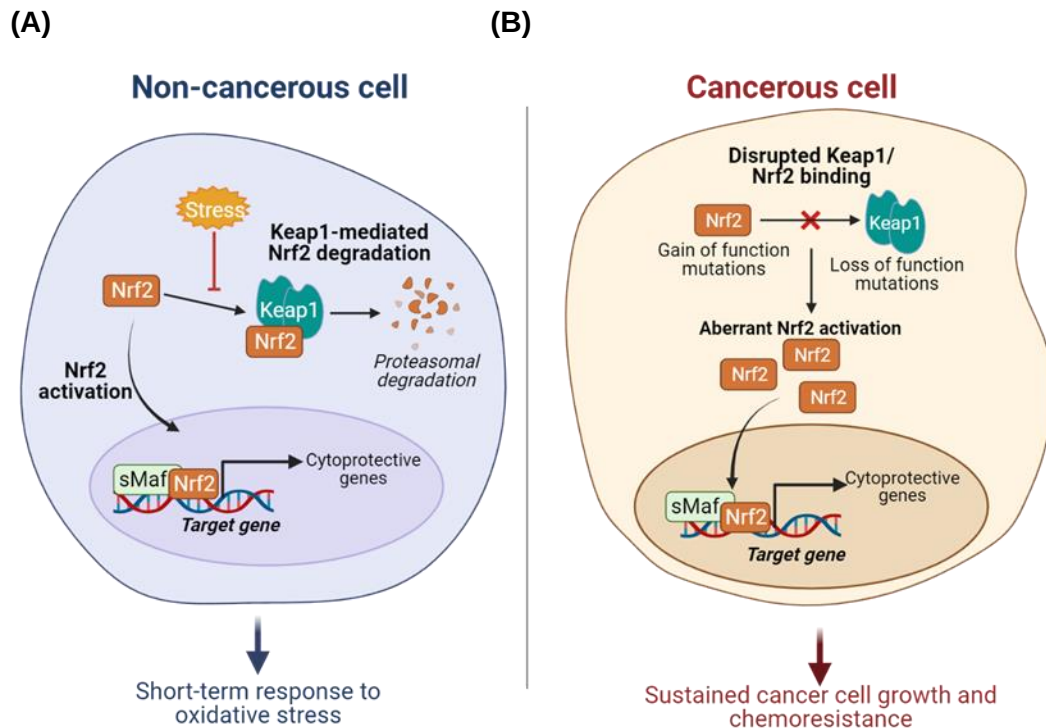


Figure 1.11 Activation of the Nrf2 pathway in normal cells compared to "Nrf2 addicted" cancer cells. (A) In normal cells, Nrf2 is tightly regulated by Keap1 to ensure low levels of Nrf2, however in cancer cells, the Nrf2/Keap1 system is disrupted, allowing greater activation of Nrf2 and increased transcription of Nrf2 target genes involved in metabolism and proliferation. **(B)** In normal cells, oxidative stress activates the Nrf2 pathway. In cancer cells, Nrf2 can provide a defence mechanism against chemotherapy and radiotherapy.

Alternatively, more recently, Nrf2 has also been found to promote cancer progression and chemoresistance in malignant cells, by providing a mechanism to induce cytoprotective gene expression: a feature originally termed the "dark side" of Nrf2 by Wang *et al.*²⁶⁸. Since this initial discovery, dysregulation of the Nrf2/Keap1 pathway and subsequent Nrf2 overexpression have been identified in several malignant neoplasms, including lung, gallbladder, head and neck, oesophageal, breast, pancreatic, ovarian, melanoma and liver cancers^{68,269-279}. Furthermore, in many of these cancers, Nrf2 overexpression is also associated with poor prognosis and increased instances of tumour metastasis^{173,272,277,279,280}. Such evidence now suggests that in Nrf2 overexpressing or "addicted" cells, Nrf2 is aberrantly activated and acts to promote tumorigenesis through the transcription of its downstream cytoprotective genes, even in the absence of oxidative stress²⁷² (**Figure 1.11B**). Furthermore, high Nrf2 expression has also been implicated in resistance to both chemotherapy and radiotherapy^{268,276,281-}

²⁸⁴, thereby indicating that Nrf2 may be a useful target in combating resistance to standard of care therapies in a broad range of cancer types²⁸⁵.

1.3.2 Nrf2 and the hallmarks of cancer

Since the discovery of the “dark side” of Nrf2, research has aimed to further elucidate the role of Nrf2 in cancer. With Nrf2 acting as such a key cell survival pathway, it is unsurprising to learn that it is involved in many of the hallmarks of cancer (**Figure 1.12**). Although explained extensively in a review by Rojo de la Vega, Chapman and Zhang 2018²⁵⁸, the role of Nrf2 in some of the cancer hallmarks is discussed briefly below.

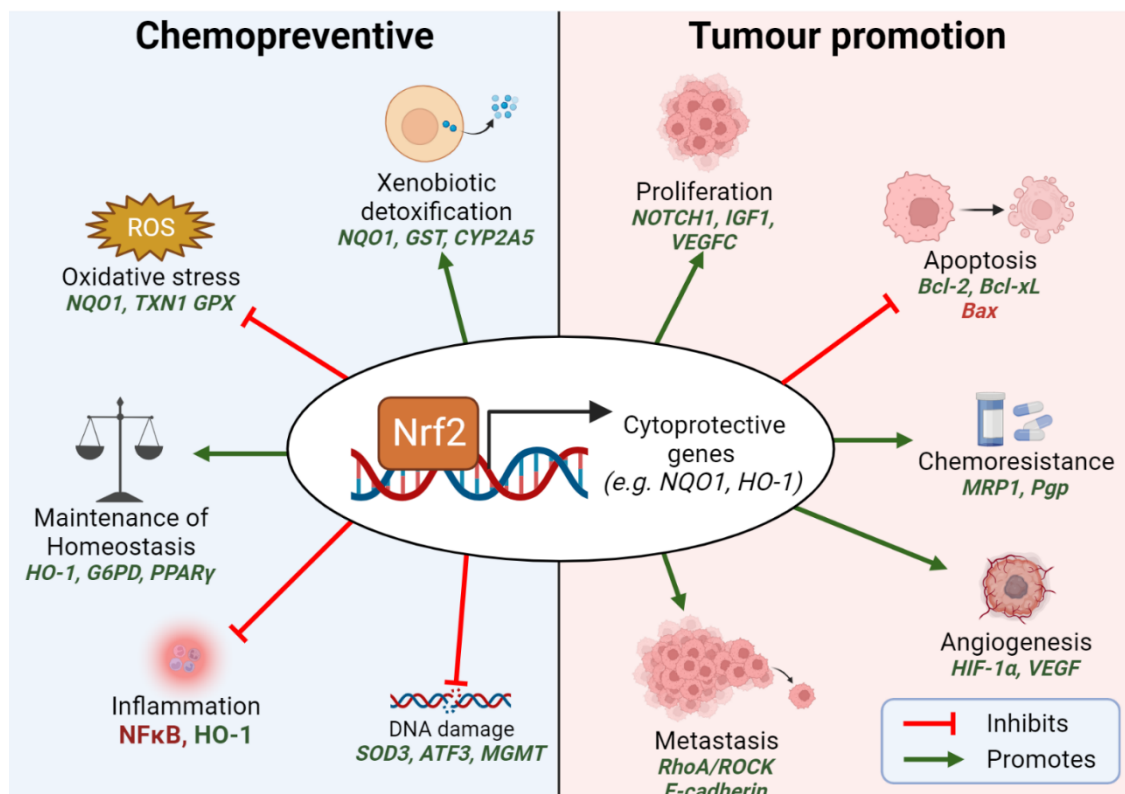


Figure 1.12 The dual roles of Nrf2 in chemoprevention and the hallmarks of cancer. In non-malignant cells (left side), Nrf2 activation promotes homeostasis and represses carcinogenesis by inducing the transcription of cytoprotective genes and repressing pro-inflammatory mediators. In cancerous conditions (right side), Nrf2 activation can promote tumorigenesis by increasing the expression of genes involved in cell proliferation, angiogenesis, and metastasis, and can suppress apoptosis. Genes that are induced and repressed by Nrf2 activation are depicted in green and red text, respectively.

As explained by Hanahan and Weinberg in 2000, cancer is defined by uncontrolled cell proliferation, often induced by the sustained activation of growth signals, such as growth factors¹⁰. Nrf2 assists in promoting proliferation through inducing the expression of a variety of genes involved in cell proliferation such as NOTCH1, IGF1 (insulin-like growth factor 1), VEGFC (vascular endothelial growth factor C), JAG1 (Jagged-1) and PDGFC (platelet-derived growth factor C)¹⁶¹. Nrf2 also cross-talks with several tumour promoting

pathways, for example, in cancer, it is induced via increased activation of the PI3K/Akt pathway, leading to increased Nrf2 activation and cell proliferation²⁸⁶. When Nrf2 is genetically inhibited using siRNA knockdown, several cancer cell lines have displayed reduced cell proliferation, including in non-small cell lung, glioblastoma, thyroid and pancreatic cell lines^{274,287-289}.

One way in which cancer cells can resist programmed cell death is by upregulating anti-apoptotic proteins and downregulating pro-apoptotic proteins^{10,12} (**Figure 1.12**). Nrf2 promotes this by increasing the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL^{290,291} and decreasing Bax expression, the release of cytochrome C release from mitochondria, and caspase 3/7 activity, following treatment with etoposide²⁹⁰. Furthermore, activation of Nrf2 in cells treated with ROS-inducing chemotherapies, such as carmustine, cisplatin, etoposide and mitoxantrone, can also confer resistance to apoptosis in cancer cells, correlated with increased ARE-driven gene transcription²⁹². Nrf2 has also been implicated in inhibiting ferroptosis (an iron-dependent mechanism of programmed cell death) in glioma cells to decrease ferroptosis cell death in response to chemotherapy treatment²⁸⁹.

A key mediator of angiogenesis is HIF-1 α (hypoxia inducible factor 1 subunit α), which induces the expression of many pro-angiogenic factors, such as VEGF (vascular endothelial growth factor). Interestingly, Nrf2 knockdown in murine tumour xenograft models showed reduced blood vessel formation, tumour growth and decreased accumulation of HIF1- α and VEGF^{293,294} (**Figure 1.12**). Furthermore, NQO1, a Nrf2 target gene, can stabilise HIF1- α and increase HIF1- α target genes²⁹⁵, suggesting that Nrf2 can induce angiogenesis through its downstream targets.

High Nrf2 expression has also been associated with increased incidence of metastatic lesions in numerous patient tumours^{279,280,296}. In order to migrate, cancer cells undergo epithelial-to-mesenchymal transition (EMT), involving decreases in E-cadherin and increases in N-cadherin in epithelial cells²⁹⁷. Arfmann-Knubel *et al.*²⁹⁸ observed that Nrf2 knockdown decreased EMT, increased E-cadherin expression and reduced N-cadherin expression in pancreatic cancer cell lines. In breast cancer, Nrf2 can also promote invasion and metastasis through increasing the RhoA/ROCK pathway²⁹⁶. Furthermore, overexpression of the Nrf2 target gene HO-1 can increase Bach1 expression through increased degradation of haem (a suppressor of Bach1), which when overexpressed has roles in glycolysis-dependent lung cancer metastasis through transcription of its own downstream target genes^{299,300}.

Metabolic reprogramming in cancer is key to providing cells with metabolites for tumour growth. The role of Nrf2 in metabolic reprogramming is extensive^{258,301,302}, however, one way that Nrf2 regulates metabolism is through its regulation of genes involved in the pentose phosphate pathway such as G6PD, PGD, TALDO1, and TKT^{164,193}, and in NADPH generation via ME1 and IDH1¹⁹³ (**Table 1.3**). Nrf2 also inhibits lipogenesis under stress conditions³⁰¹ and is involved in nucleic acid synthesis, with Nrf2 knockdown resulting in decreases in genes involved in *de novo* nucleotide synthesis, such as phosphoribosyl pyrophosphate amidotransferase (PPAT)¹⁹³.

1.3.3 Mechanisms of Nrf2 upregulation in cancer

Nrf2 has an intricate role in cancer, however, to better understand this role, the mechanisms by which Nrf2 can become upregulated must be considered. One way that cancer cells can hijack the Nrf2 pathway is through somatic mutations in Keap1, CUL3 or Nrf2. Indeed, Keap1 mutations have been observed in numerous cancers^{261,303}, with Singh *et al.* finding that 19% of non-small cell lung cancers contained a Keap1 mutation²⁶⁹. Such mutations lead to reduced levels of Keap1 and increased Nrf2 nuclear localisation, thereby increasing Nrf2 activation³⁰⁴. Mutations in Nrf2 and CUL3 have also been identified, often resulting in hyperactivation of the Nrf2 pathway and increased transcript levels of Nrf2 downstream target genes³⁰⁵. Interestingly, many of the mutations in Nrf2 have been found to occur in or near the ETGE and DLG motifs of Neh2, resulting in the disruption of Keap1-Nrf2 binding, and by extension, reducing Keap1-dependent degradation of Nrf2^{270,306}.

Nrf2 upregulation can also occur through epigenetic modification of Keap1. Hypermethylation of Keap1 has been observed in lung, colorectal, glioma and prostate cancer³⁰⁷⁻³¹⁰ and results in reduced Keap1 expression. Furthermore, deletions in exons in the NFE2L2 gene, observed in lung and head and neck cancers³¹¹, can result in transcription of Nrf2 mutants that do not associate with Keap1, thereby reducing Keap1-mediated Nrf2 degradation. Increases in Nrf2 transcription have also been associated with oncogenic alleles of the B-Raf, C-Myc and K-Ras genes, which can activate the Nrf2 response to oxidative stress³¹². Furthermore, proteins competing for Keap1-binding have also been found to indirectly upregulate the expression of Nrf2 in tumours, such as Wilms tumour gene on X chromosome (WTX), Sequestosome1 (SQSTM1), dipeptidyl peptidase 3 (DPP3) and p62^{313,314}.

1.3.4 Therapeutic targeting of Nrf2

Among its other functions in cancer, Nrf2 also plays a role in chemoresistance, for example through its regulation of MRPs involved in multidrug resistance (e.g., ABCB1), induction of anti-apoptotic proteins (e.g., Bcl-2) and antioxidant genes that can protect against chemotherapy-induced ROS damage¹⁴⁹. High Nrf2 expression has been associated with greater resistance to a variety of chemotherapies, including cisplatin, etoposide, doxorubicin, paclitaxel and 5-fluorouracil, and anti-CTLA4 and anti-PD1 immunotherapies (e.g., nivolumab, ipilimumab and pembrolizumab)^{268,315-318}.

Targeting Nrf2 overexpressing cancers with Nrf2 inhibitors therefore provides a potential method of combating such resistance. Previous studies have shown that Nrf2 knockdown sensitised cancer cells to chemotherapeutics such as etoposide, cisplatin and doxorubicin and carboplatin^{268,319}, thereby reinforcing Nrf2 inhibitors as potential adjuvant therapies alongside already approved chemotherapies. A number of inhibitors of Nrf2 have been identified (**Table 1.5**), including both natural products and small molecules, with various mechanisms of Nrf2 inhibition (**Figure 1.13**).

Table 1.5 Small molecule and natural product inhibitors of Nrf2.

Inhibitor	Class of molecule	Tested cell lines	Tested conc.	Mechanism of action	Summary of studies	Ref.
AEM1 (ARE expression modulator 1)	Small molecule	A549	<i>In vitro</i> : 1-10 μ M <i>In vivo</i> : 20 mg/kg	Unclear, but does not involve changes in Keap1 or Nrf2 protein levels.	Decreased mRNA levels of Nrf2 target genes and sensitised A549 cells to several chemotherapy drugs. <i>In vivo</i> , AEM1 reduced tumour volume.	320
<i>All-trans</i> retinoic acid	Natural product Metabolite of vitamin A	A549, AREc32, HL60, NB4	1 μ M	Binds to retinoic acid receptor- α , which associates with Nrf2 and disrupts Nrf2 DNA binding. Decreases Nrf2 nuclear import.	Reduced induction of Nrf2 target genes in AREc32 cells and reduced Nrf2 DNA binding in MCF-7 cells. Decreased Nrf2 transcriptional activity, NQO1 and HO-1 expression and increased cytotoxicity in HL60 and NB4 cells treated with arsenic trioxide.	321-324
Apigenin	Natural product flavonoid found in celery, celeriac and chamomile tea	BEL-7402/ADM	<i>In vitro</i> : 10 μ M, 20 μ M <i>In vivo</i> : 50 mg/kg	Inhibits Nrf2 via inhibition of the PI3K-Akt pathway.	Reduced mRNA and protein levels of Nrf2 and Nrf2 target genes in BEL-7402/ADM cells. Sensitised BEL-7402/ADM and BEL-7402 xenografts to doxorubicin.	325
Ascorbic acid	Natural product otherwise known as vitamin C	KCL22/SR, A549 MDA-MB 231, MDA-MB 468, MCF-7	<i>In vitro</i> : 0.1- 10 mM	Decreases nuclear import of Nrf2	Decreased Nrf2 DNA binding activity and sensitised cells to imatinib in imatinib-resistant cells. Reduced Nrf2 mRNA and protein levels in breast cancer cells and antagonised resveratrol-induced Nrf2 induction in liver cells.	326-328
Brusatol	Natural product derived from <i>Brucea javanica</i>	A549 HeLa, MDA-MB-231, Ishikawa SPEC-2 Hepa-1c1c7 HCT116CT26 A375	<i>In vitro</i> : 20-80 nM <i>In vivo</i> : 2 mg/kg	Acts post-transcriptionally to block translation of Nrf2 protein.	Reduced expression of Nrf2 in a range of cancer cells, and sensitised both <i>in vitro</i> and <i>in vivo</i> models of cancer to multiple chemotherapeutics and UVA radiation.	329-340
Chrysin	Natural product flavonoid derived from plant extracts,	BEL-7402/ADM	<i>In vitro</i> : 10 μ M	Inhibits Nrf2 via inhibition of the PI3K-Akt and ERK pathways.	Reduced expression of Nrf2 and Nrf2 downstream target genes and sensitised	341

	honey and propolis				doxorubicin-resistant cells to doxorubicin.	
Cryptotanshinone	Natural product derived from <i>Salvia miltiorrhiza</i>	A549	<i>In vitro</i> : 2.5 -10 μ M	Unknown	Inhibited Nrf2 and Nrf2 target gene expression in A549 cells, and sensitised cells to cisplatin.	342
Clobetasol propionate	Small molecule (corticosteroid)	A549 H2228	<i>In vitro</i> : 1 nM, 100 nM <i>In vivo</i> : 1 mg/kg	Promotes β -TrCP dependent degradation of Nrf2	Reduced Nrf2 protein expression and Nrf2 target gene expression in NSCLC cells and sensitised NSCLC cells to rapamycin <i>in vivo</i> .	343
EGCG (Epigallocatechin 3-gallate)	Polyphenol found in green tea	MDA-MB231	<i>In vitro</i> : 10- 50 μ M	Unknown	Sensitised breast cancer cells to cisplatin <i>in vitro</i> and <i>in vivo</i> via a Nrf2-dependent mechanism.	344
Ethionamide	Small molecule	THP-1 U937	<i>In vitro</i> : 0.1-4 mM	Unknown	Reduced Nrf2 target genes and enhanced acute myeloid leukaemia cells to arsenic trioxide.	345
Halofuginone	Small molecule	A549, KYSE70	<i>In vitro</i> : 50 nM <i>In vivo</i> : 0.25 mg/kg	Inhibits prolyl-tRNA synthetase, thereby repressing Nrf2 protein synthesis	Reduced Nrf2 protein levels and sensitised cancer cells to chemotherapeutics.	346
Isoniazid	Small molecule	Hep3B 3T3-L1	<i>In vitro</i> : 5- 20 mM	Decreases Nrf2 nuclear import	Increased cytosolic Nrf2 but decreased nuclear Nrf2 levels. Decreased expression of ARE-dependent genes.	347,348
Luteolin	Flavonoid found in celery, green pepper and chamomile tea	Caco2, A549, MCF-7	<i>In vitro</i> : 1-10 μ M <i>In vivo</i> : 10- 80 mg/kg	Increases the degradation of Nrf2 mRNA	Reduced expression of Nrf2 target genes in Caco2 cells and sensitised NSCLC cells to chemotherapeutics <i>in vitro</i> and <i>in vivo</i> .	349-353
Malabaricone-A	Prooxidant derived from <i>Myristica malabarica</i>	MOLT3, MCF-7, A549	<i>In vitro</i> : 15 μ g/mL	Uncertain	Reduced expression of Nrf2 and HO-1	354
Metformin	Small molecule	HepG2 MCF-7	<i>In vitro</i> : 1-5 mM	Reduced p-ERK1/2 to inhibit Nrf2. Induced miR-34a to suppress the Sirt1/Pgc-1 α /Nrf2 pathway.	Reduced expression of Nrf2 protein and Nrf2 downstream targets <i>in vitro</i> and <i>in vivo</i> , and sensitised MCF-7 cells to oxidative stress.	355-357
ML385	Small molecule	A549 EBC1, LK2 22Rv1, DU145	<i>In vitro</i> : 1-10 μ M	Binds to Neh1 to disrupt MafG/Nrf2 binding to the ARE.	Reduced Nrf2 and Nrf2 target gene expression in lung cancer cells and sensitised them to chemotherapeutics.	358-360

Ochratoxin-A	Mycotoxin produced by <i>Aspergillus</i> and <i>Penicillium</i>	LLC-PK1	<i>In vitro</i> : 2.5- 25 μ M	Decreases Nrf2 nuclear import and inhibits the Nrf2 via miRNA-132	Reduced Nrf2 and Nrf2 target gene mRNA levels. Inhibits PI3K/mTOR signalling in lung carcinoma cells.	361,362
Oridonin	Diterpenoid derived from <i>Rabdosia rubescens</i>	MG-63, HOS	<i>In vitro</i> : 5-15 μ M <i>In vivo</i> : 7.5-30 mg/kg	Inhibits Nrf2 and NFkB nuclear translocation.	Reduced Nrf2 levels in <i>in vitro</i> and <i>in vivo</i> models of human osteosarcoma.	363
PMF (3',4',5',5,7-Pentamethoxy-flavone)	Flavonoid isolated from <i>Rutaceae</i> plants	A549/CDDP HEK293	<i>In vitro</i> : 10- 50 μ M	Decreases levels of p-ERK	Decreased ARE transcriptional activity, reduced Nrf2 and Nrf2 target gene levels and sensitised cisplatin-resistant cells to cisplatin.	364
Trigonelline hydrochloride	Alkaloid derived from coffee	Colo357 Panc1 MiaPaCa2 H6C7 HT29	<i>In vitro</i> : 0.01- 10 μ M <i>In vitro</i> : 1 mg/kg	Disrupts Nrf2 nuclear translocation	Decreased Nrf2 activity in pancreatic and colon cancer cells and sensitised <i>in vitro</i> pancreatic cancer cells to etoposide.	365,366
Triptolide	Diterpenoid epoxide derived from <i>Tripterygium wilfordii</i> Hook f	NRK-52E U937 A549 U251 K562, HL60	<i>In vitro</i> : 80- 320 nM <i>In vivo</i> : 0.25- 1 mg/kg	Enhances GSK-3 β activity and Fyn nuclear translocation, thereby promoting Nrf2 degradation.	Decreased Nrf2 and Nrf2 target gene mRNA and protein levels and sensitised cancer cells to chemotherapeutics in a variety of <i>in vitro</i> and <i>in vivo</i> cancer models. Triptolide may also act to reduce Nrf2 mRNA via a post-translational mechanism.	367-373
Wogonin	Flavonoid derived from <i>Scutellaria baicalensis</i>	K562/A02 MCF-7 HepG2	<i>In vitro</i> : 20-60 μ M	Downregulates Nrf2 mRNA levels by suppressing the PI3K/Akt pathway	Sensitised breast and liver cancer cells to chemotherapeutics and decreased Nrf2 and Nrf2 target gene expression.	374,375
4-F	Pyrazolyl hydroxamic acid derivative	THP-1 HL60 U937	<i>In vitro</i> : 5 μ M, 10 μ M <i>In vivo</i> : 50 μ M	Unknown	Decreased Nrf2-ARE activity and mRNA of Nrf2 downstream targets in acute myeloid leukaemia cells and induced apoptosis.	376
4-Methylchalcone	Small molecule	A549 HEK293	<i>In vitro</i> : 1- 5 μ M	Inhibits the PI3K/Akt pathway	Decreased Nrf2 and NQO1 expression and sensitised A549 cells to cisplatin.	377

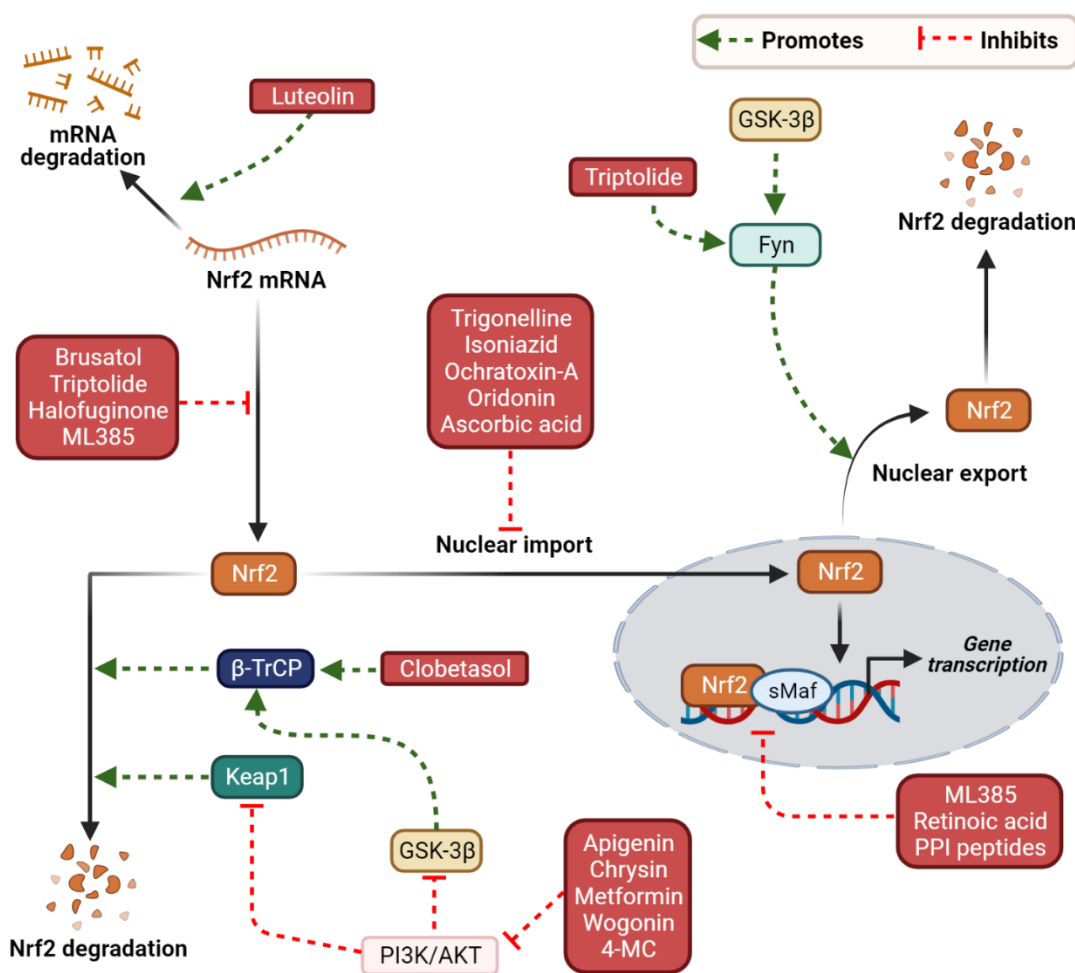


Figure 1.13 The mechanism of action of Nrf2 inhibitors on the Nrf2 signalling pathway. Nrf2 inhibitors act via several pathways, including inducing Nrf2 mRNA degradation, repressing Nrf2 nuclear translocation, promoting Nrf2 degradation and promoting Nrf2 nuclear export. Nrf2 inhibitors are displayed in red boxes. *Abbreviations: PPI; protein protein interaction.*

1.3.5 Small molecule Nrf2 inhibitors

One small molecule inhibitor, termed ARE expression modulator 1 (AEM1), was identified through a high-throughput screen and reduced mRNA levels of Nrf2 target genes, including GCLM, SOD1, TXN, HO-1 and GPX2, glutathione levels, and cell growth in A549 cells³²⁰. This study also found that AEM1 also sensitised A549 (lung adenocarcinoma) cells to etoposide, 5-fluorouracil and doxorubicin and decreased tumour volume in an A549 xenograft model in nude mice³²⁰. Another promising small molecule to be identified as a Nrf2 inhibitor is ML385, which inhibited Nrf2, glutathione, and mRNA expression of a variety of Nrf2 target genes (e.g., NQO1, GCLM, AKR1C1) in A549 cells, through binding to Neh1 and therefore disrupting Nrf2/MafG binding to ARE sequences³⁵⁹ (**Figure 1.13**). ML385 also sensitised cells to carboplatin, doxorubicin and paclitaxel in A549 and H460 (lung cancer) cells³⁵⁹.

4F (1-(4-(tert-butyl)benzyl)-3-(4-chlorophenyl)-N-hydroxy-1H pyrazole-5-carboxamide), a pyrazolyl hydroxamic acid derivative, was also identified as an Nrf2 inhibitor³⁷⁶. In acute myeloid leukaemia cells, 4F reduced Nrf2 activity and mRNA levels of GCLC and HO-1, associated with decreased tumour growth in an *in vivo* chick egg model of acute myeloid leukaemia³⁷⁶. Another small molecule reported to inhibit Nrf2 is the chalcone derivative 4-methylchalcone (4-MC)³⁷⁷, which inhibits Nrf2 activity and NQO1 expression via repression of the PI3K-Akt pathway in A549 cells (**Figure 1.13**). While 4-MC can also sensitise A549 cells to cisplatin, in HEK293 (human embryonal kidney) cells however, Nrf2 was activated, suggesting it may have a cytoprotective role in non-malignant cells, but an anti-cancer effect in tumour cells³⁷⁷.

Repurposing of previously approved clinical drugs has also been explored to identify novel Nrf2 inhibitors. For example, isoniazid (approved for use in tuberculosis) was found to inhibit the Nrf2 pathway in HepB3 (hepatocellular carcinoma) cells, where it increased cytosolic Nrf2 levels but decreased nuclear Nrf2 levels, suggesting it acted through reducing nuclear import of Nrf2³⁴⁸ (**Figure 1.13**). Isoniazid also decreased ARE activity and reduced ARE-dependent gene expression of NQO1, GCLC and HO-1 genes in murine pre-adipocyte cells³⁴⁷. Additionally, ethionamide, another antitubercular drug, inhibited Nrf2 activity and mRNA expression of GCLM and HO-1 in THP-1 (myeloid leukaemia) cells and sensitised them to standard of care arsenic trioxide treatment³⁴⁵. Furthermore, a screening of over 4000 clinical drugs identified clobetasol propionate, a glucocorticoid, as an inhibitor of Nrf2, which acted to reduce Nrf2 protein and G6PD, ME1, PGD, and GCLM expression in A549 and H2229 NSCLC cells³⁴³ (**Figure 1.13**). When combined with rapamycin treatment, clobetasol propionate further reduced tumour growth in a NSCLC xenograft model compared to rapamycin alone³⁴³.

The antidiabetic drug metformin was also found to reduce Nrf2 protein levels and nuclear import of Nrf2 in HepG2 (liver cancer) cells, through inhibition of Raf-ERK signalling³⁵⁶ (**Figure 1.13**). Metformin also reduced Nrf2, HO-1 and SOD1 expression in MCF-7 (breast cancer) cells via suppressing the Sirt1/Pgc-1 α /Nrf2 pathway³⁵⁵. Finally, another reported small molecule inhibitor is halofuginone (a synthetic derivative of the quinazolinone alkaloid, febrifugine) which was identified as a Nrf2 inhibitor through a high-throughput screen³⁴⁶. In A549 lung and KYSE70 human oesophageal cancer cells, halofuginone decreased Nrf2 protein levels via inhibiting t-prolyl synthetase and sensitised A549, KYSE70 and a KYSE70 xenograft model to cisplatin and doxorubicin³⁴⁶.

1.3.6 Natural product Nrf2 inhibitors

1.3.6.1 Vitamins

All *trans*-retinoic acid is a metabolite of vitamin A that inhibited Nrf2 binding to the ARE in MCF-7 (breast cancer) cells and decreased the expression of AKR1C1 and AKR1C2 in AREc32 cells (an MCF7-derived reporter cell line), as displayed in **Figure 1.13**³²⁴. Retinoic acid acts as an agonist of retinoic X receptor alpha (RXR α), which binds to Neh7 on Nrf2, thereby inhibiting Nrf2 binding to the ARE¹³⁶ (**Figure 1.13**). Furthermore, in HL60 and NB4 leukaemia cells with arsenic trioxide-induced activation of Nrf2, retinoic acid reduced Nrf2 transcriptional activity and the expression of NQO1 and HO-1, and sensitised cells to arsenic trioxide treatment³²⁵.

Another vitamin, ascorbic acid (vitamin C) decreased Nrf2 DNA-binding activity and expression of γ -GCS1 and glutathione, and sensitised cells to imatinib treatment in KCL22/SR (imatinib resistant chronic myelogenous leukaemia) cells³²⁶. In breast cancer cells (MDA-MB 231, MDA-MB 468 and MCF-7), ascorbic acid in combination with quercetin, reduced Nrf2 mRNA and protein levels and NQO1 and HO-1 activity³²⁸. Furthermore, ascorbic acid also antagonised resveratrol- induced Nrf2 expression in HUH7 liver cells³²⁷. Vitamins have long been explored as potential anti-cancer agents, with varying results depending on cancer type, however retinoic acid is used with some efficacy in acute promyeloid leukaemia³⁷⁸.

1.3.6.2 Flavonoids

Luteolin is a flavonoid derived from celery, green pepper, chamomile tea, and parsley. Tang *et al.*³⁴⁹ found that luteolin decreased mRNA levels of AKR1C1, HO-1, NQO1, MRP2 and GCLC in MCF-7 breast and CACO-2 colorectal cancer cells, and increased Nrf2 mRNA degradation in A549 cells (**Figure 1.13**). Furthermore, luteolin also sensitised cells to oxaliplatin, bleomycin and doxorubicin in A549 cells³⁵⁰. Furthermore, in an *in vivo* NSCLC xenograft model, luteolin decreased tumour growth and decreased expression of NQO1 and HO-1³⁴⁹. Alternatively, Kang *et al.*³⁷⁹ and Zuo *et al.*³⁵³ observed that luteolin may also activate the Nrf2 pathway in colorectal cancer, suggesting that its activity may be tissue dependent.

Apigenin and chrysin are also flavonoids found to inhibit Nrf2, which are found in celery, celeriac and chamomile, and in honey and carrots, respectively. In doxorubicin-resistant hepatocellular carcinoma cells (BEL-7402/ADM), apigenin and chrysin decreased Nrf2, AKR1B10, HO-1 and MRP5 mRNA and protein expression via inhibition of the PI3K-Akt pathway (**Figure 1.13**), and sensitised BEL-7402/ADM cells and BEL-7402 xenograft

tumours to doxorubicin^{325,341}. In contradiction to this however, some studies have found that low concentrations of apigenin can activate Nrf2 in HepG2 liver cancer cells³⁸⁰ and melanocytes³⁸¹. Apigenin, luteolin and chrysin may also induce a cytoprotective effect in primary rat hepatocytes³⁸², suggesting further research is needed to fully elucidate their potential as Nrf2 inhibitors. Another flavonoid, wogonin (found in *Scutellaria baicalensis*), has also shown potential as a Nrf2 inhibitor, with treatment in doxorubicin-resistant MCF-7 cells resulting in decreased Nrf2, HO-1 and NQO1 expression and increased sensitivity to doxorubicin³⁷⁵. HepG2 cells were also sensitised to chemotherapeutics following wogonin treatment, and had decreased expression of MRPs³⁷⁴.

1.3.6.3 Other Nrf2 inhibitors

Brusatol is a quassinoid derived from *Brucea javanica* that has shown great promise as a Nrf2 inhibitor. In A549, HeLa (cervical cancer), Ishikawa and SPEC-2 (endometrial adenocarcinoma), MCF-7 and MDA-MB-231 cells, brusatol reduced the expression of Nrf2 protein and Nrf2 target gene expression^{335,383} (**Figure 1.13**). Brusatol treatment also sensitised both a NSCLC mouse xenograft model and a mouse model of mutant Kras^{G12D}-induced lung cancer to cisplatin^{335,337} and similarly sensitised MDA-MB-231 mammosphere cells to taxol³⁸³. The promising action of brusatol was further displayed in murine Hepa-1c1c7 cells and primary human hepatocytes, where it reduced Nrf2 protein and sensitised cells to stress-inducing chemicals³³⁴. Brusatol also decreased cell viability and sensitised cells to the topoisomerase I inhibitor irinotecan in human and murine colorectal cancer cells *in vitro* and reduced tumour growth in an *in vitro* model of colorectal cancer³³². However, further research into the mechanism of action of brusatol found that brusatol acts as a general inhibitor of protein translation, and therefore also inhibits a range of other short-lived proteins^{333,384}. Hence, brusatol does not appear to be a good clinical candidate due to its wide off-target effects.

Other natural products, including cryptotanshinone, derived from *Salvia miltiorrhiza*, also inhibit Nrf2. Cryptotanshinone treatment in A549 cells reduced GCLC, GCLM, HO-1, NQO1 and MRP5 expression and sensitised cells to cisplatin³⁴². Epigallocatechin 3-gallate, a polyphenol derived from green tea, also sensitised breast cancer cells and a breast cancer xenograft model to cisplatin through an unknown Nrf2-dependent mechanism³⁴⁴. Another inhibitor, ochratoxin A, a mycotoxin, reduced Nrf2 nuclear import and reduced GST, GCL and Nrf2 mRNA levels and Nrf2 nuclear translocation in LLC-PK1 (porcine kidney tubule) cells³⁶¹. Furthermore, although ochratoxin-A also reduced Nrf2 via the upregulation of microRNA (miRNA)-132³⁶², ochratoxin A induces severe nephrotoxicity and hepatotoxicity³⁸⁵.

Oridonin, a diterpenoid derived from *Rabdosia rubescens*, has also shown promise as an Nrf2 inhibitor, reducing NQO1, HO-1 and Bcl-2 expression in MG-63 and HOS (human osteosarcoma) cells³⁶³. In an *in vivo* human osteosarcoma xenograft model, oridonin treatment resulted in a decrease in Nrf2 expression and tumour volume³⁶³.

Additionally, PMF (3',4',5',5,7-Pentamethoxy-flavone), derived from *Rutaceae* plants, reduced Nrf2, NQO1, GCLC and HO-1 levels and sensitised cisplatin-resistant A549 cells to cisplatin and reduced ARE transcriptional activity in HEK293 cells transfected with ARE plasmids³⁶⁴. Malabaricone-A, derived from the *Myristica malabarica* plant, reduced Nrf2 and HO-1 expression in MOLT3 (leukaemia), A549 and MCF-7 cells³⁵⁴, however little data is available on this inhibitor at present. Alternatively, trigonelline hydrochloride, a coffee extract, has also been identified as a promising Nrf2 inhibitor³⁶⁶. Trigonelline decreased ARE-induced gene transcription and Nrf2 nuclear translocation in MiaPaCa2, Colo357, Panc1, H6c7 (pancreatic) and HT29 (colon) cancer cells^{365,366} (**Figure 1.13**). In combination with etoposide, trigonelline also decreased the tumour growth of pancreatic cancer xenografts compared to etoposide alone³⁶⁶, hence suggesting it may show promise as a pancreatic cancer therapy.

Finally, another Nrf2 inhibitor that has shown great potential for use in cancer is triptolide, a natural product derived from *Tripterygium wilfordii* Hook f. In NRK-52E (rat renal tubular epithelial cells), nanomolar concentrations of triptolide induced apoptosis and reduced Nrf2 protein levels via activating GSK-3 β and increasing Fyn nuclear translocation, thereby enhancing Nrf2 degradation³⁷¹ (**Figure 1.13**). In leukaemia stem cells, triptolide also decreased Nrf2, NQO1, GSR and HO-1 expression and enhanced the cytotoxic effect of idarubicin³⁶⁹. Likewise, triptolide treatment also reduced mRNA and protein levels of Nrf2 and GCLC and GCLM and suppressed Nrf2/ARE activity in HepG2, A549 cells and an *in vivo* model of isocitrate dehydrogenase-mutated glioma³⁷². Triptolide also sensitised A549 and 3LL (murine lung carcinoma) cells to cisplatin, etoposide and epirubicin, and a 3LL tumour xenograft to epirubicin³⁷³. However, the promising activity of triptolide does come with systemic toxicity and has a very small therapeutic window³⁸⁶. Antibody drug conjugates of triptolide have therefore been explored, with one such therapy showing promise in *in vivo* CD26-positive cancers³⁸⁷.

1.3.7 Novel inhibitors of Nrf2

At present, Nrf2 inhibition has predominantly been explored using small molecule and natural product inhibitors of the Nrf2 pathway (**Table 1.5**), most of which rely on repressing Nrf2 signalling by either promoting Nrf2 degradation or by inhibiting its nuclear import^{256,388}. Another method of inhibiting Nrf2 signalling in cancer is to disrupt the Nrf2/sMaf protein-protein interaction (PPI) using peptides. Nrf2 and sMafs interact via their bZIP domains, wherein the α -helices of the two proteins bind together via the formation of a coiled-coil structure^{389,390}. These structures allow the two bZIP proteins to 'zip' together via interactions between hydrophobic amino acid residues, such as leucine³⁹⁰. Disruption of this interaction, for example through mutating the MafG leucine zipper domain, results in abrogated Nrf2 transcriptional activity, hence inhibitors of the Nrf2/MafG PPI could be useful in repressing Nrf2 signalling in cancer. Previously, PPIs were believed to be "undruggable" due to their large surface area and hydrophobicity, which often cannot be targeted by traditional drugs such as small molecules³⁹¹. Peptides on the other hand, are larger and can bind a wider surface area than small molecules³⁹¹. Furthermore, peptides can better mimic the native secondary structure of proteins, such as α -helices, and can also be derived from the structure of native proteins, termed peptide mimics, to specifically target certain PPIs³⁹¹. Recently, interest into peptide mimics as anti-cancer agents has increased, with one peptide-based p53 mimic currently showing promise in clinical trials of a variety of solid tumours and lymphomas³⁹².

Currently, only three studies have explored the use of peptides to target the Nrf2/sMaf interaction. Based upon the bZIP domain of Nrf2, Wiedemann *et al.*³⁹³ synthesised a series of peptides that could bind to the ARE, however these peptides were not cell permeable and showed unspecific binding for other DNA sequences. Simov *et al.*³⁹⁴ utilised a heterodimeric peptide inhibitor, derived from sections of the Nrf2 and MafG bZIP sequences, tethered together by a synthetic linker. Although the peptides in Simov *et al.*'s study could prevent native Nrf2/MafG heterodimers from binding to ARE sequences, these peptides were not cell permeable and did not inhibit Nrf2 target gene expression in permeabilised A549 non-small cell lung cancer cells³⁹⁴. In 2023, Modi *et al.*³⁹⁵ reported the design and synthesis of a peptide mimic of the Nrf2 bZIP domain, that was not only cell permeable but could also interrupt Nrf2/MafG binding and repress Nrf2 target gene expression (such as NQO1 and HO-1) in A549 non-small cell lung cancer cells. Furthermore, the peptide designed by Modi *et al.* could also sensitise A549 cells to cisplatin treatment¹⁹⁰.

Other approaches have also involved the use of PROTACs (Proteolysis-Targeting Chimeras), which consists of a ligand for a target protein linked to an E3 ligase ligand³⁹⁶.

Using this approach, PROTACs are able to specifically degrade desired proteins via the recruitment of E3 ligase complexes. Recently, a PROTAC designed against Nrf2 was synthesised, which incorporated an oligonucleotide of the ARE sequence from the NQO1 promoter, linked to a ligand for the cereblon (CRBN) and von Hippel–Lindau (VHL) E3 ligase. Using A549 cells, Ji *et al.*³⁹⁷ demonstrated that the ARE-PROTAC inhibited Nrf2 target gene transcription and subsequently sensitised cells to chemotherapeutic agents such as doxorubicin, trametinib and afatinib.

Overall, although numerous Nrf2 inhibitors have been previously reported showing promise in pre-clinical studies, none have yet to progress to the clinic, to date. Additionally, although small molecules and natural products have been the main area of interest in the Nrf2 field, emerging research is now focussing on alternative mechanisms of Nrf2 inhibition, such as with the use of peptides and PROTACs. In the present thesis, a selection of small molecule and natural product Nrf2 inhibitors were explored for their potential therapeutic use in lung and melanoma cancers. Furthermore, a novel small peptide designed to inhibit the Nrf2/MafG interaction was also developed and studied in lung cancer, as a novel method to inhibit the Nrf2/MafG PPI.

1.4 Nrf3 in cancer

The physiological role of Nrf3 is currently not well characterised, however increasing evidence suggests that it may play a role in a variety of malignant neoplasms. This was first observed in 2003, when Nrf3 was identified as one of 27 genes found to be significantly upregulated in Hodgkin's lymphoma³⁹⁸. It is possible that Nrf3 acts via a similar dual role as that observed with Nrf2, wherein it may protect against initial carcinogenesis in non-malignant cells. As identified by Chevillard, Paquet and Blank²⁰⁵, Nrf3 knockout mice are more susceptible to benzo-[a]-pyrene induced lymphoma compared to wild-type mice.

Nrf3 overexpression has since been observed in multiple tumour types, including thyroid³⁹⁹, colorectal^{212,400-402}, liver^{403,404}, kidney⁴⁰⁵⁻⁴⁰⁷, pancreatic²⁰⁹, gastric⁴⁰⁸, and bladder⁴⁰⁹ cancers, which is often associated with poorer overall survival and resistance to chemo and radiotherapy^{400,410}. On the contrary, Nrf3 upregulation has been observed to be favourable in breast cancers, wherein genetic knockdown with siRNA increased cell viability of breast cancer cell lines and prevented EMT initiation and cell migration⁴¹¹. In melanoma, Nrf3 also appears to be downregulated as disease progression continues⁴¹².

Although very few Nrf3 target genes have been validated thus far (**Table 1.4**), currently it is believed that Nrf3 mainly acts to induce tumorigenesis by promoting cell cycle progression and inducing the degradation of tumour suppressor genes. Bury *et al.* identified that Nrf3 inhibits the expression of DUX4 (a repressor of CDK1), to induce cell cycle progression in colorectal cancer cell lines (**Figure 1.14**). Furthermore, Waku *et al.* demonstrated that Nrf3 can induce the expression of POMP via binding to the POMP ARE sequence, to ultimately promote 20S proteasome assembly and mediate degradation of the tumour suppressor proteins p53 and Rb^{206,413}. This may suggest that Nrf3 acts as a mechanism in which cancer cells can regulate proteostasis and induce degradation of tumour suppressors, thereby resulting in uncontrolled proliferative signalling and resistance to apoptosis. Furthermore, Nrf3 is also able to cross-talk with the Wnt/ β -catenin pathway in liver, colorectal and pancreatic cancers, to induce Wnt signalling and subsequently enhance cancer cell migration and proliferation^{210,404}.

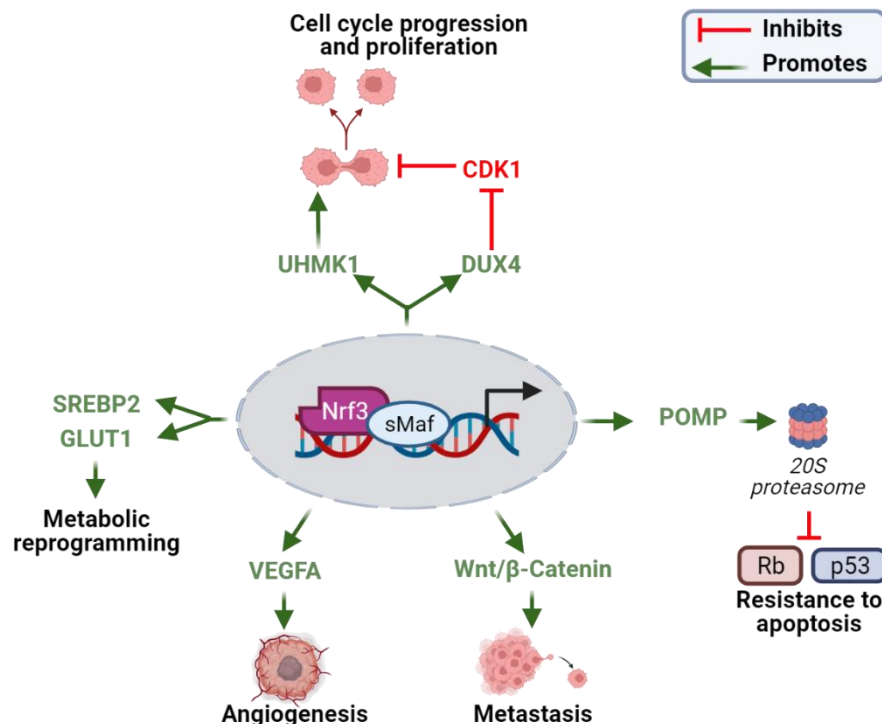


Figure 1.14 The role of Nrf3 in promoting the hallmarks of cancer. Nrf3 induces the expression of several genes that promote tumour growth via inducing metabolic reprogramming, angiogenesis, metastasis, cell proliferation and resistance to apoptosis.

Additionally, genetic inhibition of Nrf3 via knockdown with RNA interference can repress cancer cell proliferation, metastasis and induce apoptosis both in *in vitro* human bladder, colorectal, pancreatic and kidney cancer cell lines^{400,404,406,409}. Nrf3 inhibition via siRNA also inhibited the growth of an *in vivo* hepatocellular carcinoma murine xenograft tumours⁴⁰⁴, suggesting that Nrf3 inhibition may be a useful therapeutic target to repress tumour growth.

Currently, no validated Nrf3 inhibitors have been identified, besides from using RNA interference. As Nrf3 can only translocate to the nucleus once cleaved and deglycosylated, it has been suggested that Nrf3's transcriptional activity could be inhibited by either preventing Nrf3 cleavage at its NHB2 domain, or by preventing its deglycosylation at its NST domain (**Figure 1.9**). Hence, inhibitors of the DDI2 or NGYL1 proteins, which cleave and deglycosylate Nrf3, respectively, could be explored for their anti-cancer effects in cancers with high Nrf3 expression. As DDI2 possesses a retroviral-like protease (RVP) domain that is similar in structure to that found in the HIV-1 protease, HIV inhibitors that target the RVP domain may also repress DDI2 activity. It is possible therefore that HIV inhibitors could repress DDI2-dependent cleavage of Nrf3 and thus prevent its nuclear translocation and transcriptional activity. Although no research has been published on the effect of HIV inhibitors on Nrf3 activity, Fassmannová *et al.*⁴¹⁴ demonstrated that the HIV inhibitor nelfinavir could repress Nrf1 proteolytic processing, nuclear translocation and target gene transcription in multiple myeloma cells. As Nrf1 and Nrf3 share the same mechanism of activation via DDI2, further research could therefore explore nelfinavir's effects on Nrf3 proteolytic cleavage and downstream signalling.

Furthermore, Nrf3 could also be targeted by repressing its deglycosylation via inhibition of the NGLY1 enzyme. This mechanism has previously been used to inhibit Nrf1 deglycosylation and subsequent nuclear translocation using WRR139, which is a small molecule inhibitor of NGLY1⁴¹⁵. Similarly to nelfinavir, WRR139 has not been studied for its ability to also repress Nrf3, however given the shared mechanism of NGLY1-mediated deglycosylation between Nrf1 and Nrf3, it is possible that WRR139 could also repress Nrf3 signalling. Additionally, as Nrf3 must heterodimerise with sMaf proteins to exert its activity, inhibitors of the Nrf3/sMaf PPI could also repress Nrf3-driven tumorigenesis, in a similar mechanism to that observed with peptide inhibitors of Nrf2/MafG³⁹⁵.

In summary, both Nrf2 and Nrf3 are often overexpressed in cancer and can promote tumorigenesis and chemoresistance. As such both have emerged as potential therapeutic targets in cancer. In the present thesis, methods to target Nrf2 in lung and melanoma cancers, which both overexpress Nrf2 and are among the most common types of cancer in the UK, were explored. Furthermore, the role of Nrf3 in cancer was further elucidated.

1.5 Aims of this study

1. Compare the effects of seven Nrf2 inhibitors (brusatol, luteolin, ML385, retinoic acid, triptolide, trigonelline and clobetasol) on Nrf2 signalling, cell proliferation, apoptosis and chemoresistance in 2D cultures of human cancer cell lines, and in a 3D cell culture model of NSCLC.
2. Synthesise and characterise the activity of two novel Nrf2 inhibitors in NSCLC, including a Nrf2 inhibitor peptide-drug conjugate and a MafG peptide mimic to repress Nrf2/MafG-driven gene transcription.
3. Investigate the therapeutic benefit of targeting Nrf2 in melanoma using Nrf2 inhibitors, and assess their ability to repress cell proliferation, migration and to sensitise cells to standard of care BRAF inhibitors.
4. Study the differential expression of Nrf3 in patient tumours using public databases, patient-derived 3D tumouroids and cancer cell lines, and evaluate Nrf3 inhibition as an anti-cancer intervention.

Chapter 2. Materials and methods

2.1 Materials

2.1.1 General Reagents and Consumables

Dimethyl sulfoxide (DMSO), PCR grade water, brusatol, *all-trans* retinoic acid, luteolin, trigonelline hydrochloride, doxorubicin, gemcitabine hydrochloride, staurosporine, nelfinavir, WRR139 and bovine serum albumin (BSA) were purchased from Sigma Aldrich (Poole, UK). ML385 was purchased from MedChem Express (New Jersey, USA). Triptolide and clobetasol propionate were purchased from SelleckChem (Cambridge, UK). Dimethyl fumarate (DMF) was purchased from Fisher Scientific (Loughborough, UK). Human recombinant TNF- α was obtained from BD Biosciences (Oxford, UK). Vemurafenib and MG132 were purchased from Bio-Techne (Abingdon, UK). T25 and T75 culture flasks were obtained from Thermo Fisher Scientific or Greiner (Stonehouse, UK). Cryovials, pipette tips (10 μ L, 200 μ L and 1250 μ L), serological pipettes (1 mL, 5 mL, 10 mL and 25 mL) and syringe filters (0.2 μ m and 0.45 μ m) were purchased from Thermo Fisher Scientific or Starlab (Milton Keynes, UK). All other consumables were obtained from Thermo Fisher Scientific, unless otherwise stated.

2.1.2 Cell Lines

M202, A375, A2058, M263, M308, MEL501, SKMEL-5, SKMEL-28 and UACC-1273 cells were generously supplied by Dr Antoni Ribas (University of California Los Angeles, USA). HEMA-LP melanocyte cells were obtained from Thermo Fisher Scientific. All other cell lines were purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK).

2.1.3 Cell Culture

RPMI-1640, phosphate buffered saline (PBS) and DMSO Hybri-Max™ were obtained from Sigma-Aldrich. Dulbecco's Modified Eagle Medium (DMEM), McCoy's 5A medium, Eagle's Minimum Essential Medium (MEM), L-Glutamine, Penicillin Streptomycin, TrypLE Express and heat-inactivated foetal bovine serum (FBS) were obtained from Gibco (Leicestershire, UK). Melanocyte Growth Medium M2 was obtained from Promocell (Heidelberg, Germany).

2.1.4 Cell Proliferation Assay

Cell Titer 96 Aqueous One solution Cell Proliferation Assay was purchased from Promega (Hampshire, UK).

2.1.5 NQO1 Activity Assay

Tween 20, Flavin adenine dinucleotide disodium salt hydrate (FAD), glucose-6-phosphate, Nicotinamide adenine dinucleotide phosphate (NADP⁺), Yeast glucose-6-phosphate dehydrogenase, Thiazolyl Blue Tetrazolium Blue (MTT) and Menadione were purchased from Sigma Aldrich.

2.1.6 Western Immunoblotting

Glycine, sodium dodecyl sulfoxide (SDS) and Tris Base were purchased from Sigma Aldrich. Novex™ Tris-Glycine SDS Sample Buffer (2X), PageRuler™ Prestained Protein Ladder, Novex™ Tris-Glycine gel, XCell SureLock Mini Cell Electrophoresis System, XCell II™ Blot Module, SimplyBlue Safestain and Novex™ ECL Chemiluminescent Kit were all obtained from Invitrogen Life Technologies (Loughborough, UK). Polyvinylidene Difluoride (PDVF) membrane was obtained from Bio-Rad (Hemel Hempstead, UK). Marvel Original dried skimmed milk was purchased from Premier Foods (St Albans, UK).

2.1.7 Nrf2 DNA-Binding ELISA

The Nuclear Extraction Kit was purchased from Abcam (Cambridge, UK). The TransAM Nrf2 DNA-binding ELISA kit was purchased from ActiveMotif (La Hulpe, Belgium).

2.1.8 Tumouroid culture

Matrigel® Membrane Matrix was purchased from Corning (New York, USA). Menzel round coverslips (diameter 19mm, #0) were purchased from Thermo Fisher Scientific. CellEvent™ Caspase-3/7 Green Detection Reagent was obtained from Invitrogen.

2.1.9 Flow Cytometry

CytoFLEX Daily QC fluorospheres were purchased from BD Biosciences. Anti-EGFR antibody (ab30) was purchased from Abcam. The goat anti-mouse IgG Alexa Fluor-488 antibody was purchased from Invitrogen.

2.1.10 RT-qPCR

TRI-Reagent® was purchased from Invitrogen. 1-bromo-chloro-propane, 2-propanol, ethanol, SYBR® Green JumpStart™ Taq ReadyMix™ and custom-made primers (**Table 2.1**) were purchased from Sigma Aldrich. The High-Capacity cDNA Reverse Transcription kit and RNase Inhibitor were purchased from Applied Biosystems

(California, USA). DNA Away decontaminant, PCR grade water and filter pipette tips were purchased from Thermo Fisher Scientific. 0.1 mL strip tubes and caps were purchased from Qiagen (Manchester, UK).

Table 2.1. Primer sequences for RT-qPCR.

Gene	Forward (5'-3')	Reverse (5'-3')
NFE2L2	AAC CAC CCT GAA AGC ACA GC	TGA AAT GCC GGA GTC AGA ATC
NQO1	GCG AGA CCT TGT GAT ATT CCA G	TCC TAT GAA CAC TCG CTC AAA CC
HO-1	GCG AGA CCT TGT GAT ATT CCA G	TGT TGC GCT CAA TCT CCT CCT
NFE2L3	TTCA GCC AGG CTA TAA GTC AGG	GCT CAG GAT TGG TGG TAT GAG A
DJ-1	GGA GAC GGT CAT CCC TGT AGA T	GCT ACA CTG TAC TGG GTC TTT TCC A
GAPDH	AAC AGC CTC AAG ATC ATC AGC A	TGC TAA GCA GTT GGT GGT GC

2.1.11 siRNA Transfection

DharmaFECT 2 Transfection Reagent, ON-TARGETplus Non-Targeting Pool siRNA and ON-TARGETplus Human NFE2L3 SMARTPool siRNA (**Table 2.2**) were purchased from Dharmacon (Colorado, USA).

Table 2.2. siRNA targeting sequences.

Gene	Target sequences
On-Targetplus Non-targeting siRNA Pool	UGG UUU ACA UGU CGA CUA A
	UGG UUU ACA UGU UGU GUG A
	UGG UUU ACA UGU UUU CUG A
	UGG UUU ACA UGU UUU CCU A
ON-TARGETplus Human NFE2L3 siRNA SMARTpool	GAA CUG UCG UAA ACG CAA A
	GCG UAG ACC UAG AUC UUU A
	GCA AGC ACA AUG UAA CAA A
	CAA CUU UGA UCC AAU CGA U

2.1.12 Peptide Synthesis

Fmoc-amino acids were purchased from AGTC Bioproducts (Potters Bar, UK) or Novabiochem (London, UK). NovaPEG Rink Amide resin was purchased from Novabiochem. Coupling reagents (HOBt; Hydroxybenzotriazole and HBTU; Hexafluorophosphate Benzotriazole Tetramethyl Uronium) and Rink Amide MBHA resin were purchased from Sigma Aldrich or Cambridge Reagents Ltd. (Barton-upon-Humber, UK). All solvents and chemicals for peptide synthesis were purchased from VWR International (Lutterworth, UK) or Sigma Aldrich.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Preparation of Cell Culture Media

Cell culture media (RPMI-1640, DMEM, MEM) was supplemented with 2mM L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FBS. Heat-inactivated FBS was filtered through a 0.45 µm sterile filter before use in media supplementation.

2.2.1.2 Cell passaging

Cell lines and primary cells were maintained and passaged according to the conditions detailed in **Table 2.3**. All cells were maintained at 37°C, 5% CO₂ in a humidified atmosphere. Phosphate buffered saline (PBS), cell culture media and TrypLE Express were pre-warmed to 37°C prior to use. For non-adherent cell lines, cells were maintained at a cell density of between 2.5-8x10⁵ cells/mL. Cell passaging was performed every 3 days by counting the cells (see section **2.2.1.3 Cell counting**) and diluting the cell suspensions to a density of 2.5x10⁵ cells/mL with RPMI-1640 media.

Adherent cell lines (as detailed in **Table 2.3**) were passaged every 3-4 days, once cells had reached 70-80% confluency, as determined via visualisation with white light microscopy. Cells were washed with PBS then detached from the cell culture flask with TrypLE Express, as confirmed by observing the cells under white light microscopy. Once detached, appropriate cell culture media was added to the flask to neutralise the TrypLE Express, and the resulting cell suspension was diluted with additional fresh media, according to the cell line.

Table 2.3. Origins and cell culture conditions of cell lines and primary cells.

Cell Line	Origin	Culture media	Adherent (Yes/No)	Passage Procedure
A549	Lung adenocarcinoma cells derived from a 58-year-old male.	RPMI-1640 or DMEM	Y	Passaged every 3 days to between 1:10-1:20 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
H292	Lung mucoepidermoid carcinoma cells derived from a 32-year-old female.	RPMI-1640	Y	Passaged every 3 days to a 1:10 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
MIA PACA-2	Pancreatic ductal adenocarcinoma derived from a 65-year-old male.	DMEM	Y	Passaged every 3 days to between 1:10-1:20 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
BXPC3	Pancreatic ductal adenocarcinoma derived from a 61-year-old female.	RPMI-1640	Y	Passaged every 3 days to between 1:5-1:10 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
LNCAP	Lymph node carcinoma of the prostate cells derived from a 50-year-old male.	RPMI-1640	Y	Passaged every 3 days to a 1:10 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
MCF-7	Breast carcinoma cells derived from a 69-year-old female.	DMEM	Y	Passaged every 3 days to a 1:20 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
MDA-MB-231	Breast adenocarcinoma cells derived from a 51-year-old female.	DMEM	Y	Passaged every 3 days to a 1:10 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
HEPG2	Hepatoblastoma cells derived from a 15-year-old male.	DMEM	Y	Passaged every 3 days to a 1:5 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
CACO-2	Colon adenocarcinoma	MEM with non-	Y	Passaged every 3 days to a 1:5 dilution once at 70-80% confluency.

	cells derived from a 72-year-old male.	essential amino acids and sodium pyruvate.		Cells detached with TrypLE Express.
HCT116	Colon carcinoma cells derived from a 48-year-old male.	McCoy's 5A	Y	Passaged every 3 days to a 1:20 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
THP-1	Acute monocytic leukaemia cells derived from a 1-year-old male.	RPMI-1640	N	Passaged every 3 days with fresh medium to a dilution of approx. 2.5×10^5 cells per mL. Cells were kept between concentrations of 2.5 to 8×10^5 cells/mL.
HEK-293	Human embryonic kidney cells.	DMEM	Y	Passaged every 3 days to a 1:20 dilution once at 70-80% confluency. Cells were detached for 1 minute with TrypLE Express at 37°C, 5% CO ₂ .
HEMA-LP	Primary human adult epidermal melanocytes.	Melanocyte Growth Medium M2	Y	Cells were cultured in T25 flasks and were passaged once they reached 90% confluency. Media was refreshed every 3 days. Cells were not used past passage no. 6.
M202	Malignant melanoma cells (age and gender of origin is unknown).	RPMI-1640	Y	Passaged every 3 days to a 1:5 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
A375	Malignant melanoma cells derived from a 54-year-old female.	RPMI-1640	Y	Passaged every 3 days to a 1:20 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
M263	Malignant melanoma cells (age and gender of origin unknown).	RPMI-1640	Y	Passaged every 3 days to a 1:20 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
M308	Malignant melanoma cells	RPMI-1640	Y	Passaged every 3 days to a 1:5 dilution once at 70-80% confluency.

	derived (age and gender of origin unknown).			Cells detached with TrypLE Express.
MEL501	Malignant melanoma cells derived (age and gender of origin unknown).	RPMI-1640	Y	Passaged every 3 days to a 1:5 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
UACC-1273	Malignant melanoma cells derived from a 54-year old male.	RPMI-1640	Y	Passaged every 3 days to a 1:10 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
SKMEL-5	Malignant melanoma cells derived from a 24-year old female.	RPMI-1640	Y	Passaged every 3 days to a 1:10 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
SKMEL-28	Malignant melanoma cells derived from a 51-year old male.	RPMI-1640	Y	Passaged every 3 days to a 1:20 dilution once at 70-80% confluency. Cells detached with TrypLE Express.
A2058	Malignant melanoma cells derived from a 43-year old male.	RPMI-1640	Y	Passaged every 3 days to a 1:10 dilution once at 70-80% confluency. Cells detached with TrypLE Express.

2.2.1.3 Cell counting

Cell density was determined via counting with a Neubauer hemocytometer. To count the cells, 10 μ L of cell suspension was loaded onto the counting chamber and the hemocytometer was observed using light microscopy. An average cell count was determined via counting the total number of cells in four of the large squares of the hemocytometer and averaging the number according to the number of large squares counted (e.g. total number of cells counted /4). To ensure consistency, if any cells were located on the boundaries of the grid, only cells that were on the top or left-hand side boundary were counted. The cell density was then determined using the following calculation:

$$\text{Cell density (cells/mL)} = \text{Average cell count} \times 10,000$$

2.2.1.4 Freezing cells

Freezing media (10% DMSO Hybri-Max™ in FBS, see **Chapter 8.1.1** for recipe) was prepared and kept on ice. Once at 70-80% confluency, cells were detached with TrypLE Express and were centrifuged at 300 g for 7 min. The supernatant was discarded, and the cell pellet was resuspended in 1 mL cold freezing media. The resulting suspension was then decanted into a sterile vial, which was kept on ice and wrapped in paper towels for insulation. The vials were packaged into a cotton wool packed box (to allow gradual freezing) which was stored at -80°C for at least one day before being transferred to liquid nitrogen storage.

2.2.1.5 Thawing frozen cells

To thaw frozen cells, vials were defrosted at room temperature and were centrifuged at 300 g for 5 min. The supernatant was removed, and the cell pellet was resuspended in 8 mL of the appropriate media (pre-warmed to 37°C). The cell suspension was then transferred to a T25 flask and cultured at 37°C, 5% CO₂ in a humidified atmosphere. Once cells grew to 70-80% confluency, they were detached using TrypLE Express and were transferred to a T75 flask, where they were maintained at 37°C, 5% CO₂ in a humidified atmosphere (as described in **2.2.1.2 Cell Passaging**).

2.2.2 MTS Assay

Cells were seeded at the required density (**Table 2.4**) in 96-well plates and were incubated overnight at 37°C, 5% CO₂ in a humidified atmosphere to allow for cell adherence. Cells were treated with DMSO or serial dilutions of ML385, retinoic acid, luteolin (at final concentrations of 3.9-500 µM) or triptolide, brusatol, trigonelline hydrochloride, clobetasol propionate, gemcitabine hydrochloride or vemurafenib (at final concentrations of 10 pM-100 µM) for 72 hours. 10 µL of CellTiter 96 Aqueous One Solution Cell Proliferation Assay Reagent (Promega) was then added to each well and absorbance was read at 490nm on a POLARstar Optima microplate reader (BMG Labtech, Aylesbury, UK), following formation of a red/brown product.

Table 2.4 Seeding densities of cell lines used in cell proliferation assays. Cells were seeded in 96-well plates, with a total volume of 100 μ L/ well.

Cell line	Seeding density (cells /well)
A549	5×10^3
H292	8×10^3
BXPC3	6×10^3
MIAPACA-2	6×10^3
M202	8×10^3
A375	5×10^3
MCF-7	8×10^3
HEK-293	5×10^3

2.2.3 NQO1 Activity Assay

NQO1 activity was determined using an enzymatic assay, as previously described by Prochaska et al⁴¹⁶. To carry out the experiment, A549 and M202 cells were seeded in 96-well plates at a density of 8×10^3 and 4×10^4 cells/well, respectively, and were incubated overnight at 37°C, 5% CO₂ in a humidified atmosphere. Cells were then stimulated with Nrf2 inhibitors (0.1- 20 μ M) or MafG peptide mimic (25-100 μ M) for 20 hours. Media was removed, and cells were lysed with 0.8% digitonin in 2mM EDTA (pH 7.8, see **Chapter 8.1.2** for recipe). Reaction buffer was prepared (see **Chapter 8.1.2**) and kept on ice. 50 μ L NQO1 standards (15.6- 1000 μ M/mL) were prepared in 0.8% digitonin in 2mM EDTA and were added to the empty wells of the plate. A blank well (- Menadione) was prepared by adding 200 μ L reaction buffer without menadione to the well. 200 μ L of complete reaction buffer was then added to each well and the plate was read immediately at 610nm on a CLARIOstar Plus microplate reader (BMG Labtech). A linear regression was created from the NQO1 standard wells, which was then used to calculate NQO1 activity of the treatment conditions.

2.2.4 Western Immunoblotting

2.2.4.1 Preparation of whole cell lysates

Adherent cells (e.g A549, M202 and MIAPACA2) were seeded at a density of 5×10^5 cells/well in 6-well plates. All cells were incubated overnight at 37°C, 5% CO₂ in a humidified atmosphere. The following day, cells were then stimulated with either DMF (10- 30 μ M), Nrf2 inhibitors (0.2- 20 μ M), siRNA (25-100 nM), DMSO for 0-48 hours or were left unstimulated.

To collect adherent cells, the media was removed from each well and the cells were washed with 2 mL cold PBS. 200 µL 1X Tris-Glycine SDS Sample Buffer was added to each well to lyse the cells, and the resulting solution was transferred to Eppendorf tubes. To collect non-adherent cells, the cell suspension was transferred to Eppendorf tubes and was centrifuged at 300 g for 5 mins. The supernatant was removed, and the cell pellet was resuspended in 1mL cold 1X PBS. The cells were then centrifuged again, the supernatant removed, and the cells lysed in 200 µL 1X Tris-Glycine SDS Sample Buffer. The samples were transferred to ice and were homogenised using a Fisher Scientific sonic dismembrator (ThermoFisher) for 10 seconds prior to boiling for 5 mins. Samples were then stored at -80°C.

2.2.4.2 Protein Quantification

To quantify the protein concentration of each sample, a Nanodrop Spectrophotometer-1000 (ThermoFisher) was used on the A280 setting. This method detects tryptophan, tyrosine and phenylalanine residues in proteins, which absorb light at 280 nm. The concentration of protein is then determined using the Beer-Lambert Law:

$$C = \frac{A}{\epsilon L}$$

Where:

C= concentration (M)

A= UV Absorbance (AU)

ε= Extinction coefficient (M⁻¹ cm⁻¹)

L= Light path (cm)

Protein purity is calculated by determining the A260/A280 ratio, which indicates the presence of nucleic acid contaminants. To quantify protein concentration using a Nanodrop Spectrophotometer, the A280 program was selected, and the spectrophotometer was blanked with 1 µL of sample buffer. 1 µL of protein sample was loaded onto the sample pedestal and the concentration and purity of the sample was recorded. For proteins, a A260/A280 ratio of ~1.7 was considered pure.

2.2.4.3 SDS-PAGE Gel and Membrane Transfer

A XCell SureLock Mini Cell Electrophoresis System was set up according to the manufacturer's protocol using a pre-cast NuPAGE 4 to 12% Bis-Tris 1.0 mm Mini Protein Gel (either containing 9 or 12 wells). Recipes for 1X MOPS running buffer, 1X transfer buffer, 1X TBST and blocking solution are described in **Chapter 8.1.3**. 1X MOPS SDS

running buffer and 500 μ L NuPAGE antioxidant was added to the middle chamber of the system and was used to rinse the wells of the gel gently via pipette. The outer chamber of the system was then filled to $\frac{3}{4}$ full with 1X MOPS SDS running buffer.

Samples were prepared to a volume of 18 μ L or 27 μ L for 12-well and 9-well gels, respectively, with each sample containing equal amounts of protein, and 10% reducing agent. The samples were mixed thoroughly and were heated to 95°C for 5 min prior to loading onto the gel. 5 μ L of PageRuler™ Prestained Protein Ladder was loaded onto the first well of the gel, followed by the samples into the remaining wells. The electrophoresis was run at 150 V through the stacking gel, then at 200 V until protein bands reached the bottom of the gel. Protein transfer onto a Polyvinylidene Difluoride (PVDF) membrane was then performed using an XCell II™ Blot Module, according to the manufactures protocol, for 1 hour at 30 V in 1X Transfer Buffer. The membrane was then briefly stained with Ponceau S solution to confirm protein transfer. To remove the stain, the membrane was washed with 1X TBST prior to blocking overnight in blocking solution at 4°C. The gel was stained for 1 hour at room temperature with SimplyBlue Safestain to visualise protein bands.

Following overnight blocking, the membrane was cut according to size of the protein of interest, as determined via the corresponding molecular weight marker band (see **Table 2.5** for protein size and incubation details). The membrane was then incubated with the desired primary antibody at room temperature for 1.5- 2 hours on a tube roller. The membrane was then washed thoroughly with 1X TBST and blocking solution prior to incubation with anti-rabbit HRP-conjugated antibody for 45 min at room temperature (1:1000 in blocking solution). The membrane was then washed again thoroughly with 1X TBST and blocking solution. Proteins were visualised using the Novex ECL Chemiluminscent Kit and an ImageQuant LAS 4000 chemiluminescent camera (General Electric, Buckinghamshire, UK).

To re-probe a membrane for another protein of interest, the membrane was stripped with 1X Stripping Solution (see appendix for recipe) for 20 minutes at room temperature. The membrane was then washed twice with 1X PBS for 10 min and 1X TBST for 5 min prior to blocking with blocking solution for 30 min at room temperature. The membrane was then re-probed, as described above, according to table 2.5, for the protein of interest.

Table 2.5. Primary antibodies used in western immunoblotting.

Protein of interest	Approx. Protein Size	Antibody	Concentration	Incubation protocol
Nrf2	62 kDa	Rabbit anti-Human NRF2 monoclonal antibody (Abcam)	1:750 in blocking solution	1 h 30 m Room temperature
Nrf3	76 kDa	Rabbit anti-NRF3 polyclonal antibody (Prestige Antibodies)	1:500 in blocking solution	2 h Room temperature
β -Tubulin	55 kDa	Rabbit anti- β -Tubulin monoclonal antibody (Cell Signalling Technology)	1:1000 in blocking solution	1 h 30 m Room temperature
DJ-1	22 kDa	Rabbit anti-DJ-1 monoclonal antibody (Cell Signalling Technology)	1:2000 in blocking solution	1 h Room temperature

2.2.5 Nrf2 DNA-binding ELISA

2.2.5.1 Preparation of nuclear extracts

A549 and M202 cells were seeded in 6-well plates at a density of 3×10^5 cells/well. Plates were incubated overnight to allow for cell adherence before they were treated with Nrf2 inhibitors (10 μ M or 20 μ M) or MafG peptide mimic (25-100 μ M) for 4 hours. Nuclear extracts were then prepared using the Nuclear Extraction Kit (ab221978, Abcam), according to the manufacturers protocol (see **Chapter 8.1.4** for buffer preparation). Following cell stimulation, cells were washed with cold 1X PBS/ Phosphatase Inhibitor solution and were collected via scraping into 1.5 mL Eppendorf tubes in 1 mL 1X PBS/Phosphatase inhibitor solution. Cells were centrifuged at 1200 g for 5 minutes at 4°C and the cell pellet was resuspended in 200 μ L 1X Complete Hypotonic buffer. Samples were then placed on ice for 15 minutes prior to adding 30 μ L of 10% NP-40 reagent. The samples were then mixed via vortex for 10 seconds and were centrifuged at 14,000g for 1 minute at 4°C. The cell pellet was resuspended in 20 μ L 1X Complete

Nuclear Buffer and the samples were homogenised using a sonic dismembrator. Samples were incubated on ice for 30 minutes prior to centrifuging at 14,000g for 10 minutes at 4°C. The resulting supernatant was then transferred to another Eppendorf tube. Nuclear protein content was quantified using a Nanodrop Spectrophotometer using the A280 setting (see **2.2.4.2 Protein Quantification**) and samples were stored at -80°C.

2.2.5.2 Nrf2 DNA-binding ELISA assay

To quantify Nrf2 DNA-binding activity, the TransAM Nrf2 DNA Binding ELISA kit (ActiveMotif) was used according to the manufacturers protocol (see **Chapter 8.1.4** for buffer preparation). 40 µL of 1X Complete Binding Buffer was added to each well of the assay plate. Samples were each prepared to 20 µg nuclear protein in 10 µL 1X Complete Lysis Buffer and were also added to the plate (including a positive control and blank well). The assay plate was then incubated at room temperature with mild agitation (100 rpm) for 1 hour. Following this, the wells were washed 3 times with 200 µL 1X Wash Buffer. 100 µL of anti-Nrf2 antibody (1:1000 dilution in 1X Antibody Binding Buffer) was added to each well and the plate was incubated at room temperature for 1 hour (with no agitation). The wells were washed again, as before, and 100 µL of secondary HRP-conjugated antibody (1:1000 dilution in 1X Antibody Binding Buffer) was added to each well. Following 1 hour incubation at room temperature, the wells were washed 4 times. 100 µL of developing solution was then added to each well and the plate was incubated in the dark at room temperature for 15 minutes, until a medium-dark blue colour developed. To quench the reaction, 100 µL stop solution (sulfuric acid) was added to each well and the absorbance was read at 490nm on a POLARstar Optima microplate reader (BMG Labtech).

2.2.6 Tumouroid culture

2.2.6.1 Tumouroid cell seeding

Matrigel™ Membrane Matrix (Corning) was defrosted on ice prior to use and 12-well plates were prepared with round glass coverslips (19mm diameter, width 0.08-0.12 mm), sterilised in 70% ethanol. A549 cells were detached with TrypLE Express and counted (see **2.2.1.3 Cell Counting**). The required volume of cell suspension (according to the number of wells to be used, with a seeding density of 1×10^4 cells/well) was centrifuged at 300 g for 5 minutes, and all but 5 µL of the supernatant was removed. The cell pellet

was gently resuspended in the remaining supernatant and the required volume of Matrigel™ Membrane Matrix was added (volume of Matrigel required= 10 µL/well x no. of wells). The cell suspension was then mixed gently with the Matrigel™ via pipette. 10 µL of the resulting cell suspension/Matrigel mix was pipetted onto each coverslip and the plate was incubated at 37°C, 5% CO₂ for 30 minutes to allow the Matrigel™ to set. Once set, 100 µL of RPMI-1640 media was added to each well, surrounding the Matrigel™. Cells were treated with 0.1% DMSO, triptolide, staurosporine or gemcitabine hydrochloride (0.1- 10 µM in 0.1% DMSO) on either day 0 or daily from days 5-9 of incubation. Cells were maintained at 37°C, 5% CO₂ in a humidified atmosphere and media was refreshed every 3 days. White light microscopy images were taken daily using a Zeiss Primovert white light microscope (Zeiss, Cambridge, UK) and tumouroid area was calculated using ImageJ software (National Institutes of Health, Maryland, USA).

2.2.6.2 Measurement of apoptosis in tumouroids

To measure apoptosis, tumouroids were incubated with a fluorogenic substrate for activated caspase-3/7 (CellEvent™ Caspase-3/7 Green Detection Reagent), an indicator of apoptosis. Following daily treatment with triptolide or gemcitabine hydrochloride from days 5-9 of tumouroid growth, CellEvent™ Caspase-3/7 Green Detection Reagent was prepared to 5 µM in RPMI-1640 media to a total volume of 900 µL. Media was removed from the tumouroids and replaced with 100 µL/ well of the 5 µM CellEvent™ Caspase-3/7 Green Detection solution. Tumouroids were incubated at 37°C, 5% CO₂ in a humidified atmosphere in the dark for 30 min. Coverslips were then inverted onto microscopy slides and apoptosis was visualised using a Zeiss AxioVert 200M epifluorescent microscope (Cambridge, UK). Microscopy images were then analysed using ImageJ software.

2.2.7 Flow Cytometry

A549, HEK-293 and M202 cells were counted and 1x10⁶ cells per treatment were collected into Eppendorf tubes. Cells were centrifuged at 300 g for 5 minutes at 4°C and the cell pellet was washed with cold PBS. The cells were then centrifuged again at 300 g for 5 minutes at 4°C and the cell pellet was resuspended in either 100 µM FITC-AEYLRK peptide or mouse anti-EGFR monoclonal antibody (1:1000 dilution) in 500 µL PBS 0.5% BSA, or in 500 µL PBS 0.5% BSA alone (negative control). Cells were incubated on ice for 1 hour prior to washing three times with cold PBS. Cells were then centrifuged at 300 g for 5 minutes at 4°C. For the EGFR expression experiments, cells were then incubated for a further hour on ice with a secondary goat anti-mouse IgG Alexa Fluor-488 antibody (1:100 dilution) in 500 µL PBS 0.5% BSA and cells were centrifuged

again. Prior to analysis, the cell pellet was then resuspended in 300 μ L PBS 0.5% BSA. Peptide binding and EGFR expression were then analysed using a CytoFlex Flow Cytometer (Beckman Coulter, California, USA).

2.2.8 Reverse Transcriptase quantitative PCR (RT-qPCR)

2.2.8.1 Sample Preparation

Cells were seeded at 1×10^6 cells/ well in 6-well plates and were incubated overnight at 37°C, 5% CO₂ in a humidified atmosphere. The following day, the media was removed, and the cells were washed with 1 mL of PBS. Cells were then incubated in 1mL of Tri-Reagent for 5 minutes at room temperature to allow the cells to lyse. For the MafG peptide mimic experiments in A549 cells, cells were first stimulated with 25-100 μ M MafG peptide mimic for 2-48 hours prior to cell lysis with Tri-Reagent. The lysed cells were then transferred to DNase and RNase-free Eppendorf tubes, which were kept on ice prior to storing at -80°C.

2.2.8.2 RNA Isolation

Samples in Tri-Reagent were thawed at room temperature and 100 μ L 3-bromo-3-chloro-propane was added to each tube. The samples were shaken by hand and incubated at room temperature for 10 minutes prior to centrifuging at 12,000 g for 20 minutes at 4°C. The top aqueous layer was then transferred to fresh Eppendorf tubes and 500 μ L 2-propanol was added to precipitate the RNA. The samples were mixed via vortex for 10 seconds and were subsequently incubated at room temperature for 10 minutes. Samples were then centrifuged at 12,000 g for 15 minutes at 4°C and the resulting supernatant was discarded. 1 mL of 70% ethanol was then added to each tube to wash the cell pellet. The samples were mixed via pulsing on a vortex prior to being centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was removed, and any remaining ethanol was evaporated at room temperature. 20 μ L of RNase-free water was then added to the pellet and mixed via vortex. The quantity and purity of RNA was determined using a Nanodrop Spectrophotometer-1000 on the 'Nucleic Acid' setting. All RNA samples were then diluted with additional RNase-free H₂O to a concentration of 200 ng/ μ L and samples were stored at -80°C.

2.2.8.3 cDNA Synthesis

200 ng of RNA was prepared to a total volume of 5 μ L in RNase-free water. A cDNA synthesis Mastermix solution was prepared from the High-Capacity cDNA Reverse

Transcription Kit (Applied Biosystems) using the following recipe: 32% H₂O, 20% 10X RT Buffer, 20% random hexamers, 10% RNase Inhibitor, 10% reverse transcriptase and 8% dNTPs. 5 µL of cDNA synthesis Mastermix was then added to each RNA sample and mixed gently via pipette. Prepared samples were loaded into a PTC-100 Thermal Peltier Cycler (Biorad), which was set to incubate the samples at 25°C for 10 minutes, 37°C for 2 hours, 85°C for 5 minutes, and finally hold at 4°C. Samples were then further diluted with RNase-free water to a concentration of 0.6-10 ng/µL and were stored at -20°C.

2.2.8.4 RT-qPCR protocol

RT-qPCR Mastermix was prepared using the following recipe: 66.67% SYBR Green, 26.67% RNase-free H₂O and 6.67% primers (5 µM forward + reverse). Primer sequences are described in **Table 2.1**. For each sample to be tested, 5 µL of cDNA was mixed with 15 µL of RT-qPCR Mastermix via pipette in 0.1mL strip tubes. All samples were run in duplicate and non-template controls (RNase-free H₂O) were used for each gene being studied. cDNA calibration curves were produced for each cell line and gene being studied and amplification efficiencies were calculated for each set of primers used (see **Chapter 8.2.1.1** and **8.2.1.2**). Samples were loaded into a Rotor Gene-Q Series 5-Plex Real Time PCR Cycler (Qiagen) and were cycled on either a two-step or three-step program. Two-step RT-qPCR (for NFE2L2, HO-1, NQO1, GAPDH, DJ-1) was carried out with the following cycling settings: 2 minutes at 95°C followed by 40 cycles of [15 seconds at 95°C then 40 seconds at 60°C] and a melt curve stage ramping from 60°C -94°C. Three-step RT-qPCR (for NFE2L3, GAPDH) was conducted using the following cycling settings: 2 minutes at 95°C followed by 40 cycles of [15 seconds at 95°C, 20 seconds at 60°C then 30 seconds at 72°C] and a melt curve stage ramping from 60°C -94°C (see **Chapter 8.1.1.3** for melt curves). Relative gene expression was then analysed via the $2^{-\Delta\Delta Ct}$ method (as displayed below), using cycle threshold values determined by the Rotor-Gene Q Series Software 2 (Qiagen).

$2^{-\Delta\Delta Ct}$ Method:

$$\Delta Ct = Ct (\text{gene of interest}) - Ct (\text{housekeeping gene})$$

$$\Delta\Delta Ct = \Delta Ct (\text{treatment condition}) - \Delta Ct (\text{control/ untreated condition})$$

$$\text{Relative gene expression} = 2^{-\Delta\Delta Ct}$$

2.2.9 Wound Scratch Assay

12-well plates were prepared by drawing 2 parallel horizontal lines on the bottom of each well, and 1 mL PBS was placed in the 4 corner wells. A375 and M202 cells were seeded at 3×10^5 cells/ well in 12 well plates and were incubated at 37°C, 5% CO₂ overnight in a humidified atmosphere to allow cell adherence. The following day, the media was removed, and cells were washed gently with 500 µL warm PBS. In each well, 3 vertical scratches were made through the cell monolayer with a 200 µL pipette tip. Cells were washed again twice with 500 µL PBS prior to adding 1 mL of serum-free RPMI media to each well. Images were taken of each scratch between the two pre-marked horizontal lines, using a Leica DMIL white light microscope (Leica Microsystems, Wetzlar, Germany). Cells were treated with DMSO or 0.01-10 µM Nrf2 inhibitors or vemurafenib and were incubated for 24h at 37°C, 5% CO₂. After 24h, the cells were washed with 500 µL PBS, the media refreshed, and white light microscopy images were taken. Migration rate (µm/hour) was calculated by measuring the width of the scratch area using an ImageJ plugin⁴¹⁷, using the following equation:

$$\text{Migration rate} = \frac{(\text{Width in } \mu\text{m at 0 h}) - (\text{Width in } \mu\text{m at 24 h})}{\text{Treat time (h)}}$$

2.2.10 siRNA Transfection

2.2.10.1 General siRNA transfection protocol

MIAPACA-2 cells were seeded in antibiotic-free DMEM media at the appropriate density for the plating format desired, according to **Table 2.6**. Cells were then incubated overnight at 37°C, 5% CO₂ overnight in a humidified atmosphere. The following day, siRNA treatments and DharmaFECT 2 Reagent were prepared in separate tubes in serum-free medium, as described in **Table 2.6**, and were incubated at room temperature for 5 minutes. siRNA treatments were prepared from 5 µM stock concentrations of either ON-TARGETplus Non-Targeting Pool, ON-TARGETplus Human NFE2L3 SMARTPool siRNA (see **Table 2.2** for sequences) or RNase-free H₂O (for the untreated control). A 1:1 solution of the DharmaFECT 2 reagent and siRNA treatments was then prepared and mixed together via pipette, prior to incubation at room temperature for 20 minutes (for total volumes of 20 µL, 200 µL or 400 µL, depending on the plate format). Antibiotic-free medium was then added to the DharmaFECT/siRNA solution to a final concentration of 100 nM siRNA in a total volume of 100 µL, 1000 µL or 2000 µL/well for 96, 12 and 6 well plates, respectively. The media was then removed from the cells and was replaced

with the DharmaFECT/siRNA solution. Cells were incubated with siRNA treatments for 48 hours prior to RNA or protein extraction, which was carried out as previously described in sections **2.2.4.2** and **2.2.8.2** respectively.

Table 2.6. Volumes of reagents used in siRNA transfection.

		Tube 1: Diluted siRNA (μL/well)		Tube 2: diluted DharmaFECT (μL/well)			
<i>Plate format</i>	<i>Seeding density (cells/well)</i>	<i>siRNA (μL)</i>	<i>Serum-free medium (μL)</i>	<i>Volume of DharmaFECT reagent (μL)</i>	<i>Serum-free medium (μL)</i>	<i>Complete medium (μL/well)</i>	<i>Total transfection volume (μL/well)</i>
96 well	6x10 ³	0.5	9.5	0.25	9.75	80	100
12 well	1x10 ⁵	5	95	2.5	97.5	800	1000
6 well	2.5x10 ⁵	10	190	5	195	1600	2000

2.2.10.2 MTS assay following siRNA transfection

MIAPACA-2 cells were seeded in 96-well plates in antibiotic free DMEM media and siRNA transfection was conducted as described in **2.2.10.1 General siRNA transfection**, with each treatment being carried out in triplicate. After 48 hours of incubation with untreated, non-targeting and targeting siRNA treatments, 10 μL of CellTiter 96 Aqueous One Solution Cell Proliferation Assay Reagent (Promega) was added to each well and the plate was incubated at 37°C, 5% CO₂ in a humidified atmosphere until a dark red product formed. Absorbance was subsequently read at 490nm on a POLARstar Optima microplate reader.

2.2.10.3 Wound scratch assay following siRNA transfection

12-well plates were prepared as described in section **2.2.9. Wound Scratch Assay**, MIAPACA-2 cells were then seeded in the prepared plates in antibiotic-free DMEM media and siRNA transfection was carried out as above, with all treatments being set up in duplicate. Following incubation with the siRNA treatments for 48 hours, the cells were washed with warm PBS and three vertical scratches were made per well using a P200 pipette tip. The cells were washed once more and 1 mL of serum-free DMEM was added to each well. The scratch area was imaged at 0 hours, 24 hours and 48 hours using a Leica DMIL microscope and the migration rate was calculated using ImageJ analysis, as previously described in section **Chapter 2.2.9**.

2.2.11 Peptide Synthesis

All peptides were synthesised using standard Fmoc-SPPS, using either a NovaPEG Rink Amide or Rink Amide MBHA resin and a Biotage Syro I peptide synthesiser (Biotage, Hengeod, UK) or Liberty Blue Microwave peptide synthesiser (CEM Microwave Technology Ltd., Buckingham, UK). To synthesise the AEYLRK and RALELK peptides studied in **Chapter 4.3.1**. 100 mg of NovaPEG Rink Amide resin was swelled in *N,N*-Dimethylformamide (*N,N*-DMF) for 30 min prior to amino acid coupling with 4 equivalents of amino acid (dissolved in *N*-Methyl-2-pyrrolidone; NMP or *N,N*-DMF), 4 equivalents of HBTU, 3.9 equivalents of HOBT and 8 equivalents of DIPEA. Coupling reactions were carried out twice for 45 min for each amino acid, with excess reagents washed away with *N,N*-DMF. Deprotection of the Fmoc protecting group was carried out via shaking with a 20% piperidine solution in *N,N*-DMF for 10 min, twice. Acetylation of the peptides was achieved via addition of 4 equivalents of acetyl chloride, 4 equivalents of DIPEA in DMF for 45 min. Each acetylation reaction was carried out twice and completion was measured via Kaiser test. The peptides were cleaved from the resin using a solution of 45% TFA (trifluoroacetic acid), 45% DCM (dichloromethane) and 10% TIPS (triisopropylsilane), which was added to the resin for 3 hours. The resulting solution was evaporated under reduced pressure and the crude peptide was triturated with cold diethyl ether. Peptides were then characterised via HPLC (high-performance liquid chromatography) and MALDI-TOF (Matrix-assisted laser desorption/ionization-Time of Flight).

2.2.12 Analysis of Gene Expression Omnibus and The Cancer Genome Atlas databases

Analysis of datasets from the Gene Expression Omnibus (GEO) was carried out using the GEO2R web server (www.ncbi.nlm.nih.gov/geo/geo2r/)⁴¹⁸ for the following publicly available datasets; GSE15605, GSE44076, GSE15471, GSE70947, GSE14520, as detailed in **Table 2.7**. Datasets included were selected according to the number of samples included and were excluded if < 12 samples were available. All studies selected included patient-matched tissue, asides from the GSE15605 melanoma dataset, which, due to the lack of patient-matched data available in published melanoma databases, did not include patient-matched data, however contained the greatest sample numbers ($N \geq 12$) for each sample type, compared to other GEO melanoma datasets. Data from The Cancer Genome Atlas (TCGA) studies, matched with normal TCGA and GTEx (Genotype-Tissue Expression) data, was analysed using the GEPIA web server

(www.gepia.cancer-pku.cn/index.html)⁴¹⁹. Statistical analysis was carried out using GEPIA via analysis of variance (ANOVA).

Table 2.7 Study design and sample types of GEO datasets.

Dataset / Cancer type	Study design	Sample types	Ref.
GSE15605/ Melanoma	Microarray analysis of gene expression in normal skin, primary melanoma and metastatic melanoma tissue samples of melanoma patients, collected from the University of Michigan Multidisciplinary Melanoma Clinic, USA. Participant age ranged from 20-92 years (median age 59.4).	Normal skin (N=16), primary melanoma (N=46), metastatic tissue (lymph nodes, subcutaneous soft tissue, spleen and small intestine, N=12)	⁴²⁰
GSE44076/ colon adenocarcinoma	Microarray analysis of colorectal tumours and paired, adjacent non-cancerous colon tissue, collected from the Bellvitge University Hospital, Spain. Participant age ranged from 28-88 years (median age 70).	Patient-matched samples of non-tumour tissue (N=97) and colon adenocarcinoma tissue (N=97).	⁴²¹
GSE15471/ pancreatic ductal adenocarcinoma	Microarray gene expression analysis in patient-matched non-cancerous pancreatic tissue and pancreatic ductal adenocarcinoma tumours, collected at the Fundeni Clinical Institute, Romania. Participant age ranged between 42-85 years (average 67.1, median not stated).	Patient-matched non-cancerous pancreatic tissue (N=38) and adjacent pancreatic tumour tissue (N=38).	⁴²²
GSE70947/ breast adenocarcinoma	Microarray analysis in patient-matched non-cancerous breast tissue and adjacent breast adenocarcinoma tumours, collected at the Oslo University Hospital, Norway. Participant age data not publicly available.	Patient-matched non-cancerous breast tissue (N=142) and adjacent breast adenocarcinoma tissue (N=142).	⁴²³
GSE14520/ hepatocellular carcinoma	Gene expression data (collected via microarray) of hepatocellular carcinoma patients, collected at the Liver Cancer Institute and Zhongshan Hospital, China. Participant age data not publicly available.	Patient-matched non-cancerous liver tissue (N=212) and adjacent hepatocellular carcinoma tumour tissue (N=212).	⁴²⁴

2.2.13 Analysis of RNA sequencing data of patient-derived and patient matched colorectal organoid and tumouroids

Previously generated RNAseq data of patient matched organoids and tumouroids derived from 6 colorectal cancer patients (N=6) was provided and analysed by Dr Mark Williams' laboratory (University of East Anglia, UK). Isolated RNA from the samples was sent to the Earlham Institute (Norwich, UK) for sequencing using Illumina RNAseq (175bp PE HiSeq 4000; 100 million reads/sample) technology. The Williams laboratory, in collaboration with Prof. Dan Brewer's lab, processed the resultant FastQ files using the nf-core/RNA-seq workflow (v1.4.2)⁴²⁵. A differential gene expression analysis on the raw counts was performed using the DESeq2 package (v1.36.0)⁴²⁶ by the Williams laboratory.

2.2.14 Alphafold protein interaction prediction model analysis

The Nrf2/MafG leucine zipper interaction was modelled using ColabFold⁴²⁷, an Alphafold⁴²⁸-based user interface which predicts protein interactions. For this model, the following sequences of the Nrf2 and MafG leucine zipper were queried: MafG; KEELEKQKAELQQEVEKLAENASMKLELDALRSKYEALQ and Nrf2; IVELEQDLHLKDEREKLLKEKGENDRNHLLKRRRLSTLYL. The predicted interaction was then modelled in PyMOL (Schrödinger, Inc., New York).

2.2.14 Statistical analysis and data presentation

All experiments, unless otherwise stated, were carried out to N=3 and results are expressed as the mean $\pm$ SEM. GraphPad Prism 9 (GraphPad Software Inc, California, USA) or RStudio (Posit, Massachusetts, USA) was used to perform statistical analyses, and to calculate IC₅₀ values for the cell viability assays. To test whether data followed a normal distribution, a Shapiro-Wilk test was applied. For normally distributed data, analysis was conducted via one-way or two-way ANOVA and post-hoc Tukey multiple comparison test or Dunnett's test. For data that were not normally distributed, a Kruskal-Wallis and post-hoc Dunn test was applied. For all tests, P values of ≤ 0.05 were considered statistically significant. For **Figure 6.2**, differential gene expression analysis on microarray data was conducted using Limma⁴²⁹ and the Benjamini & Hochberg adjustment via the GEO2R webserver⁴¹⁸. For **Figure 6.3**, a log-rank test was carried out using the GEPIA webserver⁴¹⁹. All graphical figures (**Figures 1.1-1.13** and **Figure 7.1**) were produced using Biorender.

Chapter 3. Evaluating the effects of Nrf2 inhibitors in cancer cells

Acknowledgments:

Gabrielle Adekola and Caroline Antonios collected the data for the MCF-7 cells in **Figure 3.2**. Sean Tattan assisted with data visualisation for **Figure 3.2**. Dr Griselda Awanis performed the caspase-3 labelling experiments for **Figure 3.14**.

3.1 Introduction

The transcription factor Nrf2 has been widely researched for its role in coordinating and regulating the oxidative stress response following exposure of cells to a variety of stressors, including ROS, UV radiation and infection. In the presence of stress, Nrf2 exerts its action through regulating the expression of hundreds of genes involved in cytoprotection, xenobiotic detoxification and metabolism¹¹⁹. As oxidative stress is correlated with the pathogenesis of a variety of chronic conditions⁴³⁰, Nrf2 has therefore emerged as a promising therapeutic target in several of these diseases, including inflammatory disease and cancer⁴³¹. Several activators of the Nrf2 pathway have been identified in the literature, only one compound: dimethyl fumarate (DMF) is currently approved for clinical use in the UK for multiple sclerosis and psoriasis²⁴³.

While activators of Nrf2 continue to be explored in the context of inflammatory disease, therapeutic targeting of Nrf2 in cancer appears to be more complex. Although Nrf2 activators have been shown to be useful in protecting cells against tumorigenesis, via the prevention of DNA damage and ROS accumulation, Nrf2 activation in cancer may not always prove beneficial⁴³². Certainly, Nrf2 does prevent carcinogenesis, with several chemopreventive compounds, such as sulforaphane and oltipraz, known to act via Nrf2-dependent pathways⁴³³. However, increasing evidence suggests that Nrf2 may possess a 'dark side' in cancer, with overactivation of Nrf2 signalling providing a protective effect for tumours²⁷². Aberrant activation of Nrf2, associated with loss of function mutations in Nrf2's regulator, KEAP1, and gain of function mutations in NFE2L2, have been identified in a variety of patient tumours including breast, bladder, melanoma, head and neck, pancreatic, and lung cancers^{269,270,434,435}.

Furthermore, dysregulation of the Nrf2 pathway in cancer, caused by Nrf2 overexpression, promotes several of the hallmarks of cancer, including uncontrolled proliferation, angiogenesis, metastasis, and resistance to cell death mechanisms²⁵⁸. Overexpression of Nrf2 in patient tumours also correlates with poor prognosis and increased resistance to standard of care chemotherapy²⁷², likely due to Nrf2's role in detoxification and its regulation of several of the common drug efflux pumps implicated in chemoresistance, such as MRP1 (multidrug resistance protein 1)⁴³⁶.

While Nrf2 activators are therefore unsuitable for use in cancer, there is potential therapeutic benefit in inhibiting Nrf2 signalling in 'Nrf2 addicted' tumours. Experimental evidence has shown that RNA interference of Nrf2 can decrease cancer cell proliferation and increase sensitivity of numerous cancer cells to chemotherapeutic agents, including

in pancreatic, cervical, and thyroid cancer cells^{274,288,437}. Since the discovery of the 'dark side' of Nrf2 in cancer, numerous inhibitors of the Nrf2 pathway have now been reported²⁵⁶, including both small molecule and natural products that target Nrf2 via repressing its transcription, inducing its degradation (via the 26S proteasome) and by preventing its nuclear accumulation²⁸⁵ (as shown in **Figure 1.13**). Although the majority of these inhibitors have shown some anti-cancer activity in the literature (**Table 1.5**), their efficacy has not been compared, with some inhibitors only being reported in single studies³⁴³. Here, several Nrf2 inhibitors that were reported in the literature (**Table 3.1**) have been compared for their effects on cancer cell proliferation and the Nrf2 signalling pathway in a variety of cancer cell lines in both 2D and 3D cell culture models.

Table 3.1. Mechanism of action of the Nrf2 inhibitors explored in the current study.

Inhibitor name	Class of molecule	Mechanism of action	Ref.
Brusatol	Triterpene lactone derived from <i>Brucea javanica</i>	Inhibits Nrf2 protein synthesis	331,334-336,339,340,384,438-440
Luteolin	Flavonoid derived from chamomile, celery and green peppers	Induces Nrf2 mRNA turnover	349-352,379
ML385	Small molecule	Inhibits Nrf2-MafG interaction	358,359
All-trans Retinoic acid	Vitamin A derivative	Complexes with RXR α , to inhibit Nrf2 at Neh7	321-324
Triptolide	Diterpenoid epoxide derived from <i>Tripterygium wilfordii</i>	Inhibits Nrf2 mRNA synthesis and induces GSK3 β /Fyn-mediated Nrf2 nuclear export	371-373,441
Trigonelline hydrochloride	Alkaloid derived from fenugreek, hemp and coffee	Prevents Nrf2 nuclear translocation	365,366,442,443
Clobetasol propionate	Small molecule corticosteroid	Induces β -TrCP-dependent degradation of Nrf2	343

3.2 Aims

1. Compare Nrf2-expression status in a panel of human lung, breast, melanoma, liver, leukaemia, and pancreatic cancer cell lines.
2. Study the anti-proliferative effects of seven reported Nrf2 inhibitors in a panel of high, moderate, and low- Nrf2 expressing cell lines.
3. Evaluate the ability of the reported inhibitors to suppress the Nrf2 pathway and sensitise cells to chemotherapy in a high-Nrf2 expressing cancer cell line.
4. Utilise 3D cancer cell culture techniques to test the efficacy of the Nrf2 inhibitors on tumouroid growth and apoptosis.

3.3 Results

3.3.1 Protein expression profile of Nrf2 in cancer cell lines

The expression of Nrf2 in healthy cells is a tightly regulated process, with Nrf2 possessing a very short half-life of approximately 13-30 mins¹³⁹. Such rapid turnover ensures that Nrf2 maintains homeostasis by providing a quick, yet transient response to oxidative stress. However, in some cancers, the Nrf2 pathway can become hijacked, leading to Nrf2 overexpression and dysregulated induction of genes that contribute to cytoprotection and chemoresistance⁴³². To explore the expression of Nrf2 in a panel of cancer cell lines, basal Nrf2 protein expression was compared in a panel of melanoma, pancreatic, lung, breast, liver and monocytic leukaemia cells via western blot analysis. As shown in **Figure 3.1**, cell lines with the highest Nrf2 protein expression were the A549 non-small cell lung cancer, MDA-MB-231 breast cancer, and HEPG2 liver cancer cell lines. Conversely, the lowest expressing cell lines were the A375 melanoma, H292 lung, and BXPC3 pancreatic cancer cell lines.

Interestingly, Nrf2 was detected as a doublet in the majority of cell lines studied in **Figure 3.1**, with bands appearing at around ~100 kDa. While the exact reason for the doublet was not characterised in the present study, several previous studies have reported the appearance of an Nrf2 doublet via SDS-PAGE^{444,446,447}. One explanation for this could be a lack of specificity of the EP1808Y antibody utilised in our study, however several studies with the EP1808Y antibody have demonstrated that the intensity of both bands is diminished following Nrf2 knockdown, suggesting that both bands are forms of Nrf2^{444,445}. Furthermore, we have also observed that in cell lines where only a singlet is typically seen under basal conditions (such as THP-1), stimulation with the Nrf2 activator DMF induces a second (upper) band via SDS-PAGE (data not shown), potentially suggesting that the second band may be a modified form of Nrf2 with a higher molecular weight, for example a phosphorylated form. This is supported by Mikac *et al.* who observed that treatment of A549 cells with a lambda protein phosphatase (to remove phosphate groups), resulted in a decrease in the observed molecular weight of the upper, but not lower, band of the doublet⁴⁴⁵.

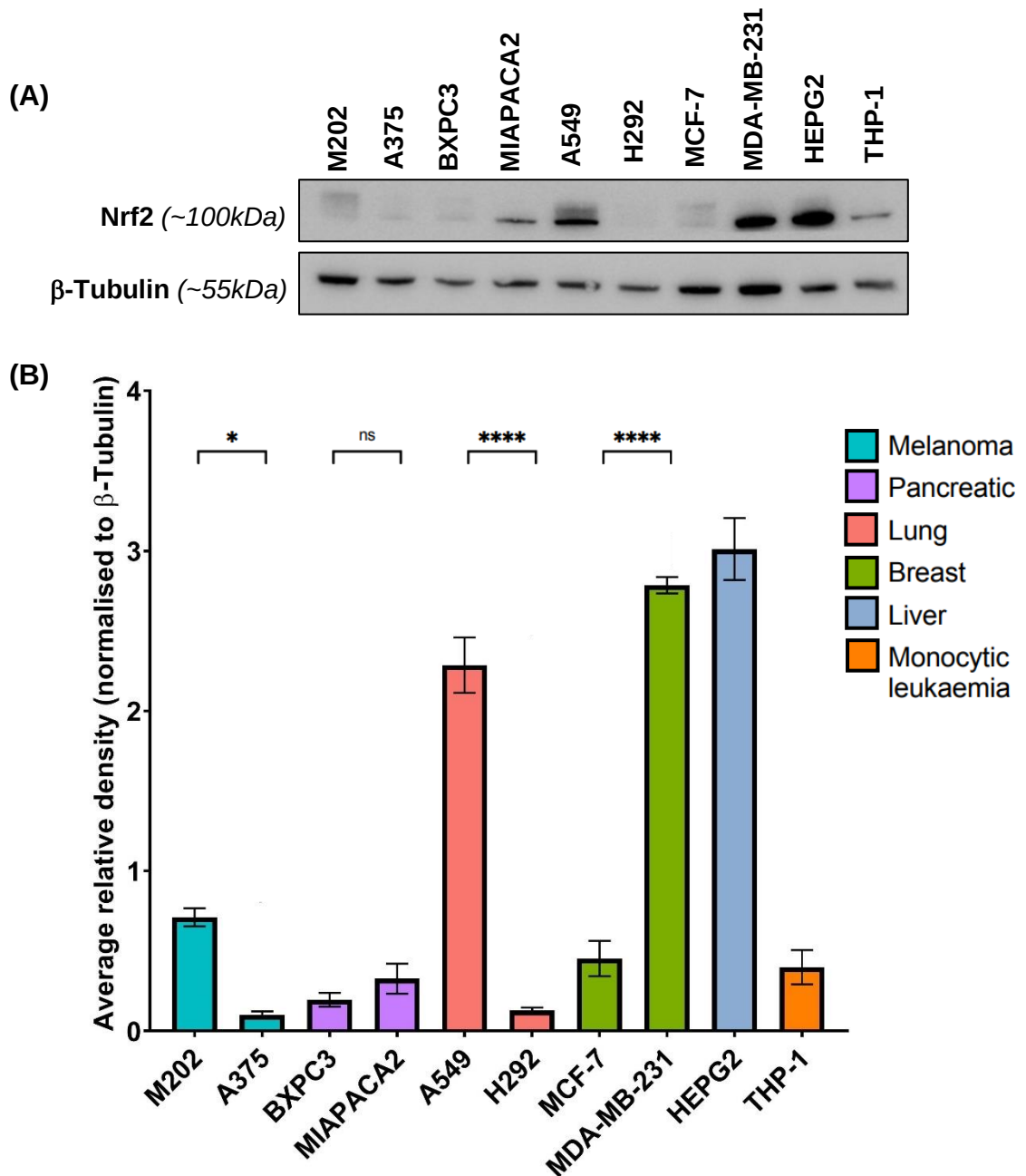


Figure 3.1. Nrf2 protein expression in lung, pancreatic, monocytic leukaemia, and melanoma cell lines. Nrf2 protein expression was explored via western blot using whole cell lysates prepared from 1×10^6 cells from melanoma (M202, A375), pancreatic (MIAPACA2, BXPC3), lung (A549, H292), breast (MCF-7, MDA-MB-231), liver (HEPG2) and monocytic leukaemia (THP-1) cancer cell lines. (A) Representative western blot images of Nrf2 expression in cancer cell lines, with β -Tubulin included as a loading control. (B) Densitometry analysis of western blot images presented as average relative density, normalised to β -Tubulin control. Data are presented as mean $\pm$ SEM (N=3) and statistical analysis was conducted via one-way ANOVA and post-hoc Tukey test $P^* \leq 0.05$, $P^{****} \leq 0.0001$, ns= not significant.

Furthermore, our results showed that cancer cell lines of the same organ origin displayed different Nrf2 protein expression profiles; for example, the MDA-MB-231 breast cancer cells displayed significantly higher expression of Nrf2, compared to the MCF-7 breast cancer cells (**Figure 3.1B**). A similar effect was observed in the lung cancer cell lines, with the H292 cells expressing significantly lower levels of Nrf2 protein than the A549

cells (**Figure 3.1B**). Among the melanoma cell lines, the M202 cells displayed much higher levels of Nrf2 compared to the A375 cells (**Figure 3.1B**). Subtler differences were observed in the pancreatic cancer cell lines, with the MIAPACA2 cells having slightly higher levels of Nrf2 compared to the BXPC3 cells, although such differences were not statistically significant (**Figure 3.1B**).

3.3.2 Comparison of anti-proliferative effects of reported Nrf2 inhibitors in cancer cell lines

Nrf2 has been shown to contribute to many of the hallmarks of cancer including ‘uncontrolled proliferative signalling’, in part due to its ability to crosstalk with proliferation-promoting signalling pathways such as the Notch1 and PI3K/Akt pathways²⁵⁸. Nrf2 inhibitors may therefore be useful in preventing cell proliferation, to ultimately reduce tumour growth and progression. Hence, a selection of reported Nrf2 inhibitors (**Table 3.1**) from the literature were selected and their effects on cell proliferation in lung, pancreatic, melanoma and breast cancer cells were explored via MTS assay (**Figure 3.2**).

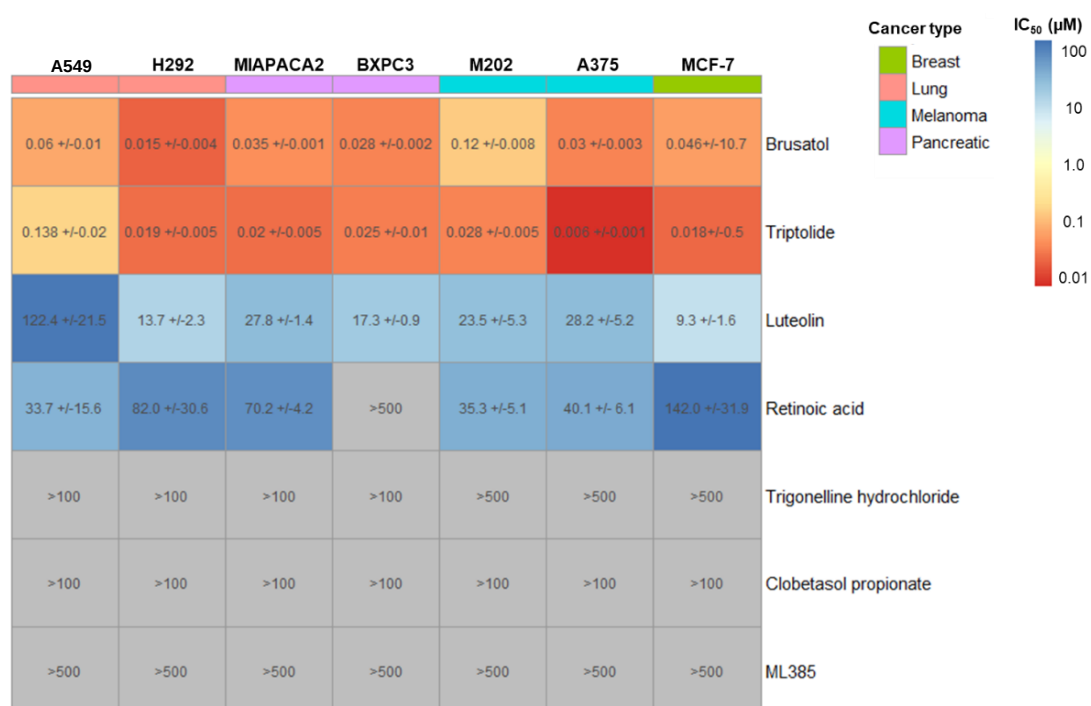


Figure 3.2 Heat map of the anti-proliferative effects of Nrf2 inhibitors in a panel of lung, pancreatic, melanoma and breast cancer cell lines. Cell proliferation of A549, H292, MIAPACA2, BXPC3, M202, A375 and MCF-7 cells was determined via MTS assay, following incubation of each cell line with serial dilutions of Nrf2 inhibitors for 72 h. IC₅₀ values were calculated using Graphpad Prism 9, and are presented in μM as mean ±SEM (N=3). The heat map colour scale was generated using Log (IC₅₀) values.

Among the Nrf2 inhibitors studied, brusatol and triptolide had the greatest inhibitory effects on cell proliferation across all cell lines tested, with IC₅₀ (half maximal inhibitory concentration) values ranging from 15 nM-120 nM and 19 nM-138 nM, respectively (**Figure 3.2**). Luteolin also showed some inhibitory activity on cell proliferation, possessing IC₅₀ values of <41 µM in most cell lines. Interestingly however, A549 cells were much more resistant to luteolin (IC₅₀ of 122.37 µM) and to triptolide (IC₅₀ of 138 nM), than other cell lines tested (**Figure 3.2**). Another inhibitor, retinoic acid, demonstrated moderate anti-proliferative activity in the majority of cell lines tested, with IC₅₀ values of 33.73 µM- 142 µM. However, no activity was observed in the BXPC3 pancreatic cancer cells (**Figure 3.2**). On the other hand, ML385, trigonelline hydrochloride and clobetasol propionate showed the poorest anti-proliferative effects, with IC₅₀ values of >100 µM in lung and pancreatic cancer cells, and >500 µM in melanoma and breast cells (**Figure 3.2**).

3.3.3 Nrf2 inhibitors differentially affect Nrf2 protein expression in high Nrf2-expressing A549 cells

To further study the use of Nrf2 inhibitors in cancer, the effect of Nrf2 inhibitors on Nrf2 protein expression was studied. For these experiments, A549 non-small cell lung cancer (NSCLC) cells were selected due to their high expression of Nrf2, as displayed in **Figure 3.1**. Following treatment of the A549 cells with 20 µM of the inhibitors for 4 hours, Nrf2 protein expression was then determined via western blot analysis and subsequent densitometry analysis of protein bands (**Figure 3.3**).

Figure 3.3A shows that in A549 cells, the greatest inhibitory effects on Nrf2 expression were observed with brusatol and triptolide, which significantly reduced Nrf2 protein expression to approximately 22% and 27% of DMSO vehicle control, respectively (**Figure 3.3B**). Luteolin and clobetasol also markedly reduced Nrf2 protein expression to 68% and 61% of DMSO control, respectively (**Figure 3.3B**), while retinoic acid and trigonelline displayed very little inhibitory effects. Interestingly, ML385 treatment induced a slight increase in Nrf2 protein expression (**Figure 3.3B**).

Furthermore, while the Nrf2 activator DMF was initially included as a control to induce Nrf2 protein expression, we observed that treatment with 30 µM DMF unexpectedly reduced Nrf2 protein expression in A549 cells to that of 68% of DMSO control (**Figure 3.3B**), suggesting that it may also have a role in suppressing the Nrf2 pathway. As this was unexpected, this result was further investigated using a range of concentrations of

DMF (10, 20 and 30 μ M) and several treatment time points (**Figure 8.2**). While higher DMF concentrations (e.g., 20 and 30 μ M) at a treatment time of 4 h could repress Nrf2 protein expression, 10 μ M treatment at 4 h and 20 h could induce Nrf2 protein expression, as discussed in **Chapter 8.2.2**. Hence a concentration of 10 μ M was utilised to induce Nrf2 signalling in later experiments.

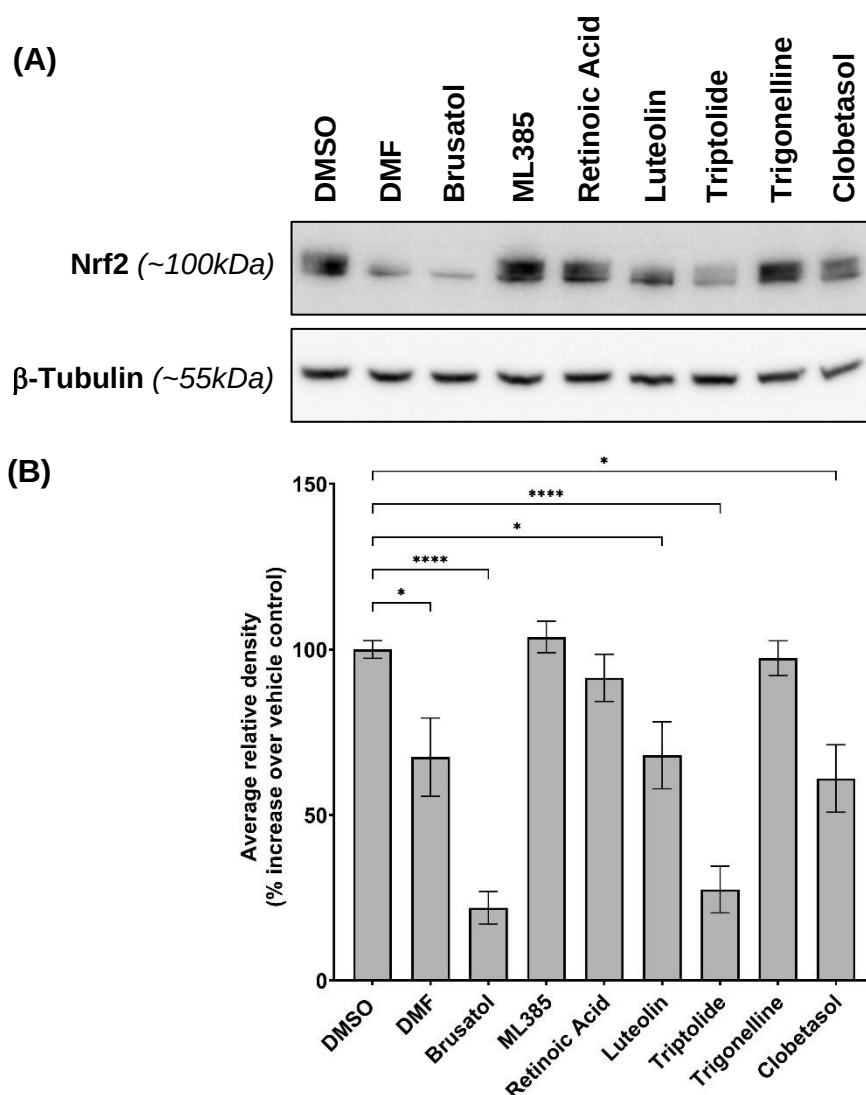


Figure 3.3 Nrf2 inhibitors differentially inhibit Nrf2 protein expression in A549 cells. 1×10^6 A549 cells were treated with 20 μ M Nrf2 inhibitors or 30 μ M DMF for 4 h and Nrf2 expression was measured via western blot analysis. **(A)** Representative western blot images of Nrf2 protein expression with β -Tubulin included as a loading control. **(B)** Densitometry of western blot images presented as average relative density (% increase over DMSO vehicle control). Data are presented as mean $\pm$ SEM (N=3) and statistical analysis was conducted via one-way ANOVA and post-hoc Dunnett's test ($P^* \leq 0.05$, $P^{**} \leq 0.01$, $P^{***} \leq 0.001$, $P^{****} \leq 0.0001$).

As both brusatol and triptolide treatment induced a significant reduction in Nrf2 protein expression in high Nrf2-expressing A549 cells, we next investigated whether the effects of brusatol and triptolide treatment on Nrf2 protein expression were concentration-

dependent. A549 cells were hence treated with a range of concentrations (0-20 μM) of brusatol or triptolide, which were selected in line with their activity in repressing Nrf2 signalling in other reports^{334,335,370,371}, for 4 hours and protein expression was measured via western blot (**Figure 3.4**).

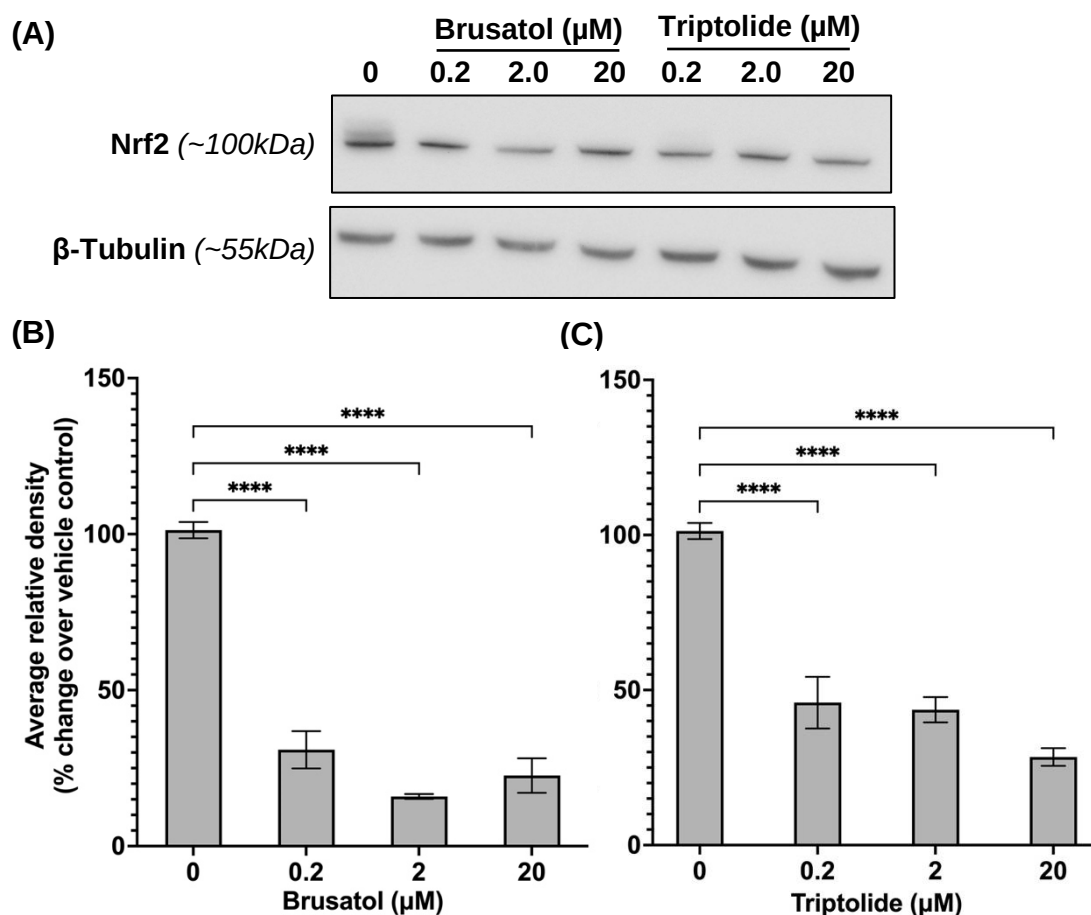


Figure 3.4 Brusatol and triptolide potentially inhibit Nrf2 expression in A549 cancer cells in a concentration-dependent manner. 1×10^6 A549 cells were treated with 0.2-20 μM brusatol or triptolide for 4 h and Nrf2 expression was measured via western blot analysis. **(A)** Representative western blot images of Nrf2 expression, with β -Tubulin included as a loading control (N=3). Densitometry of western blot images was analysed, normalised to β -Tubulin and presented as average relative density compared to DMSO vehicle control $\pm$ SEM for **(B)** brusatol and **(C)** triptolide. Statistical analysis was conducted via one-way ANOVA and post-hoc Dunnett's test ($P \leq 0.05$, $P^{***} \leq 0.001$, $P^{****} \leq 0.0001$).

As shown in **Figure 3.4A**, we observed that brusatol's inhibitory effects do not appear to follow a typical concentration response, as the greatest inhibition was observed at 2 μM (16% of DMSO control), rather than at the highest concentration of 20 μM (22% of DMSO control). However, the lowest concentration of 0.2 μM , did still induce a significant reduction in Nrf2 protein expression to that of 31% of DMSO control. On the other hand, **Figure 3.4C** shows that Nrf2 protein expression decreased as triptolide concentration increased, suggesting that triptolide's effect on Nrf2 protein expression is concentration dependent. Triptolide induced its greatest inhibitory effects at 20 μM treatment (28% of DMSO control).

To explore the effect of brusatol and triptolide treatment time on Nrf2 protein expression, A549 cells were treated with 2 μ M brusatol or triptolide for either 2, 4, 6 or 8 hours prior to protein extraction. As shown in **Figure 3.5**, brusatol's effects were most effective at suppressing Nrf2 expression at 2 hours. These effects were maintained between 2-4 hours; however, protein expression began to increase significantly between 2-8 hour treatment (**Figure 3.5B**). On the other hand, while triptolide did reduce Nrf2 protein expression at 2 hours, the greatest effects were seen with 6-8 hours of treatment (**Figure 3.5C**). Notably, in **Figure 3.5C**, Nrf2 protein expression appears to decrease between 2–6-hour triptolide treatment, however, begins to plateau after 6 hours. These results therefore suggest that both brusatol and triptolide can rapidly suppress Nrf2 protein expression at low concentrations and can sustain this suppression for several hours, despite the very quick turnover of Nrf2.

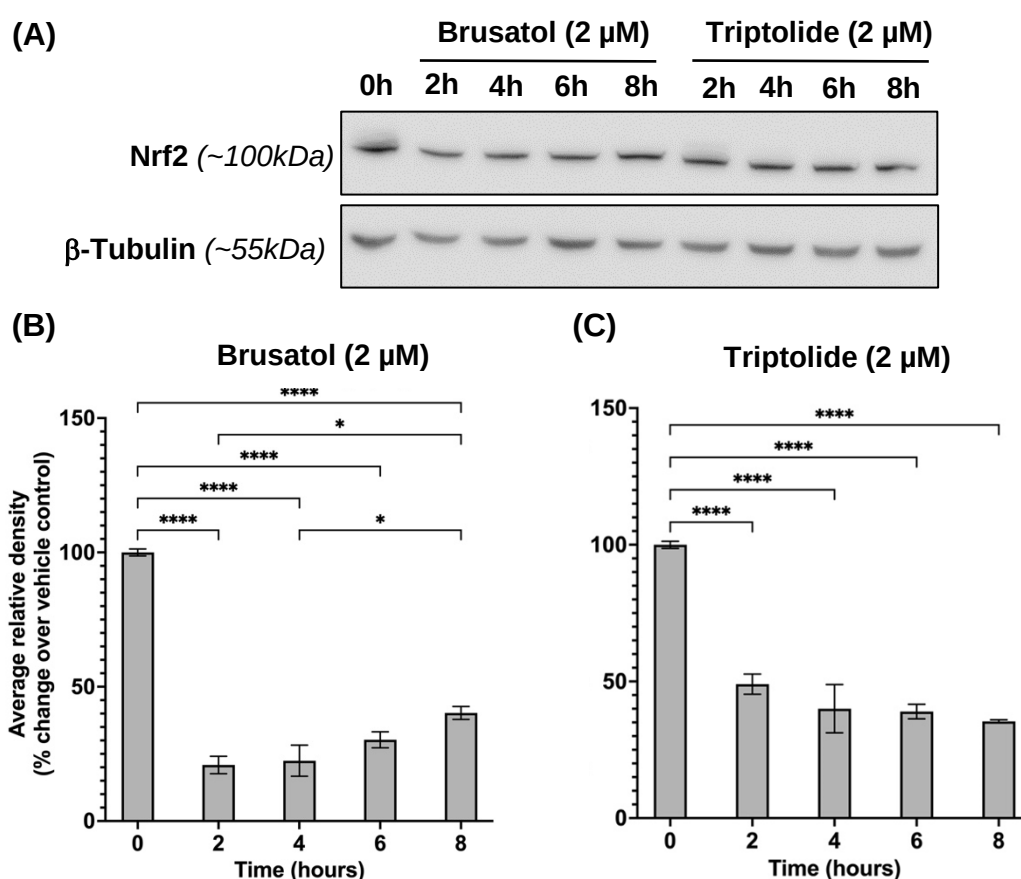


Figure 3.5 Brusatol and triptolide inhibit Nrf2 expression in A549 cells in a time-dependent manner. (A) Representative western blot images of Nrf2 protein expression in 1×10^6 A549 cells treated with 2 μ M brusatol or triptolide for 2-8 h. Images are representative of three individual experiments (N=3), with β -Tubulin included as a loading control. Densitometry of western blot images was analysed, normalised to β -Tubulin and presented as average relative density compared to DMSO vehicle control for (B) brusatol and (C) triptolide. Statistical analysis was conducted via one-way ANOVA and post-hoc Tukey test ($P^* \leq 0.05$, $P^{***} \leq 0.001$, $P^{****} \leq 0.0001$).

3.3.4. Effect of Nrf2 Inhibitors on Nrf2 DNA Binding activity in A549 cells

To elicit changes in gene transcription, Nrf2 binds to ARE sequences within the promoter region of its target genes. One method of assessing the DNA-binding activity of Nrf2 is to use an ELISA-based assay, utilising the Nrf2 consensus binding sequence (5' – GTCACAGTGA CT CAGCAGAATCTG – 3') to detect nuclear Nrf2 protein bound to DNA. Hence, to assess the effect of the inhibitors on Nrf2 DNA-binding activity, nuclear protein extracts of A549 cells treated with Nrf2 inhibitors for 4 hours were prepared and Nrf2 DNA-binding activity was quantified via an Nrf2 DNA-binding ELISA.

As shown in **Figure 3.6A**, inclusion of a recombinant Nrf2 protein as a positive control (+) significantly increased Nrf2-DNA binding activity to 650.0% DMSO control. Meanwhile, the Nrf2 inhibitors had varying effects on Nrf2 DNA-binding activity, with brusatol having the greatest inhibitory effect, significantly reducing activity to 22.0% of DMSO control (**Figure 3.6B**).

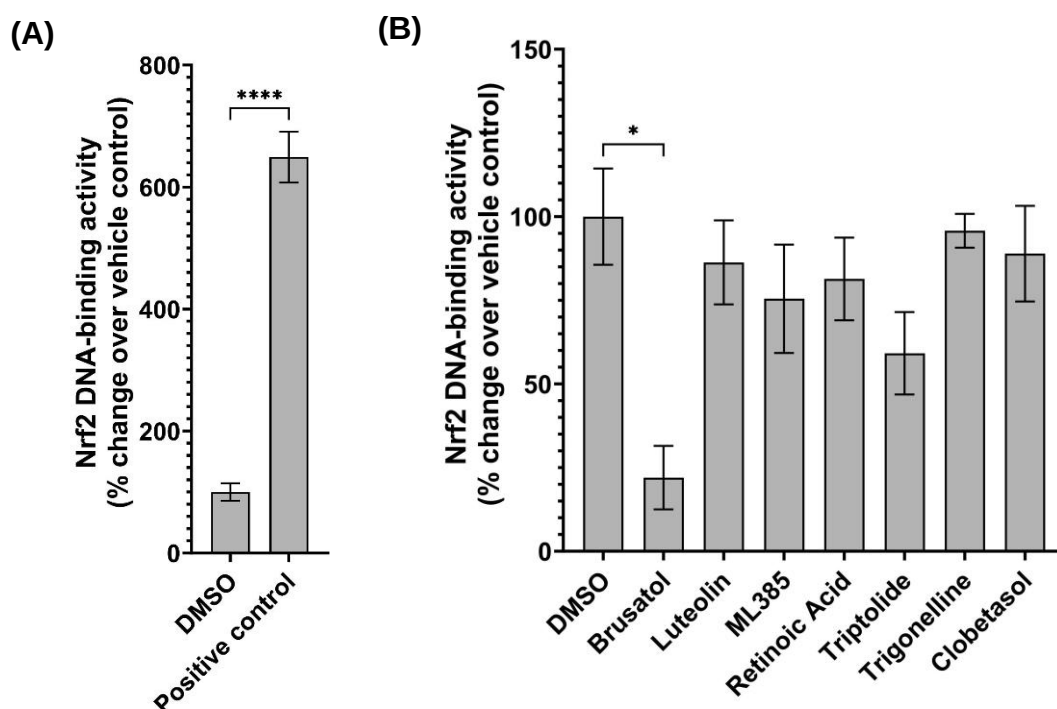


Figure 3.6 The effect of Nrf2 inhibitors on Nrf2 DNA-binding activity in A549 cells. 3×10^5 A549 cells were treated with DMSO or 20 μ M Nrf2 inhibitors for 4 h prior to preparation of nuclear extracts. Nrf2 DNA-binding activity was then determined via an Nrf2 DNA-binding ELISA assay with samples prepared from (A) 5 μ g Nrf2 positive control ((+); recombinant Nrf2 protein)) or (B) 20 μ g nuclear extracts of A549 cells treated with Nrf2 inhibitors or DMSO. Results are presented as mean $\pm$ SEM (N=3) and statistical analysis was conducted via one-way ANOVA and post-hoc Dunnett's test (* $P \leq 0.05$, $P^{****} \leq 0.0001$).

While not significant, triptolide reduced Nrf2 DNA-binding activity to 59.2% of DMSO control (**Figure 3.6B**). Other inhibitors such as ML385 and retinoic acid demonstrated a moderate reduction in Nrf2 DNA-binding activity, to that of 75.4% and 81.4% of DMSO control (**Figure 3.6B**). The poorest activity was seen with trigonelline treatment, which only demonstrated a slight reduction in Nrf2 DNA-binding activity, which was not significant (95.7% of DMSO control, **Figure 3.6B**).

3.3.5. Effect of Nrf2 Inhibitors on Nrf2 downstream gene NQO1 in A549 cells

NQO1 is a detoxification enzyme and downstream target gene of Nrf2 that has been shown to be correlated with advanced metastatic states and poorer overall survival rates in many cancer types, including in NSCLC⁴⁴⁸. As NQO1 is a downstream target gene of Nrf2, knockdown or inhibition of Nrf2 can decrease NQO1 activity. Hence, the ability of the Nrf2 inhibitors to suppress NQO1 activity was investigated using an enzymatic assay previously described by Prochaska and Santamaria⁴¹⁶, following A549 cell treatment with 10 or 20 μ M DMF or Nrf2 inhibitors for 20h (**Figure 3.7**).

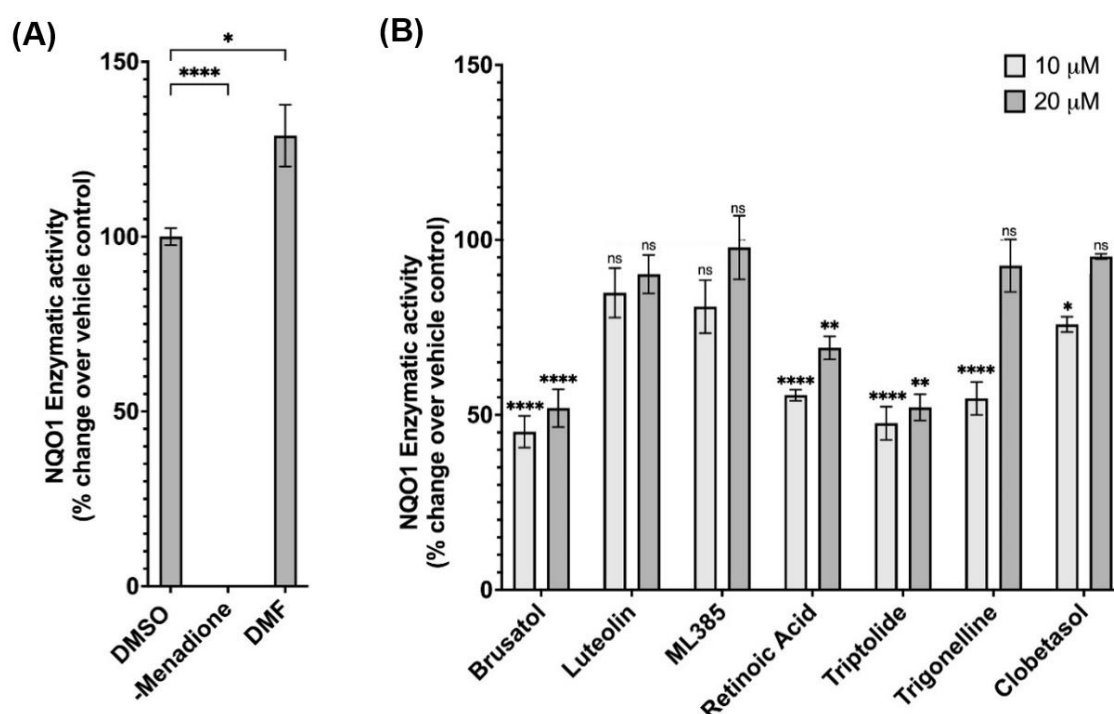


Figure 3.7 Nrf2 inhibitors differentially suppress NQO1 enzymatic activity in A549 cells. 8×10^3 A549 cells were treated with (A) DMF (10 μ M) or (B) Nrf2 inhibitors (10 or 20 μ M) for 20 h and NQO1 activity was measured by enzymatic assay. Reaction buffer without substrate (-Menadione) was included as a blank control. Results are presented as mean $\pm$ SEM (N=3). Statistical analysis was conducted via one way-ANOVA (A) or two-way ANOVA (B) and post-hoc Dunnett' test compared to DMSO ($P^* \leq 0.05$, $P^{**} \leq 0.01$, $P^{***} \leq 0.001$, $P^{****} \leq 0.0001$, ns= not significant).

As shown in **Figure 3.7A**, treatment of the cells with 10 μM DMF resulted in a significant increase in NQO1 enzymatic activity to 144% of DMSO control, while cells incubated with reaction buffer without menadione (-Menadione) did not display any enzymatic activity. A lower concentration of DMF was included in this experiment, compared to that used in **Figure 3.3**, as lower concentrations of DMF (such as 10 μM) are shown to induce Nrf2 activity in A549 cells in the literature⁴⁴⁹ and induced Nrf2 protein expression at 20 h in our own optimisation experiments (**Figure 8.2**).

Figure 3.7B shows that treatment of A549 cells with Nrf2 inhibitors at two concentrations, 10 μM and 20 μM resulted in varying effects on NQO1 activity. Among the inhibitors studied, brusatol and triptolide (10 μM) consistently possessed the greatest inhibitory effects, significantly decreasing NQO1 activity to 45.2% and 47.6% of DMSO control, respectively (**Figure 3.7B**). Similarly, trigonelline hydrochloride and retinoic acid (10 μM) significantly reduced NQO1 enzymatic activity to 54.8% and 55.7% of DMSO control, respectively. While retinoic acid maintained its inhibitory activity at 20 μM , the activity of trigonelline hydrochloride was lost at 20 μM (**Figure 3.7B**).

On the other hand, luteolin, ML385 and clobetasol displayed very little effects on NQO1 enzymatic activity, however all three inhibitors showed their greatest activity at 10 μM , reducing NQO1 enzymatic activity to 82.9%, 80.9% and 75.9% of DMSO control, respectively (**Figure 3.7B**). As brusatol and triptolide were some of the most efficacious inhibitors at reducing NQO1 enzymatic activity in **Figure 3.7**, these inhibitors were further studied for any concentration dependent effects (**Figure 3.8**).

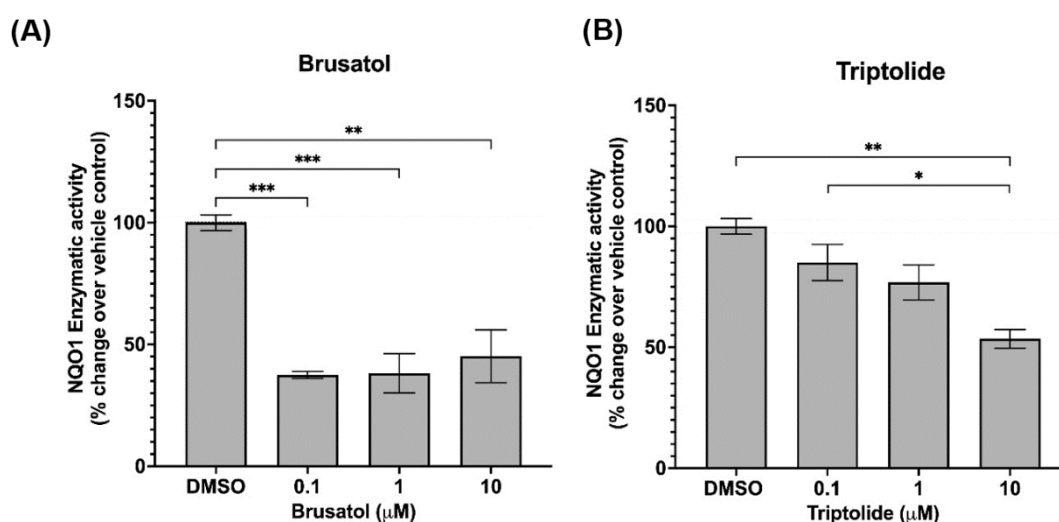


Figure 3.8 Triptolide inhibits NQO1 enzymatic activity in a concentration-dependent manner. A549 cells (8×10^3 cells) were treated with 0.1-10 μM **(A)** brusatol or **(B)** triptolide for 20 h and NQO1 activity was measured by enzymatic assay. Reaction buffer without substrate (-Menadione) was included as a blank control. Results are presented as mean $\pm$ SEM (N=3) and statistical analysis was conducted via one way-ANOVA and post-hoc Tukey test ($P^* \leq 0.05$, $P^{**} \leq 0.01$, $P^{***} \leq 0.001$).

When concentration-response experiments were conducted, triptolide displayed a typical concentration-dependent inhibitory effect on NQO1 enzymatic activity, with the greatest effects being displayed at a concentration of 10 μ M (**Figure 3.8B**). Interestingly, brusatol did not show a typical concentration response, with 10 μ M displaying the weakest effects to repress NQO1 activity, and very little difference observed between concentrations of 0.1 μ M and 1 μ M (**Figure 3.8A**).

3.3.6 ML385, trigonelline and clobetasol do not sensitise A549 cells to gemcitabine hydrochloride

Three of the inhibitors (ML385, trigonelline and clobetasol) were reported to sensitise cancer cells to the anti-proliferative effects of chemotherapeutic agents yet possessed IC_{50} values >100 μ M in cell proliferation assays (**Figure 3.2**). As it has been suggested in the literature that these inhibitors may be useful as adjuvant therapies to standard of care chemotherapy^{343,359,365,442,443}, we next studied whether these inhibitors could sensitise A549 cells to the anti-proliferative effects of gemcitabine hydrochloride, a chemotherapeutic agent commonly used in the treatment of NSCLC cancer. Brusatol was also included as it has previously been shown to sensitise NSCLC cells to chemotherapeutic agents³³⁵. A549 cells were co-incubated with the inhibitors and gemcitabine for 72 h and cell proliferation was measured by MTS assay (**Figure 3.9**).

As shown in **Figure 3.9**, at a non-cytotoxic concentration (0.01 μ M, as determined from IC_{50} values via MTS assay), brusatol did appear to slightly increase the anti-proliferative effects of gemcitabine, however these effects were very minimal and were not statistically significant. Non-cytotoxic concentrations of ML385, trigonelline and clobetasol, as determined by MTS assay, also appeared to slightly potentiate the anti-proliferative effects of gemcitabine (0.01 μ M), compared to gemcitabine alone (**Figure 3.9B-D**), although such effects were not significant.

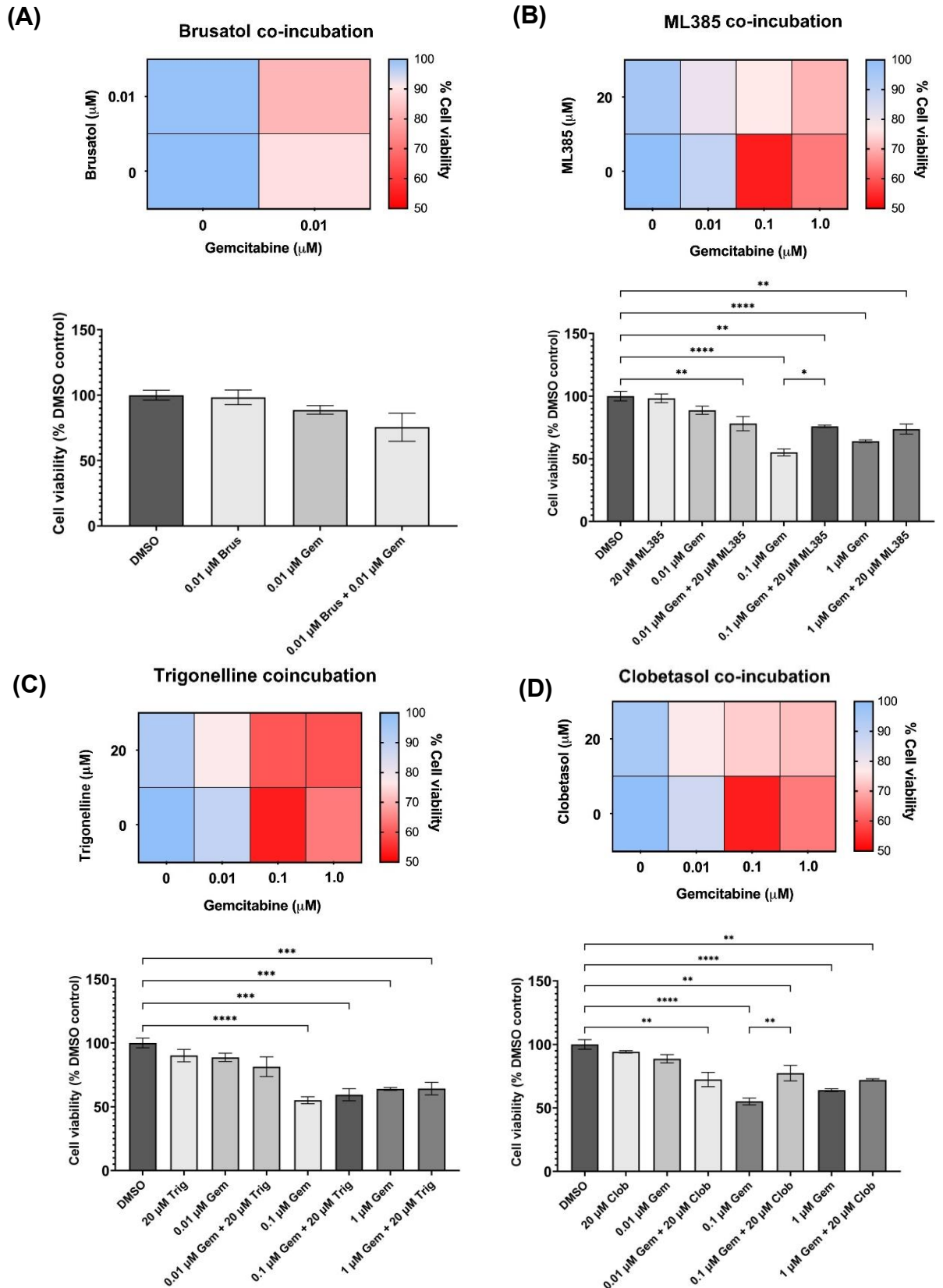


Figure 3.9 Brusatol, trigonelline, clobetasol and ML385 do not sensitise A549 cells to gemcitabine. 5×10^3 A549 cells were treated with either 0.01 μM or 20 μM of (A) brusatol (Brus) (B) ML385 (ML) (C) trigonelline (Trig) or (D) clobetasol (Clob) in combination with 0.01 μM, 0.1 μM or 1 μM gemcitabine hydrochloride (Gem) for 72 h. Cell viability was measured via MTS assay. Results are presented as mean % cell viability $\pm$ SEM (N=3) as both heat maps (top) and bar plots (bottom). Statistical analysis was conducted via one way-ANOVA and post-hoc Tukey test ($P \leq 0.05$, $P^{**} \leq 0.01$, $P^{***} \leq 0.001$, $P^{****} \leq 0.0001$).

3.3.7 Triptolide inhibits formation and growth of A549 3D tumouroids

A major drawback of experiments conducted using 2D cell culture is that it does not accurately mimic physiological conditions as cancer cells grow in a 3D environment supported by extracellular matrix components. As such, 3D cell culture techniques are a valuable tool in drug screening, in lieu of *in vivo* models. One method of 3D cell culture is to seed cells in a basement membrane, such as Matrigel Matrix, in which the cells form 3D tumouroid structures over several days of culture, as displayed in the DMSO control images in **Figure 3.10**.

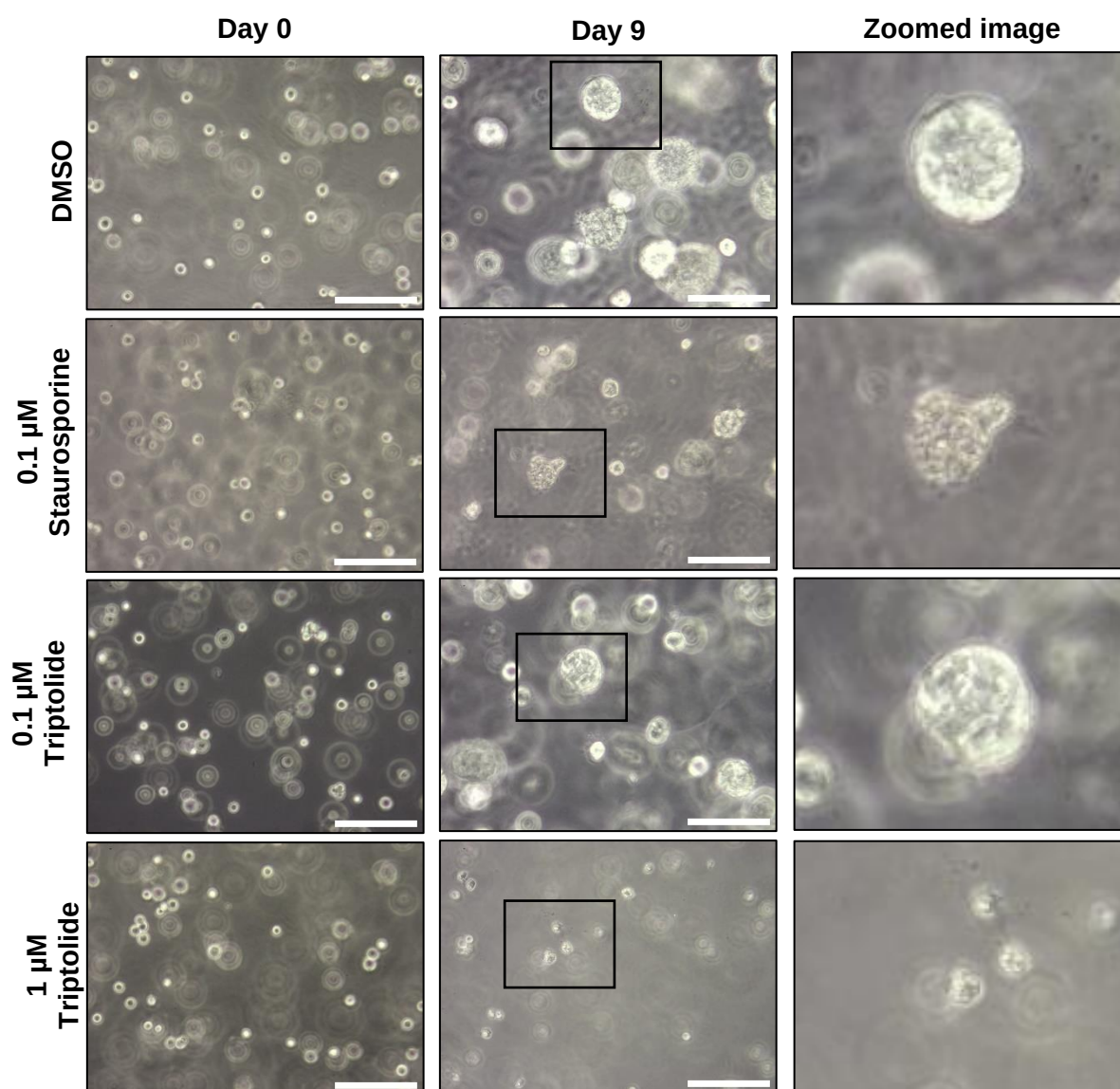


Figure 3.10 Triptolide suppresses A549 3D tumouroid formation. Representative white light microscopy images of A549 3D tumouroids treated with 0.1-1 μM triptolide or 0.1 μM staurosporine on days 0 (left) and day 9 (middle) of culture. Regions of interest displaying tumouroids from day 9 images are zoomed in and presented on the right-hand side in black boxes. 1×10^4 A549 cells were suspended in Matrigel matrix and were treated on day 0 of culture with triptolide or staurosporine. Cells were cultured for 9 days, with white light microscopy images taken daily. Images are representative of three individual experiments (N=3). Scale bar 100 μm .

Among the Nrf2 inhibitors tested here, both brusatol and triptolide consistently showed potent effects at inhibiting both cell proliferation (**Figure 3.2**) and Nrf2 signalling in A549 cells (**Figures 3.3-3.8**). However, brusatol treatment has been shown to have several off-target effects³⁸⁴ (as discussed further in **Chapter 3.4**), hence triptolide was instead selected for further experiments. To explore the effects of triptolide in a 3D cell culture environment, A549 tumouroids were generated (see **Chapter 8.2.3** for experiment optimisation details) and cells were treated with 0.1- 1 μM triptolide on day 0 of growth (**Figure 3.10**). Tumouroid area was then charted over 10 days of culture via analysis of white light microscopy images (**Figure 3.11**).

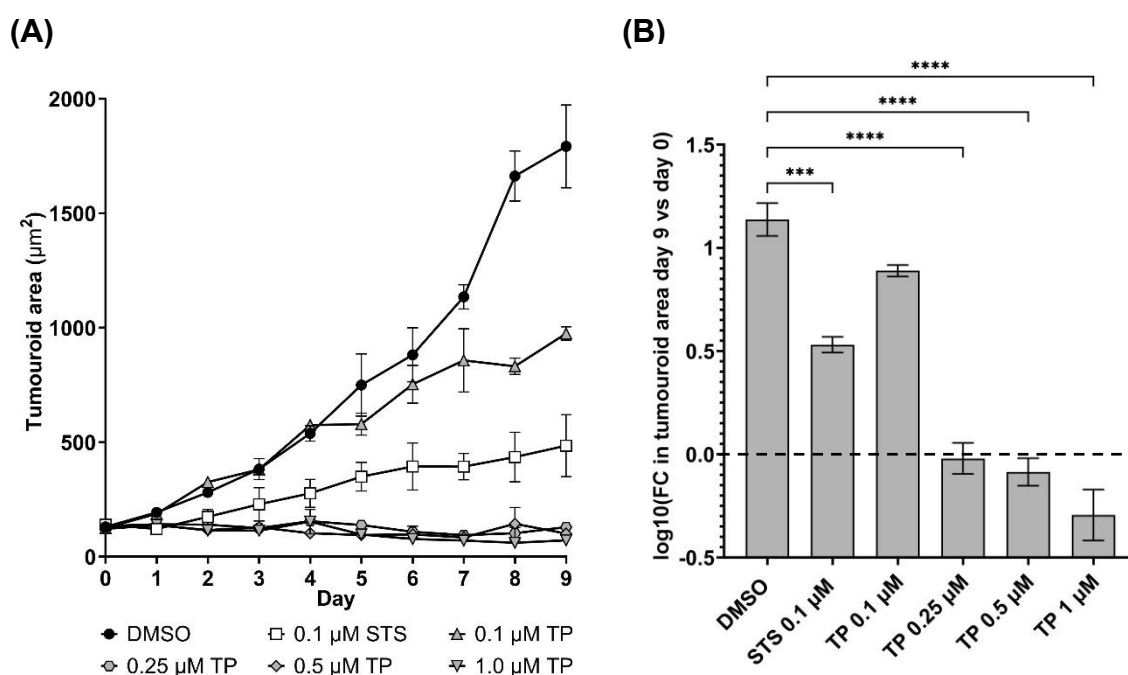


Figure 3.11 Triptolide suppresses A549 3D tumouroid formation in a concentration dependent manner. (A) Average area of A549 tumouroids stimulated with 0.1-1 μM triptolide (TP) or 0.1 μM staurosporine (STS) on day 0 of culture. Cells were incubated for 9 days and tumouroid area was quantified via analysis of white light microscopy images. Results are presented as mean area (μm^2) $\pm$ SEM (N=3). **(B)** Fold change (FC) in tumouroid area on day 9 vs day 0. Results are presented as $\log_{10}(\text{FC}) \pm$ SEM with a $\log_{10}(\text{FC}) < 0$ indicating a decrease in tumouroid area compared to day 0. Statistical analysis in **(B)** was conducted via one-way ANOVA and post-hoc Dunnett's test ($P^{**} \leq 0.01$, $P^{***} \leq 0.001$, $P^{****} \leq 0.0001$).

As indicated by the black boxes in **Figure 3.10**, when cultured in Matrigel Matrix, A549 cells treated with DMSO vehicle control form large tumouroid structures by day 9 of growth. Here, staurosporine, a non-specific kinase inhibitor that has potent inhibitory effects on 2D A549 cell growth⁴⁵⁰, was included as a positive control. For these experiments, a concentration of 0.1 μM staurosporine was selected according to concentration response experiments on A549 tumouroid growth (see **Chapter 8.2.3**). As shown in **Figure 3.10** and **Figure 3.11B**, treatment with 0.1 μM staurosporine on day 0

of culture impaired tumouroid formation and significantly reduced tumouroid growth over 9 days compared to that of the vehicle (DMSO) control. Similarly, treatment with 0.1- 1 μ M triptolide significantly inhibited the growth of the tumouroids in a concentration-dependent manner (**Figure 3.11A and B**).

As we found that triptolide could suppress tumouroid formation, next we explored the effect of triptolide on the growth of already-established A549 tumouroids. To achieve this, cells were cultured in Matrigel for 5 days to allow sufficient growth, prior to treatment with triptolide. Once again, white light microscopy images were taken daily (**Figure 3.12**), which were subsequently analysed to calculate tumouroid area (**Figure 3.13**). For these experiments, staurosporine was again included as a positive control. As determined by initial optimisation experiments (data not shown), it was found that a ten-fold increase in concentration of staurosporine was required to repress tumouroid growth (1 μ M), compared to that used in **Figure 3.10** (0.1 μ M), likely due to the larger tumouroid area at time of initial treatment. A higher concentration of both staurosporine and triptolide was therefore selected for these experiments. Hence, tumouroids were treated daily with triptolide (0.1- 10 μ M) or 1 μ M staurosporine from days 5-9 of culture, and growth was measured via analysis of white light microscopy images (**Figure 3.12**).

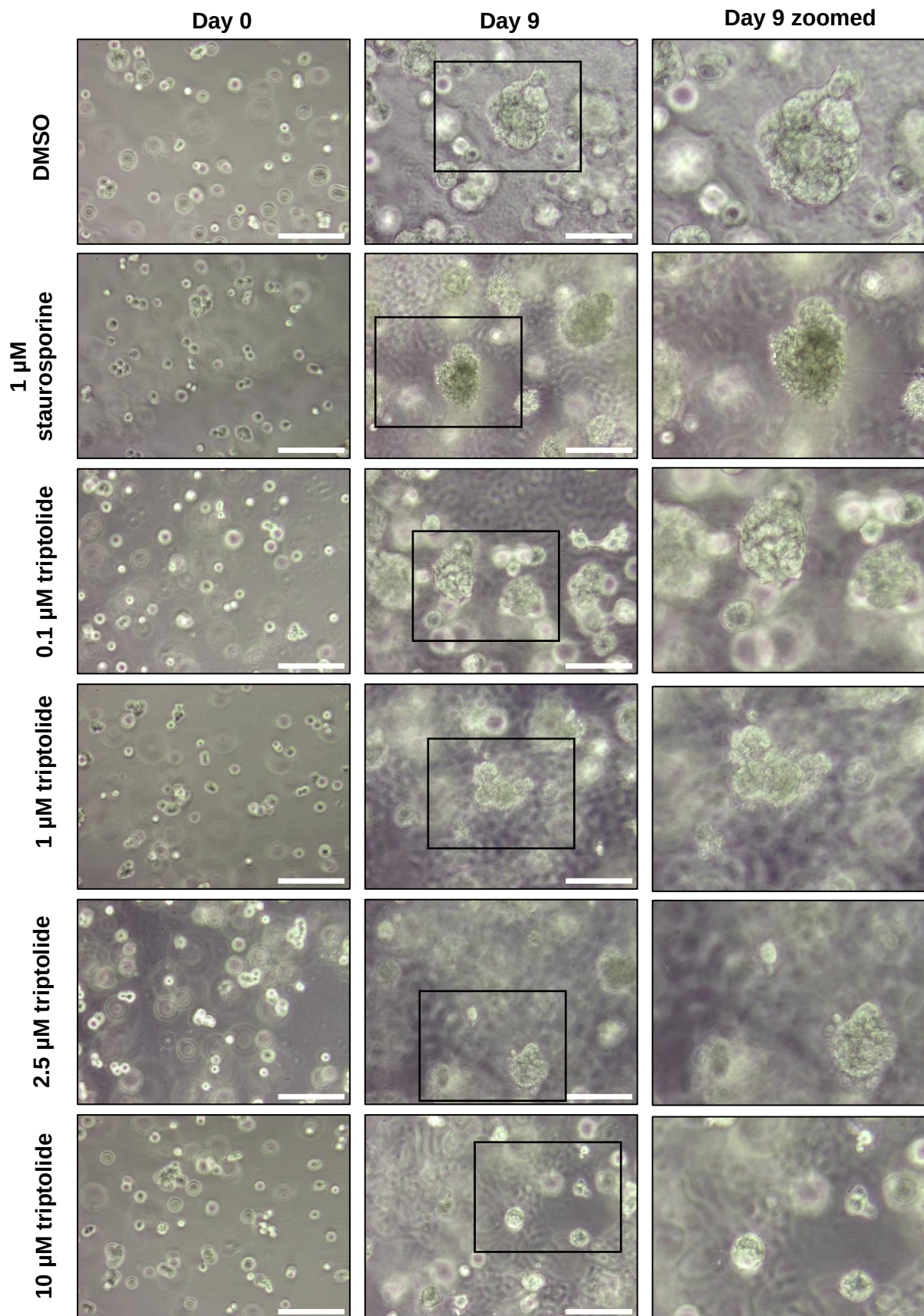


Figure 3.12 Triptolide suppresses A549 3D tumouroid growth. Representative white light microscopy images of A549 tumouroids stimulated with 0.1-10 μ M triptolide or 1 μ M staurosporine on days 0 (left) and 9 (middle) days of culture. Regions of interest displaying tumouroids from the day 9 images are zoomed in on the right-hand side to show tumouroid morphology. Cells were seeded in Matrigel Matrix and were cultured for 5 days prior to daily stimulation. Images are representative of 3 individual experiments (N=3). Scale bar 100 μ m.

Treatment with 1 μM staurosporine significantly reduced tumouroid growth compared to vehicle control (**Figure 3.13B**), and tumouroids also underwent morphological changes including induction of membrane-blebbing (**Figure 3.12**). Furthermore, treatment with 1-10 μM triptolide significantly reduced tumouroid area compared to vehicle control in a concentration-dependent manner (**Figure 3.13B**). Moreover, the tumouroids treated with triptolide also began to show signs of membrane blebbing and formation of apoptotic bodies (**Figure 3.12**).

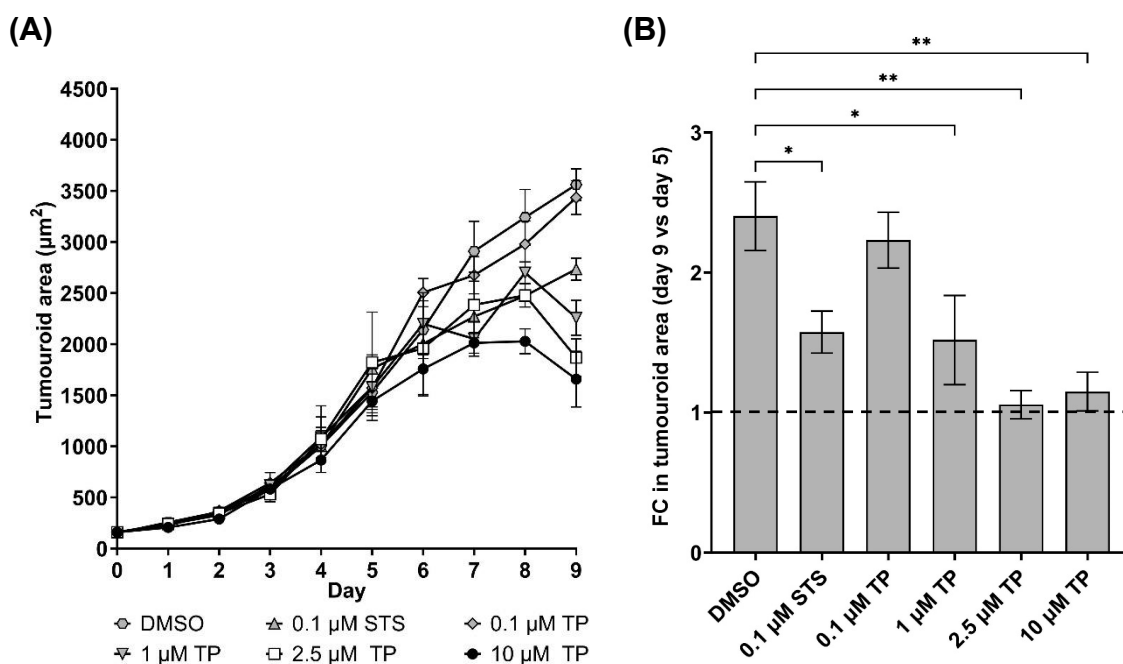


Figure 3.13. Triptolide inhibits A549 3D tumouroid growth. (A) Average area of A549 tumouroids over 9 days when treated daily with 0.1-10 μM triptolide (TP) or 1 μM staurosporine (STS) on days 5-9 of culture. Tumouroid area was measured via analysis of white light microscopy images. Results are presented as mean tumouroid area (μm^2) $\pm$ SEM (N=3). **(B)** Average fold change (FC) in tumouroid area on day 9 vs day 5. FC <1 indicates a decrease in tumouroid area compared to day 5. Statistical analysis in **(B)** was conducted via one-way ANOVA and post-hoc Dunnett's test ($P^* \leq 0.05$, $P^{**} \leq 0.01$).

Additionally, compared to the tumouroids treated on day 0 (**Figure 3.10**), those treated on day 5 (**Figure 3.12**) required higher concentrations of triptolide to elicit a significant decrease in tumouroid area. For example, a 10-fold increase in concentration was required to see a 50% reduction in tumouroid area compared to vehicle control, in the day 5-stimulated cells than in the day 0-stimulated cells. This result was expected however, as the day 5-stimulated tumouroids were able to form much larger structures prior to treatment with triptolide.

3.3.8 Triptolide induces apoptosis in A549 3D tumouroids

Apoptosis is a form of programmed cell death that occurs through a signalling cascade involving the activation of caspase-3 and caspase-7. As we consistently observed morphological changes that are typically associated with apoptosis in the triptolide-treated tumouroids (e.g., membrane blebbing), we next investigated whether triptolide was inducing apoptosis using live-cell fluorescent labelling of the tumouroids with a fluorogenic substrate for activated caspase-3/7 (**Figure 3.14**), following triptolide treatment (as described in **Figure 3.12**).

As shown in **Figure 3.14**, treatment of tumouroids with 1-10 μ M triptolide treatment induced activation of caspase-3/7, while no activated caspase-3/7 was observed in those treated with DMSO control. Furthermore, consistent with our results in **Figure 3.12**, tumouroids displaying higher amounts of activated caspase-3/7 also appeared much smaller and were undergoing membrane blebbing (**Figure 3.14**). As shown in the black boxes in **Figure 3.12**, many of the tumouroids in the highest treatment conditions also appeared to be in the very late stages of apoptosis, as indicated by the formation of apoptotic bodies and cell fragmentation. Together, these results suggest that triptolide does induce cell death via apoptosis in 3D A549 tumouroids.

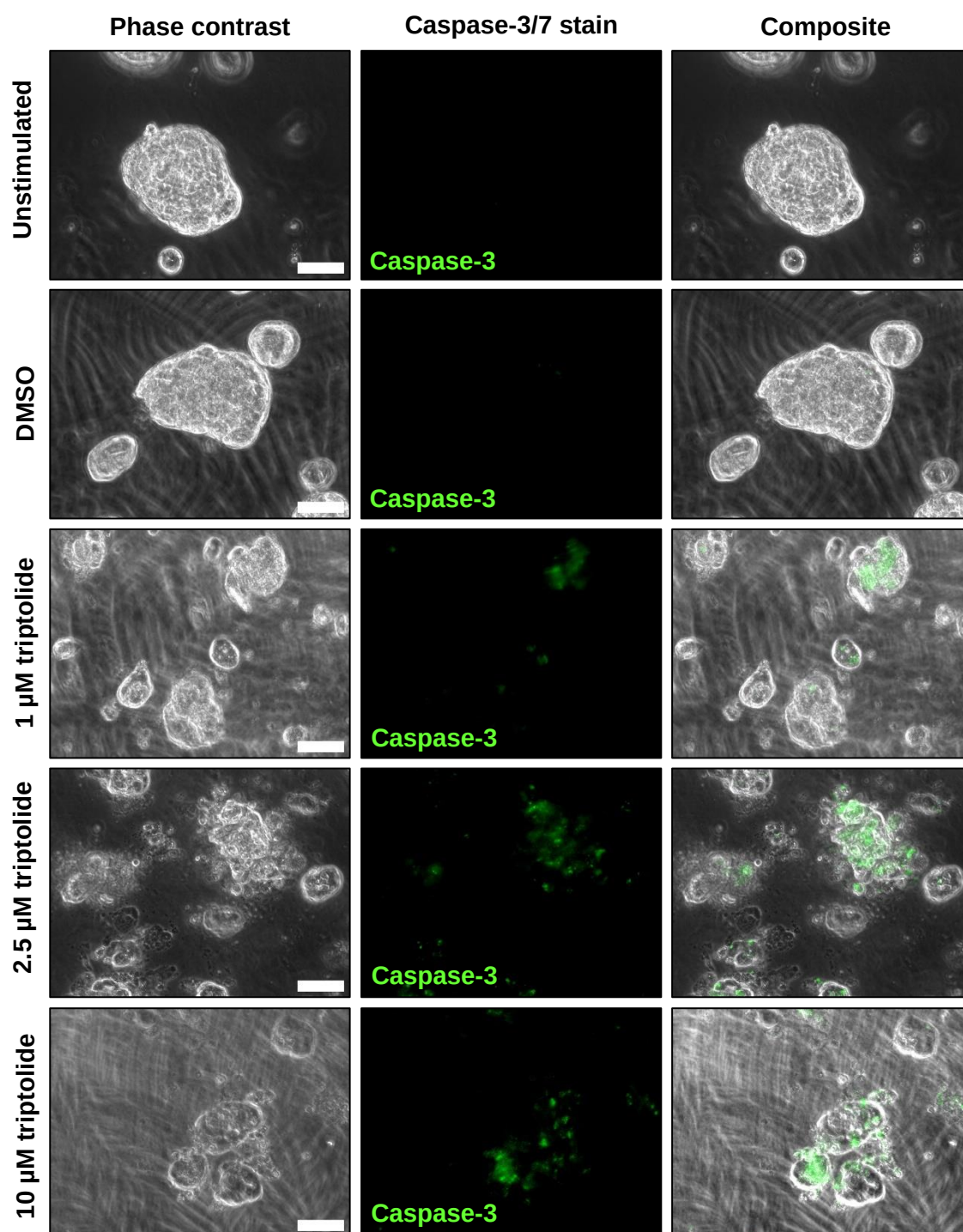


Figure 3.14. Triptolide induces apoptosis in A549 3D tumouroids. Representative images of tumouroids labelled with a fluorogenic substrate for activated caspase-3/7 following treatment with 1-10 μ M triptolide. Tumouroids were cultured for 5 days prior to treatment. On day 9, CellEvent™ Caspase-3/7 Detection Reagent was incubated with the tumouroids for 30 min and caspase-3/7 activation was visualised via epi-fluorescent microscopy (N=3). Scale bar 100 μ m.

3.3.9 Effect of triptolide and gemcitabine hydrochloride co-incubation in both 2D and 3D cell culture of A549 cells

As Nrf2 has been shown to promote chemoresistance in many cancer types, Nrf2 inhibitors may sensitise tumours to already existing chemotherapeutic agents. Hence, we next investigated whether triptolide could sensitise A549 cells to gemcitabine hydrochloride, a chemotherapeutic drug used in the treatment of NSCLC. Firstly, we investigated the effects of triptolide co-incubation with gemcitabine hydrochloride in 2D cell culture, via MTS assay (**Figure 3.15**).

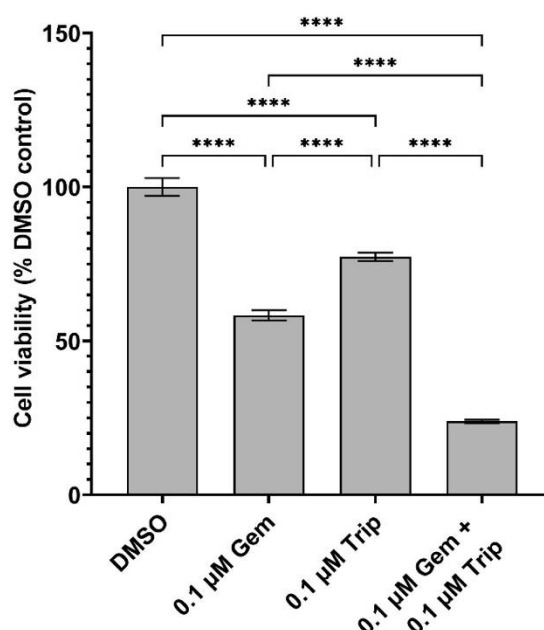


Figure 3.15. Triptolide sensitises A549 cells to gemcitabine in 2D cell culture. A549 cells (5×10^3 cells/well) were treated with 0.1 µM triptolide or 0.1 µM gemcitabine, alone or in combination, for 72h and cell viability was measured via MTS assay. Data is presented as mean $\pm$ SEM and statistical analysis was performed via one-way ANOVA with post-hoc Tukey test (N=4, ****P $\leq$ 0.0001).

For these experiments, a concentration of 0.1 µM of gemcitabine was selected based upon previous experiments from MTS assays (data not shown). As shown in **Figure 3.15**, 0.1 µM gemcitabine reduced cell viability by 46% compared to DMSO control. When combined with triptolide at a sub-IC₅₀ concentration (**Figure 3.2**), cell viability was reduced by 77%, compared to DMSO control, suggesting that triptolide sensitises A549 cells to the anti-proliferative effects of gemcitabine (**Figure 3.15**).

To identify whether similar effects are observed in 3D cell culture, A549 tumouroids were generated and treated with triptolide and gemcitabine, alone or in combination, from days 5-9 of culture (**Figure 3.16**). For these experiments, several concentrations of triptolide and gemcitabine were first tested, to determine the best concentration range to use

(outlined in **Chapter 8.2.3**). As before, tumouroid area was measured for each day of growth (**Figure 3.17**).

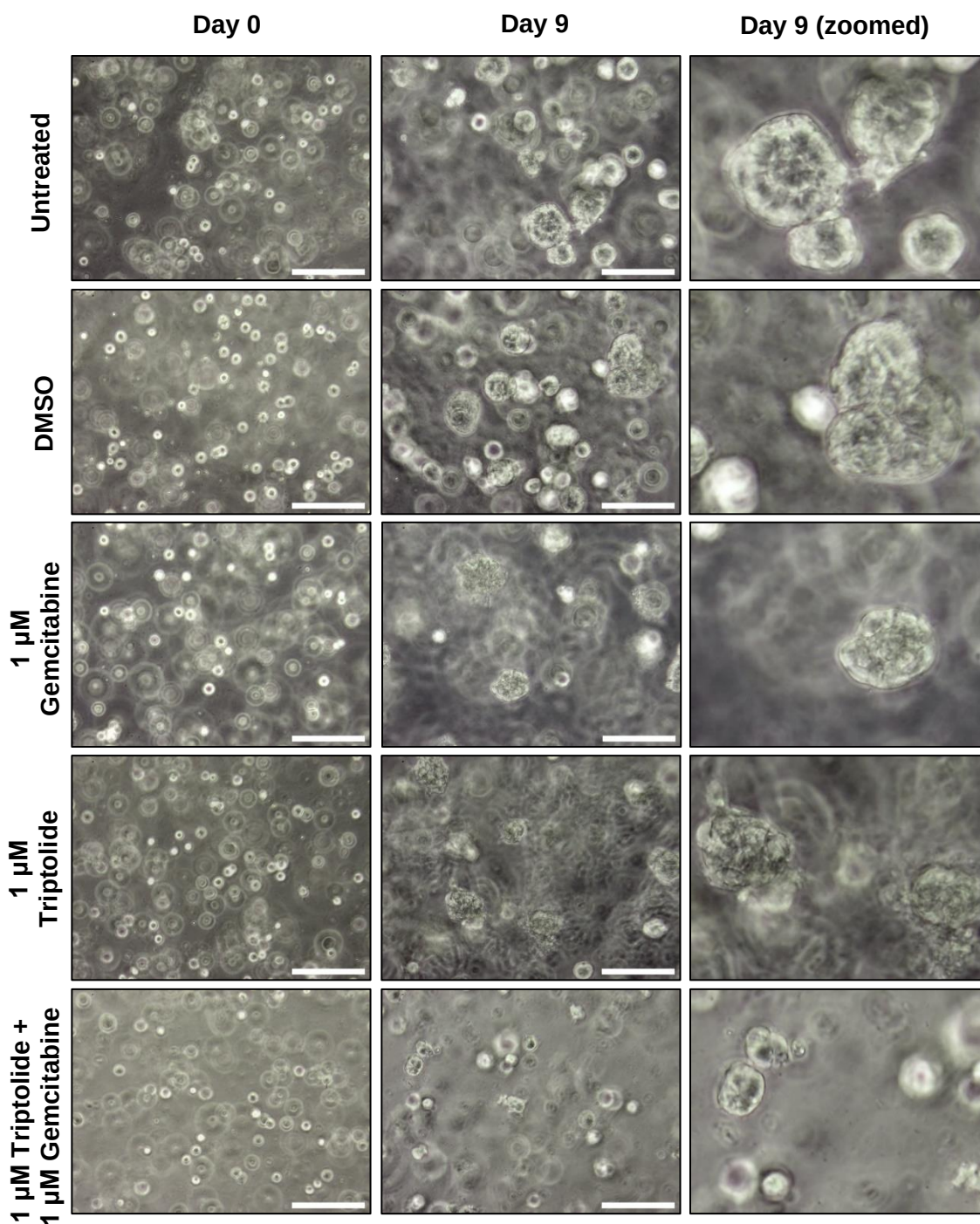


Figure 3.16. Representative images of A549 tumouroids treated with triptolide and gemcitabine alone and in combination. Representative white light microscopy images of A549 tumouroids treated with 1 μ M triptolide and 1 μ M gemcitabine, alone or in combination on days 0 (left) and 9 (middle) of culture. Regions of interest are displayed on the right-hand side showing zoomed sections of tumouroids on day 9 of culture. Cells were seeded in Matrigel Matrix and were cultured for 5 days prior to treatment. Images were taken daily and are representative of 3 individual experiments (N=3). Scale bar 100 μ m.

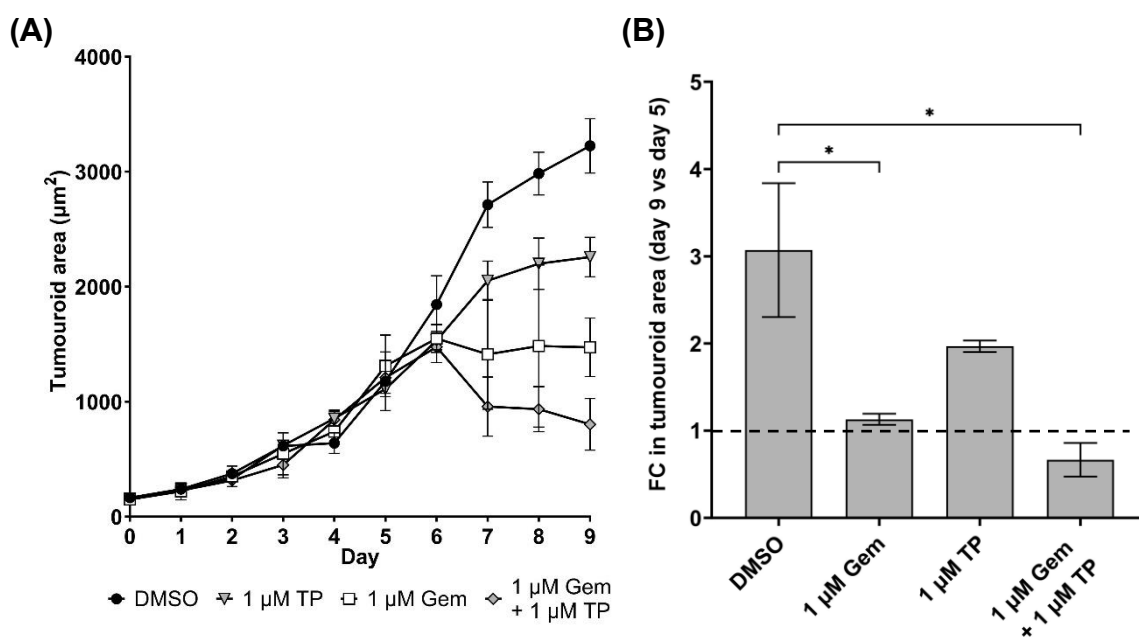


Figure 3.17. Co-incubation of triptolide with gemcitabine reduces A549 tumouroid growth. **(A)** Average area over 9 days of tumouroids treated with 1 μM triptolide (TP) and 1 μM gemcitabine (Gem), alone or in combination from days 5-9 of growth. White light microscopy images were taken daily, and growth was measured via analysis of white light microscopy images. Data is presented as mean area $\pm$ SEM (N=3). **(B)** Fold change (FC) in area of tumouroids on day 9 vs day 5. FC <1 indicates a reduction in tumouroid area compared to day 5. Statistical analysis in **(B)** was carried out via one-way ANOVA and post-hoc Tukey test (* $P \leq 0.05$).

Co-incubation of 1 μM triptolide and 1 μM gemcitabine resulted in a significant reduction in tumouroid area compared to vehicle control, however, no significant difference was observed compared to gemcitabine alone (**Figure 3.17B**). Despite this, tumouroids co-incubated with both triptolide and gemcitabine were the only condition to achieve a fold change in area of <1 over the treatment period of 4 days (**Figure 3.17B**), which is indicative of an area decrease. As indicated by the black boxes shown in the white light microscopy images (**Figure 3.16**), the tumouroids incubated with the combination of triptolide and gemcitabine also displayed indications of membrane blebbing, suggesting that apoptosis may be occurring.

As such, we next investigated whether gemcitabine in combination with triptolide induced apoptosis (as previously described in **Figure 3.14**). As shown in **Figure 3.18**, in the untreated and vehicle control conditions, no induction of apoptosis was observed. However, both 1 μM gemcitabine and 1 μM triptolide induced apoptosis as single agents in A549 tumouroids (**Figure 3.18**). Co-incubation of gemcitabine with triptolide also induced apoptosis in A549 tumouroids (**Figure 3.18**). Interestingly, greater levels of caspase-3/7 activity appeared to be induced in this condition and tumouroids were of a smaller size compared to triptolide or gemcitabine alone (**Figure 3.18**).

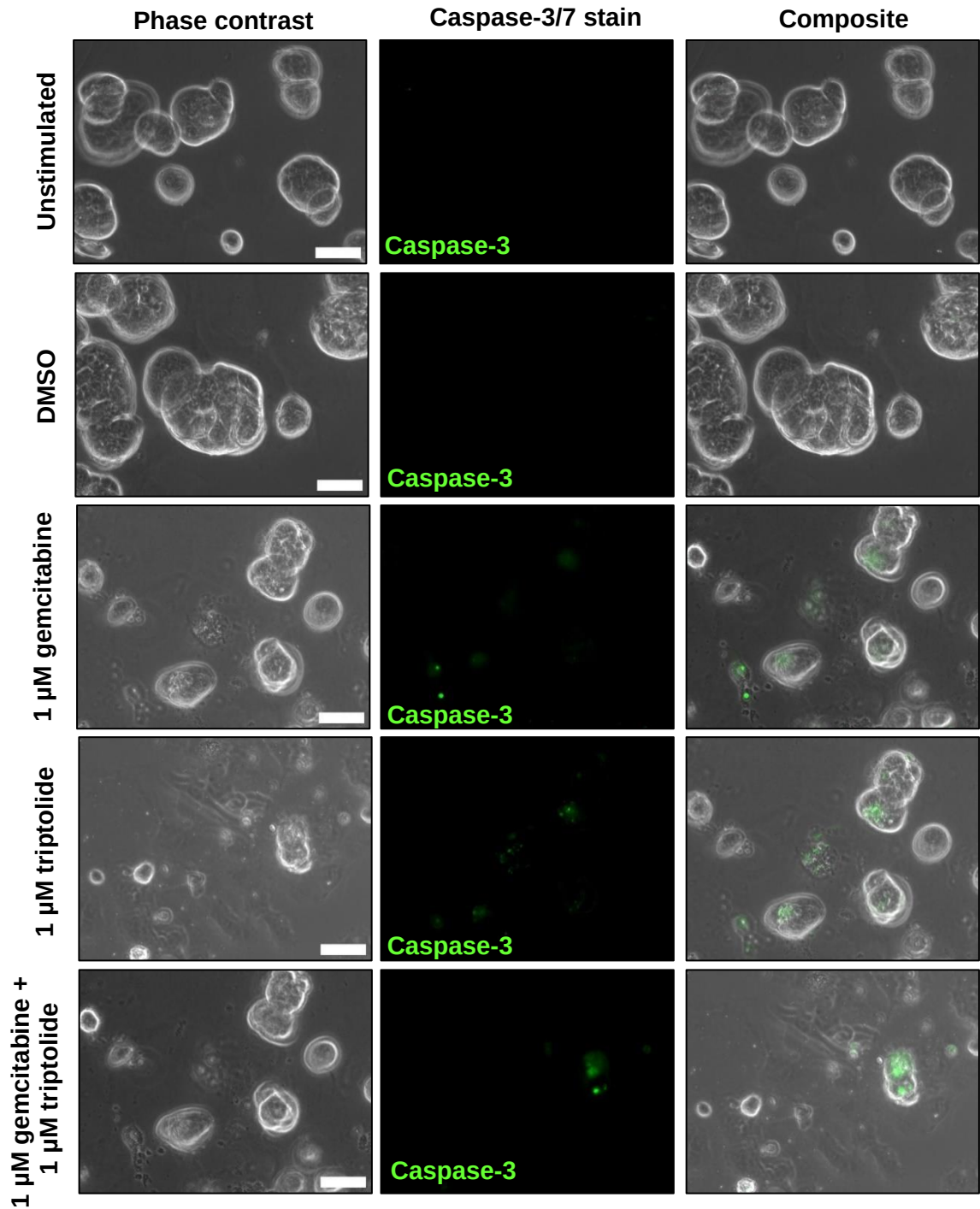


Figure 3.18. Triptolide and gemcitabine alone and in combination induce apoptosis in 3D A549 tumouroids. Tumouroids were cultured and treated with 1 μ M triptolide and gemcitabine, alone or in combination from days 5-9 of growth. Caspase-3/7 activation was visualised via epi-fluorescent microscopy following incubation with 5 μ M CellEvent™ Caspase-3/7 Detection Reagent for 30 min. Results and images are representative of 3 individual experiments (N=3). Scale bar 100 μ m.

3.4. Discussion

Acting as the ‘master-regulator’ of the oxidative stress response, Nrf2 has many roles in maintaining homeostasis, regulating inflammation, and preventing carcinogenesis. In non-pathological conditions, Nrf2 provides a rapid and transient response to stress, via the induction of cytoprotective genes with detoxification, antioxidant and anti-inflammatory effects (e.g., NQO1 and HO-1). In the context of cancer however, the Nrf2 pathway can be hijacked, resulting in Nrf2 overexpression. High levels of Nrf2 in cancer may then promote chemoresistance, angiogenesis, metastasis, and tumour growth²⁵⁸. Nrf2 may therefore be a useful target in cancer chemotherapy for patients with high Nrf2-expressing tumours. While, several Nrf2 inhibitors have been reported in the literature, this is the first comparative study exploring the effects of Nrf2 inhibitors on cancer cell proliferation and Nrf2 signalling in a variety of cancer cell lines in both 2D and 3D culture.

Many cancer types have been shown to exhibit high expression of Nrf2, such as lung, breast, melanoma, colorectal, ovarian and liver cancers^{68,269,279,451-455}. High expression of Nrf2 in patient tumours is also typically associated with poor prognosis and the development of chemoresistance²⁷². Studies on Nrf2 signalling in cancer are typically carried out using cancer cell lines, however many of these studies only explore Nrf2 expression in a small number of cell lines, and usually focus only on one cancer type. Hence, for the first time to our knowledge, Nrf2 expression was examined in a panel of 11 cancer cell lines of different tissue origin, including cell lines derived from lung, melanoma, breast, prostate, pancreatic, monocytic leukaemia, liver and colorectal tumours (**Figure 3.1A**). These results showed that among the cell lines tested, the highest expression of Nrf2 was observed in the A549 NSCLC, MDA-MB-231 triple negative breast cancer and HEPG2 hepatocellular carcinoma cell lines (**Figure 3.1B**).

While the mechanism underpinning the high expression of Nrf2 in the HEPG2 and MDA-MB-231 cells is unknown, Nrf2 expression in both cell lines appears to promote chemoresistance. In the MDA-MB-231 cells, treatment with doxorubicin and mitoxantrone induces Nrf2 expression, which is associated with decreases in ROS levels, and increases in expression of the anti-apoptotic protein Bcl-2, and the drug efflux pump P-gp, thereby providing a protective response to the cells⁴⁵⁶. In HEPG2 cells, Nrf2 protects against ferroptosis in response to exposure to the chemotherapeutic agents sorafenib and erastin via the induction of HO-1 and NQO1⁴⁵⁷. While these cell lines have value in screening for inhibitors and understanding the role of Nrf2 in these cancer subtypes, further studies identifying their mechanisms of Nrf2 upregulation could be explored.

On the other hand, the mechanism of Nrf2 overexpression in the A549 cells is well-characterised, having a known somatic mutation in the Kelch domain of KEAP1 at G333C, in addition to Keap1 promoter hypermethylation²⁶⁹. As the Kelch domain is essential for KEAP1-mediated degradation of Nrf2, mutations in this domain may therefore contribute to the high expression of Nrf2 in the A549 cells. Such mutations are also reflected in patient tumours, with dysregulation of the KEAP1/Nrf2 axis reported in many NSCLC cases²⁶⁹. Similarly to the A549 cell line, many of these patient tumours also exhibit point mutations in the Kelch or intervening region domains of KEAP1²⁶⁹, associated with higher expression of Nrf2. The A549 cells therefore serve as a useful *in vitro* model of NSCLC tumours with Nrf2 overexpression.

As Nrf2 has such a key role in many cancers, several natural product and small molecule inhibitors of the Nrf2 pathway have been identified in the literature, with varying anti-cancer effects in different cancer types²⁵⁶. In this study, a selection of these inhibitors: brusatol, luteolin, ML385, retinoic acid, triptolide, trigonelline and clobetasol (**Table 3.1**) were compared for their effects on cell proliferation, Nrf2 protein expression, and Nrf2 downstream signalling activity in both 2D and 3D cell culture models of high Nrf2-expressing A549 NSCLC cells.

Interestingly, while initially included as a control to induce Nrf2 activity, the Nrf2 activator DMF appeared to have repressive effects on Nrf2 protein expression in A549 cells (**Figure 3.3**). As reported by Saidu *et al.*⁴⁴⁹, at certain concentrations, DMF may act as an inhibitor of protein deglycase DJ-1 (DJ-1), which is a small multifunctional protein with roles in redox sensing, apoptosis, and metabolism. As DJ-1 typically acts to stabilise Nrf2 by inhibiting Nrf2's interaction with Keap1⁴⁵⁸, the inhibitory effect of DMF on DJ-1 may contribute to its ability to repress Nrf2. Furthermore, studies have reported that DMF possesses cytotoxic effects and inhibits nuclear Nrf2 expression in several cancer cell lines, such as NSCLC, melanoma, colon, pulmonary and ovarian cells, but not in non-malignant cells^{449,459,460}. To further investigate the effects that we observed with DMF on Nrf2 and DJ-1 protein expression in A549 NSCLC cells, a concentration and time response of DMF was conducted. As discussed in **Chapter 8.2.2**, DMF had varying effects on A549 Nrf2 protein expression, depending on the concentration and stimulation time (**Figure 8.2**). In general, higher concentrations (20 μ M and 30 μ M) of DMF resulted in a reduction in Nrf2 expression, while lower concentrations (10 μ M) induced Nrf2 protein expression (**Figure 3.2**). Furthermore, the effects of DMF also appeared to be time-dependent, with the higher concentrations of DMF having their greatest inhibitory effects on Nrf2 protein expression at 8h. However, DMF did not show any effects on DJ-

1 protein expression (see **Chapter 8.2.2, Figure 8.2**), which contradicts Saidu *et al.*'s hypothesis. While these results suggest a potential dual role of DMF in Nrf2 modulation, such effects seem to be highly dependent on the correct dosage and treatment conditions, and do not appear to be solely dependent on DJ-1, as previously hypothesised⁴⁴⁹. Although DMF is gaining interest as a potential anti-cancer agent, further research should better elucidate its mechanism of action in cancerous cells.

Brusatol, a quassinoid derived from the *Brucea javanica* plant, is perhaps the most widely studied of the Nrf2 inhibitors in the literature. Initially reported as an inhibitor of Nrf2 protein expression by Ren *et al.*³³⁵ in NSCLC cells, brusatol not only inhibits Nrf2 downstream signalling and cell proliferation, but also induces apoptosis and sensitises cancer cells to chemotherapeutic agents in a variety of *in vitro* and *in vivo* models of pancreatic³³⁹, colorectal^{329,331,332}, hepatocellular⁴⁶¹, lung^{335,336,462}, breast³⁴⁰ and leukaemic⁴⁶³ cancers. In the present study, among a panel of high (A549), medium (M202, MCF-7 and MIAPACA2) and low (A375, H292 and BXPC3) Nrf2-expressing cancer cell lines (**Figure 3.1**), brusatol exhibited the most potent anti-proliferative effects of the Nrf2 inhibitors, with IC₅₀ values of <120 nM (**Figure 3.2**). These results are consistent with other reports that brusatol possesses potent anti-proliferative effects at nanomolar concentrations in several cancer cell lines^{331,332,334,335,339,438}.

Furthermore, in A549 NSCLC cells, compared to the other Nrf2 inhibitors, brusatol also demonstrated the greatest repressive effects on Nrf2 protein expression (**Figure 3.3**), Nrf2-ARE binding (**Figure 3.6**) and NQO1 activity (**Figure 3.7**). Brusatol rapidly repressed Nrf2 protein expression within 2 h of stimulation, with repressive effects maintained over the course of 8 h (**Figure 3.5**). Interestingly, brusatol did not appear to show typical concentration-response effects on Nrf2 protein expression, exerting the greatest inhibitory effects at 2 µM, compared to that of 0.2 µM or 20 µM (**Figure 3.4**). The inhibitory effects of brusatol on NQO1 activity also did not show a typical concentration response (**Figure 3.8**), with the lowest concentration (0.1 µM) displaying the greatest inhibitory effects, which may suggest that brusatol works best at sub-micromolar concentrations in A549 cells.

Although the results presented here further support the anti-cancer effects of brusatol observed in the literature, brusatol's mechanism of action remains under debate. Despite initially being reported to selectively induce ubiquitination of Nrf2 and reduce Nrf2 protein synthesis^{334,335}, brusatol also appears to exert inhibitory effects on several oncogenic pathways, including the c-Myc⁴³⁹, PI3K/Akt/mTOR^{461,464}, STAT3⁴³⁸ and RhoA/ROCK⁴⁶² pathways. While such effects could be explained by Nrf2's ability to crosstalk with

members of these pathways, a comprehensive study of the A549 proteome following brusatol stimulation suggests that brusatol acts as a general inhibitor of protein synthesis, with greater effects at degrading short-lived proteins, such as Nrf2³⁸⁴. Moreover, following construction of a biotin-brusatol conjugate and use of a streptavidin affinity assay and mass spectrometry analyses, Xing *et al.*⁴⁴⁰ identified 793 brusatol-binding candidate proteins, including Skp1, which acts as an adapter for the SCF E3 ligase complex known to mediate Nrf2 degradation. While it is clear that brusatol does act as an inhibitor of Nrf2, and can suppress proliferation in NSCLC, the viability of this compound being licenced as an anti-cancer agent appears unlikely at present, due to its off-target effects. As shown in this study however, brusatol remains one of the most robust and potent inhibitors of Nrf2 currently identified, hence is still useful as a control in studying the mechanisms of Nrf2 in cancer and inflammation.

Luteolin on the other hand, appears to have a more complex effect on the Nrf2 pathway in cancer. Previous studies have observed that in NSCLC, colon and breast cancers, luteolin promotes Nrf2 mRNA turnover, represses Nrf2 protein and Nrf2-downstream gene expression³⁵⁰, and sensitises NSCLC cells *in vitro* and *in vivo* to several anti-cancer agents, such as cisplatin, doxorubicin and bleomycin³⁴⁹. Conversely, luteolin has also previously been shown to induce Nrf2 and its downstream genes, HO-1 and NQO1, in several diseases⁴⁶⁵, including colorectal cancer^{353,379} hepatocellular carcinoma⁴⁶⁶, diabetic cardiomyopathy⁴⁶⁷ and intracerebral haemorrhage⁴⁶⁸. In the present study, while luteolin treatment induced a significant reduction in Nrf2 protein expression in A549 cells, compared to other inhibitors tested (**Figure 3.3**), its effects on Nrf2-DNA binding and NQO1 activity were much less prominent (**Figure 3.6** and **3.7**).

Luteolin did inhibit cell proliferation of cancer cell lines however, possessing IC₅₀ values of ≤28.2 μM for all cell lines besides the high Nrf2-expressing A549 cells, which possessed a much higher IC₅₀ value of 122.5 μM (**Figure 3.2**). When the IC₅₀ values of the A549 and H292 lung cancer cells were compared, a near 10-fold decrease in potency was observed in the A549 cells. One potential explanation for this could be that the A549 cells express higher levels of Nrf2 than that of the H292 cells (**Figure 3.1**), therefore may be more resistant to Nrf2 inhibition with luteolin. Furthermore, while observed here to inhibit cell proliferation of a variety of cancer cell lines, and inhibit Nrf2 protein expression in NSCLC cells, the conflicting role of luteolin in the literature sheds some doubt on its use as an Nrf2 inhibitor. While further studies could be performed to better elucidate the potential of luteolin as a chemotherapeutic, its role in modulating Nrf2 may be highly dependent on cell type.

Another Nrf2 inhibitor that showed varying effects on the Nrf2 pathway in cancer in the present study was the small molecule inhibitor, ML385. Originally reported to interrupt the interaction between Nrf2 and MafG, ML385 has gained interest due to its apparent ability to reduce cell proliferation and Nrf2 downstream gene expression, (such as GSH) in Keap1-mutant, but not wild type NSCLC cells and lung squamous cell carcinoma cells^{358,359,469}. As many of the Nrf2-overexpressing tumours possess mutations in Keap1, an inhibitor with specific selectivity for Keap1-mutant tumours would be highly beneficial. However, contrary to the literature, in the present study, ML385 did not show any effects on cell proliferation at concentrations of $\leq 500 \mu\text{M}$ in a panel of cancer cell lines, including the Keap1 mutant A549 cells (**Figure 3.2**). Furthermore, although ML385 did show some inhibitory effects on Nrf2-ARE binding (**Figure 3.6**), minimal effects on Nrf2 protein expression and NQO1 activity were observed (**Figures 3.3** and **3.7**), contrary to previous reports in A549 cells³⁵⁹. Additionally, ML385 also did not appear to sensitise A549 cells to gemcitabine (**Figure 3.9B**).

Possible explanations for why such effects were not observed in our study could be that the stimulation time was not sufficient, as one previous study used treatment times of 24 h for Nrf2 protein expression and 72 h for reduction of NQO1 activity³⁵⁹. Furthermore, after observing cells using phase contrast microscopy, it appeared that at high concentrations, ML385 precipitated out of solution in cell culture media, suggesting that the lack of effect observed in ML385 could be due to insolubility. Although attempts were made to remedy this, including a slow dilution of ML385 in foetal bovine serum and cell culture media, and testing of ML385 from several commercial sources, the solubility issue persisted at concentrations of $>50 \mu\text{M}$. While results from this study regarding ML385 were therefore inconclusive, ML385 is often cited in the literature as an Nrf2 inhibitor, primarily in studies of inflammation⁴⁷⁰. Evidence supporting its use in cancer is still emerging, with most studies focussing on its potential as a sensitising agent to chemotherapy and radiotherapy^{360,471}, although such effects were not seen in this study.

Another Nrf2 inhibitor that has shown promise in the literature is trigonelline hydrochloride, a plant alkaloid found in *Coffee arabica* and *Trigonella foenum-graecum*. Although not widely researched, trigonelline has been reported to inhibit Nrf2 nuclear import and repress expression of Nrf2 downstream genes, such as NQO1 and HO-1 *in vitro* in both pancreatic cancer and NSCLC cells^{365,442}. In the present study, while trigonelline did not appear to have any anti-proliferative effects in A549 cells at concentrations $<100 \mu\text{M}$ (**Figure 3.2**), such results are supported in the literature, in which trigonelline may sensitise NSCLC, head and neck cancer and pancreatic ductal adenocarcinoma cells to cisplatin and etoposide but do not possess anti-proliferative

activity as a single agent⁴⁴³. Furthermore, when the effects of trigonelline on Nrf2 signalling in NSCLC were explored, 20 μ M trigonelline had very little effect on Nrf2-ARE binding (**Figures 3.6**) and did not suppress Nrf2 protein expression in A549 cells (**Figure 3.3**). While trigonelline did induce a significant reduction in NQO1 activity in A549 cells at a concentration of 10 μ M, such effects were lost at a concentration of 20 μ M (**Figure 3.7**).

While reported by Fouzder *et al.*⁴⁴² to suppress nuclear Nrf2 and NQO1 protein expression at concentrations as high as 50 μ M in A549 cells, trigonelline has also demonstrated biphasic effects on the Nrf2 pathway in HT29 colorectal cancer cells³⁶⁶, with concentrations <0.01 μ M and >10 μ M having little or no activity. Although in contrast to the results presented by Fouzder *et al.*⁴⁴², it is possible that trigonelline also exhibits biphasic modulation of Nrf2 in A549 cells, perhaps explaining the lack of effect on NQO1 activity observed at 20 μ M compared to 10 μ M in our study. Furthermore, while the mechanism through which trigonelline inhibits Nrf2 signalling remains unclear, evidence suggests that trigonelline may act to inhibit phosphorylation of Nrf2, thereby keeping Nrf2 localised to the cytosol⁴⁴². Hence, while trigonelline does appear to have some effects on Nrf2 downstream signalling in NSCLC in the present study, its lack of effects on unphosphorylated Nrf2 could be expected. To further elucidate trigonelline's effects, additional concentrations could therefore be tested to explore both nuclear and cytosolic expression of Nrf2 and phosphorylated Nrf2.

Another natural product that has been reported to inhibit Nrf2 is the vitamin A derivative: *all-trans* retinoic acid. Acting as an agonist for RXR α , retinoic acid promotes binding of RXR α to the Neh7 domain of Nrf2, thereby preventing Nrf2 from associating with the ARE³²⁴. Retinoic acid has therefore shown activity in repressing expression of Nrf2 downstream genes such as NQO1, HO-1, AKR1C1 and AKR1C2 in NSCLC, acute myeloid leukaemia (AML) and breast cancer cells^{323,324}. Additionally, retinoic acid also sensitises AML cells to arsenic trioxide³²³, a common treatment in AML, and NSCLC cells to cisplatin³²². In this study, although retinoic acid did not significantly inhibit Nrf2 protein expression, it did reduce Nrf2-ARE binding and NQO1 activity (**Figures 3.3, 3.6, 3.7**) in A549 cells, consistent with previous reports³²². As retinoic acid inhibits the interaction of Nrf2 with the ARE, the results on Nrf2-ARE binding and NQO1 activity were expected. However, the effect of retinoic acid on Nrf2 protein expression in cancer cell lines is not clear. Although originally reported to only affect Nrf2-ARE binding, studies have since demonstrated repressive effects on both Nrf2 mRNA and Nrf2 nuclear protein expression in cholangiocarcinoma cells³²¹ and NSCLC cells³²² with retinoic acid treatment, potentially due to the autoregulatory role of Nrf2⁴⁷². A possible explanation for

the lack of effect observed in the present study is that a decrease in nuclear Nrf2 may not result in a significant decrease in Nrf2 in whole cell lysates. To address this, the effect of retinoic acid on Nrf2 mRNA and nuclear and cytosolic protein levels could be studied.

Furthermore, though retinoic acid did show some anti-proliferative effects, such effects were in the micromolar range, with IC₅₀ values ranging between 33.7-142 μ M (**Figure 3.2**). Contrary to triptolide and luteolin however, the greatest effects were observed in the high Nrf2-expressing A549 cells (**Figure 3.2**). Additionally, retinoic acid did not induce any anti-proliferative effects in the low Nrf2-expressing BXPC3 pancreatic cancer cells, contrary to other reports^{473,474}. These results suggest that retinoic acid's effects on proliferation do not seem to be correlated with Nrf2 expression status, as in the case of other inhibitors such as brusatol, triptolide and luteolin. Although retinoic acid's effects on cell proliferation vary across different cancer cell types and were not as potent at reducing cell proliferation and Nrf2 signalling as inhibitors such as brusatol and triptolide (**Figure 3.2**), retinoic acid is already well established in the clinic in AML treatment, with fewer off-target effects than that of brusatol, hence may have potential in treating NSCLC.

Another Nrf2 inhibitor that could be repurposed is the corticosteroid clobetasol propionate, which is currently used to treat skin conditions such as eczema and psoriasis. Initially identified as an Nrf2 inhibitor among a drug screen of ~4000 compounds in the clinic, clobetasol is reported to inhibit the expression of several Nrf2 downstream genes, including GCLM (glutamate-cysteine ligase modifier subunit) and ME1 (malic enzyme 1)³⁴³. To date, only one study has been published on clobetasol in the context of Nrf2, in which Choi *et al.*³⁴³ identified that in NSCLC cells, clobetasol induces GSK-3 β -mediated phosphorylation of Nrf2 at the Neh6 domain, thereby promoting nuclear export of Nrf2. As shown here, clobetasol treatment significantly reduced Nrf2 protein expression in A549 cells (**Figure 3.3**), however had little effect on Nrf2-ARE binding (**Figure 3.6**) and NQO1 activity (**Figure 3.7**) at 20 μ M. At 10 μ M however, clobetasol did slightly reduce NQO1 activity (**Figure 3.7**).

An explanation for the differences in effects observed in our study compared to that in the literature is that Choi *et al.* selected a longer treatment time, with inhibition of Nrf2 target genes only being observed after 5 days of treatment. Furthermore, while we did not observe any effects on A549 cell proliferation with <100 μ M clobetasol, the previous study by Choi *et al.* observed that, at concentrations of 0.01-100 nM, clobetasol propionate did not inhibit cell proliferation in A549 cells, however, did sensitise cells to the cytotoxic effects of rapamycin³⁴³. Further studies to test additional treatment times

with clobetasol and confirm its mechanism of action to induce GSK-3 β -mediated Nrf2 degradation could be carried out, to further assess its efficacy as an Nrf2 inhibitor in cancer.

The final Nrf2 inhibitor explored in the present study was triptolide, a natural product isolated from *Tripterygium wilfordii* that has been used in traditional Chinese medicine for many years, with several anti-cancer, anti-inflammatory and immunosuppressive effects⁴⁷⁵. Triptolide has previously been shown to suppress Nrf2, NQO1, GSR and HO-1 mRNA and protein expression in leukaemia cells, associated with the induction of ferroptosis, apoptosis, and increased sensitivity of cells to doxorubicin^{368,441}. Also, triptolide induces apoptosis and interrupts glutathione metabolism via suppression of Nrf2 downstream target gene expression, including GCLC, GCLM, SLC7A11 in glioblastoma cells³⁷². Conversely, triptolide has also been shown to induce Nrf2 activation and repress NF κ B signalling in murine models of acute pancreatitis⁴⁷⁶. In the present study, triptolide displayed the second-best inhibitory effects on Nrf2 protein expression (**Figure 3.3**), Nrf2-ARE binding (**Figure 3.6**) and NQO1 activity (**Figure 3.7**) in A549 cells, second only to brusatol. Such effects on Nrf2 protein expression were also shown to be concentration and time-dependent (**Figures 3.4** and **3.8**), with triptolide inducing a reduction in Nrf2 protein levels to <50% vehicle control within just 2 h of treatment (**Figure 3.5**). Likewise, triptolide also exerted concentration-dependent inhibitory effects on NQO1 activity in A549 cells, with effects observed at concentrations as low as 0.1 μ M (**Figure 3.8**).

Furthermore, triptolide also induced potent anti-proliferative effects across the panel of cancer cell lines studied, with IC₅₀ values ranging between 6-138 nM (**Figure 3.2**). Interestingly, similarly to brusatol and luteolin, sensitivity to triptolide also appears to be correlated with Nrf2 status of each cell line, with the high Nrf2-expressing A549 cell line demonstrating the highest IC₅₀ value (138 nM, **Figure 3.2**). While this may suggest that cells with higher Nrf2 expression levels may be slightly more resistant to triptolide, the ability of triptolide to induce anti-proliferative effects at concentrations in the nanomolar range observed here further emphasises its potential as an anti-cancer agent^{475,477}. Triptolide acts via several mechanisms including repressing Nrf2 mRNA and protein expression, inhibiting Nrf2 nuclear translocation, and inducing GSK-3 β /Fyn-mediated Nrf2 degradation^{370,371,373}. While we did not study effects on Nrf2 mRNA, our results support the hypothesis that triptolide inhibits Nrf2 protein expression in A549 cells³⁷³. In contrast to these findings however, Nam *et al.*³⁷⁰ observed that triptolide did not reduce total Nrf2 protein and mRNA expression in A549 cells, but increased cytosolic localisation of Nrf2. As we found that triptolide exerts a concentration-dependent

inhibitory effect on Nrf2 protein expression at concentrations of >100 nM, a possible explanation for these conflicting results could be that at very low concentrations of 20 nM, such as those used by Nam *et al.*, triptolide does not affect Nrf2 mRNA or protein levels.

With one of the best suppressive effects on proliferation and the Nrf2 pathway among the Nrf2 inhibitors in this study, triptolide was therefore selected for further studies, to evaluate its potential as an anti-cancer agent in NSCLC. Although studies in 2D cell culture are useful in providing a platform for quick and cost-efficient screening of potential Nrf2 inhibitors, they do not accurately mimic the 3D environment of NSCLC tumours. To better address this, we investigated the ability of triptolide to suppress cell proliferation and induce apoptosis in a 3D culture model of NSCLC, using A549 tumouroids.

As expected from the anti-proliferative effects observed previously in 2D cell culture (**Figure 3.2**), triptolide displayed concentration-dependent effects to inhibit formation (**Figure 3.11**) and growth (**Figure 3.13**) of A549 tumouroids. Consistent with several *in vitro* studies in breast cancer, leukaemia, glioblastoma and NSCLC cells^{367-369,372,373,478}, triptolide also induced apoptosis of A549 tumouroids via caspase-3/7 activation (**Figure 3.14**). Treatment with triptolide in combination with gemcitabine also significantly inhibited A549 cell growth in 2D culture, compared to gemcitabine alone (**Figures 3.15**). In 3D culture of A549 tumouroids, co-incubation of gemcitabine with triptolide did not significantly inhibit tumouroid growth compared to gemcitabine alone (**Figure 3.17B**). Despite this, the co-incubation of triptolide with gemcitabine was the only treatment condition that resulted in a fold change in tumouroid area of below 1, indicating a decrease in tumouroid area compared to day 5. This poses the question as to whether additional combinations of triptolide and gemcitabine concentrations may result in a statistically significant additive effect. Further experiments may also further validate our observation that triptolide in combination with gemcitabine induces apoptosis (**Figure 3.18**), to further explore reports in the literature that suggest that triptolide acts as a chemosensitizer^{368,369,372,373,441,475,476}. Although higher concentrations of triptolide and gemcitabine were required to induce growth-suppressive effects in 3D than 2D culture, such effects were expected, as 3D spheroids are typically more resistant to chemotherapy than cells cultured in a 2D monolayer⁴⁷⁹.

While the current research supports the application of triptolide as an anti-cancer agent in high Nrf2-expressing NSCLC tumours, increasing evidence suggests that triptolide may also inhibit *de novo* mRNA synthesis of a variety of genes, including the c-myc and Src oncogenes, by depleting expression of RPB1 (the main subunit of RNA polymerase

II)^{480,481}. Furthermore, the advancement of triptolide into the clinic has largely been hampered by its low solubility, hepatotoxicity, and cardiotoxicity⁴⁷⁷. Despite this, the potential of triptolide as an anti-cancer agent and Nrf2 inhibitor remains promising, with current approaches focussed on targeted delivery of triptolide⁴⁸². Additionally, several triptolide analogues are also being studied, with improved safety and pharmacokinetic profiles, such as Minnelide, a pro-drug of triptolide, currently being investigated in phase II trials for refractory pancreatic cancer [ClinicalTrials.gov Identifier: NCT03117920⁴⁸³].

One limitation of this study is that we did not study the effect of the Nrf2 inhibitors on Nrf2 mRNA expression, which may be useful in validating the mechanism of action of inhibitors such as brusatol, triptolide, luteolin and trigonelline. Furthermore, this study also could have explored the nuclear and cytosolic localisation of Nrf2 in each treatment condition by western blot. As several of the inhibitors are believed to act on a variety of signalling pathways, further research could also utilise RNA interference of Nrf2 to validate whether the anticancer activity of each of the inhibitors is Nrf2-dependent.

In conclusion, among a panel of cancer cell lines, Nrf2 expression was highest in A549 NSCLC, HEPG2 hepatocellular carcinoma and MDA-MB-231 breast cancer cells. When compared among 7 natural product and small molecule Nrf2 inhibitors reported in the literature (**Table 3.1**), brusatol and triptolide showed the most promising and potent effects to inhibit proliferation of several lung, breast, pancreatic and melanoma cell lines, and suppress the Nrf2 pathway. In NSCLC tumouroids, triptolide also inhibited growth and induced apoptosis both as a single agent and in combination with gemcitabine. These results therefore suggest that triptolide may be potentially useful as a chemotherapeutic agent. Despite its reported off-target effects, the potency of triptolide as an anti-proliferative agent and Nrf2 inhibitor make it a prime candidate for use in targeted therapies, such as antibody or peptide-drug conjugates.

Chapter 4. Novel methods of targeting Nrf2 in cancer

Acknowledgments

Dr Marco M D Cominetti synthesised the triptolide-peptide conjugate in **Figure 4.4**. Dr Andrew Beekman synthesised the peptide studied in **Figures 4.7-13**.

4.1 Introduction

As discussed in **Chapter 3**, a number of Nrf2 inhibitors have been reported in the literature, which show varying activity in repressing Nrf2 signalling in Nrf2-addicted cancers²⁵⁶. Several of these inhibitors have emerged as potential drugs of interest for use in cancer, however as some of these inhibitors possess off-target effects^{384,481}, more targeted methods of inhibiting Nrf2 could be explored. In this study, two novel methods of targeting Nrf2 were developed and studied in NSCLC, including a peptide-drug conjugate utilising the Nrf2 inhibitor triptolide and a peptide mimic of the MafG bZIP domain to block the Nrf2/MafG interaction.

Triptolide, a natural product inhibitor of Nrf2, has potent inhibitory effects on Nrf2 signalling and cancer cell proliferation *in vitro* at nanomolar concentrations both within the literature^{367-370,372,373} and as discussed in the present thesis (**Chapter 3** and **Chapter 5**), and also displays anti-cancer activity *in vivo*⁴⁷⁵. Despite its initial promise, triptolide has struggled to progress into clinical trials due to its systemic toxicity⁴⁷⁷. However, owing to its high cytotoxicity and potency in repressing cancer cell growth and its ability to sensitise cancer cells to a variety of chemotherapies^{373,441}, triptolide is a potentially suitable candidate for use in a targeted therapy in Nrf2-overexpressing tumours⁴⁸². Targeted therapies are becoming increasingly used in the field of chemotherapy and are designed to deliver a potent cytotoxic agent (also referred to as a cytotoxic payload) specifically to cancer cells, thereby reducing systemic side effects and increasing the therapeutic window of the cytotoxic drug⁴⁸⁴.

Currently, the leading type of targeted therapy that exists in the clinic is that of antibody-drug conjugates (ADCs), with 14 approved for clinical use and over 100 in clinical trials for a variety of diseases, including solid and haematological tumours⁴⁴. ADCs specifically target cancer cells via the inclusion of monoclonal antibodies which possess high binding specificity to antigens overexpressed on the cell surface of cancer cells⁴⁴. Cytotoxic payloads are conjugated to the antibody via the use of a chemical linker, which allows for specific delivery of the payload to the tumour microenvironment. Although these are the leading form of targeted therapy⁴⁴, they do possess some drawbacks. Most notably, ADCs are expensive to manufacture due to the complexities of antibody production and purification⁴⁶. Additionally, ADCs may also induce immunogenic responses including the formation of anti-drug antibodies, which can inactivate the administered cytotoxic and therefore decrease overall response to the ADC⁴³. Finally, ADCs typically have a large molecular weight of >150 kDa, which restricts their ability to penetrate the tumour stroma and enter cells⁴⁶.

Due to the issues associated with antibodies, interest has shifted towards the use of small cell-targeting peptides, to form peptide-drug conjugates (PDCs). These are designed to work via a similar mechanism to ADCs, however, require a small peptide as the targeting moiety instead of antibodies. A key benefit to this approach is that peptides are much cheaper and easier to synthesise than antibodies, with most techniques involving the use of cheap reagents and standard solid phase peptide synthesis protocols⁴⁵. Furthermore, peptide synthesis can easily be automated via the use of robotic peptide synthesisers to streamline their production. As peptides are very small molecules compared to antibodies (<20 kDa), PDCs may also better penetrate the tumour stroma and have been shown to possess lower immunogenicity compared to ADCs⁴³. Although many PDCs are currently in development, two already exist in the clinic; Lu¹⁷⁷ dotatate and Melflufen, which are indicated for neuroendocrine cancer⁴⁷ and multiple myeloma⁴⁸, respectively.

In PDCs, a wide variety of cell targets may be taken advantage of, similarly to ADCs. For example, the Lu¹⁷⁷ dotatate PDC is composed of a radiotherapeutic agent linked to a somatostatin analogue, which can target the somatostatin receptor-2 that is commonly overexpressed in neuroendocrine cancers⁴⁷. Typically, targets are selected based on their overexpression in cancer cells compared to normal cells. One example of such a target is the epidermal growth factor receptor (EGFR), which potentiates sustained proliferative signalling and is commonly overexpressed in a variety of cancer types, including NSCLC, prostate adenocarcinoma and breast carcinomas¹⁹. Several anti-EGFR therapies have been investigated clinically and are approved for use in solid tumours, such as cetuximab, panitumumab, nimotuzumab, and necitumumab⁴⁸⁵. Such therapies bind EGFR and block its ligand-interaction, leading to repressed EGFR signalling, receptor internalisation and degradation, which in turn suppresses cell proliferation⁴⁸⁵. As a commonly overexpressed target in cancer, EGFR has therefore emerged as a valuable target in both ADCs and PDCs.

While previous research has largely focussed on the development of anti-EGFR antibodies in ADCs⁴⁸⁶, recently several small peptides that specifically bind to EGFR have been identified⁴⁸⁷⁻⁴⁹⁰, that may be suitable for inclusion in an EGFR-targeting PDC. Currently, no PDCs that target Nrf2 have been developed, hence this study aimed to both synthesise a novel EGFR targeting PDC incorporating the Nrf2 inhibitor triptolide and subsequently study its potential use as an anti-cancer agent in EGFR and Nrf2-overexpressing tumours.

Aside from using natural products, such as triptolide, to inhibit Nrf2, this study also explored other novel mechanisms of Nrf2 inhibition in cancer. One such method is to target the Nrf2/MafG protein-protein interaction (PPI) via the use of peptides. PPIs were previously believed to be 'undruggable' targets. However recent advances have identified novel ways of inhibiting these interactions. One issue in targeting PPIs is that the interface between the two proteins is typically very hydrophobic and lacks potential binding pockets with which small molecules could interact³⁹¹. Although 'hot spots' can be identified within the PPI interface that could be targeted, the large surface area of these hot spots far exceeds that typically observed with the ligand-binding domains of traditional drug targets (such as enzymes and receptors), making it difficult for small molecules to bind and disrupt the PPI⁴⁹¹.

In contrast to small molecules, peptides are much larger and can span a wider area of the PPI interface, making them ideal molecules to target PPIs. Additionally, peptides can also be derived from the native protein sequence (known as peptide mimics), which can imitate the secondary structure of proteins (such as α -helices) to promote strong binding to the hydrophobic core of PPIs⁴⁹¹. Peptides are therefore emerging as promising molecules to target PPIs in cancer, with several peptide-based PPI inhibitors currently in early development for use in chemotherapy³⁹¹. One such therapy that disrupts both the p53/MDMX and p53/MDM2 PPIs via the use of a p53 peptide mimic has now entered Phase I clinical trials for patients with leukemic cancers³⁹².

The PPI between the Nrf2 and sMaf proteins (e.g., MafG, MafK and MafF) is well known to be necessary for Nrf2's ability to bind the antioxidant response element (ARE) and induce target gene transcription^{79,150}. Both Nrf2 and sMaf proteins contain basic region leucine zipper (bZIP) domains that comprise of several leucine residues that 'zip' together with other bZIP proteins to form homo and heterodimers^{122,492}. In the case of Nrf2/sMaf heterodimers, the two proteins interact via a coiled coil PPI between their ZIP domains which promotes association with the ARE¹²⁶.

Coiled coils are a structural motif that involve the supercoiling of two α -helical proteins to form a stable and highly regular structure. This supercoiling is mediated by the presence of 'heptad repeats' consisting of 2 amino acids with hydrophobic residues (e.g., leucine and valine) separated by polar amino acids³⁸⁹. Hydrophobic residues of one coil can interact with the corresponding hydrophobic residues of the other coil, forming a hydrophobic core that stabilises the homo or heterodimer complex³⁹⁰. Not only does the Nrf2/MafG leucine zipper interaction facilitate binding of Nrf2 to the ARE to induce transcription of Nrf2 target genes^{126,493}, binding of the MafG ZIP domain to Nrf2 masks a

nuclear export signal (NES) located in the ZIP domain of Nrf2 (termed NESzip)^{126,493}. By masking Nrf2's NESzip motif, MafG prevents nuclear export of Nrf2 by retaining it in the nucleus to induce transcription¹²⁶. The Nrf2/MafG PPI is therefore an invaluable potential target for Nrf2 inhibition in Nrf2-overexpressing cancers.

To inhibit the Nrf2/MafG interaction in cancer, a novel small peptide derived from the sequence of the MafG ZIP domain was synthesised, with the hypothesis that it could inhibit the formation of the native Nrf2/MafG coiled coil PPI and subsequently repress Nrf2 signalling in cancer cells. Here, this MafG peptide mimic was studied for the first time for its effects on Nrf2-ARE binding activity, Nrf2 downstream gene expression and activity, and on its ability to sensitise Nrf2-addicted lung cancer cells to standard of care chemotherapy.

4.2 Aims

1. Synthesise a peptide-drug conjugate that incorporates the Nrf2 inhibitor triptolide to target EGFR overexpressing cancer cells using solid-phase peptide synthesis.
2. Characterise the anti-proliferative effects of the triptolide peptide-drug conjugate on cancer cell lines.
3. Investigate the effects of disrupting the Nrf2-MafG protein-protein interaction on the Nrf2 pathway in cancer cell lines via the use of a MafG peptide mimic.
4. Explore the potential therapeutic benefit of disrupting the Nrf2-MafG interaction with a MafG peptide mimic in cancer cell lines.

4.3 Results

4.3.1 Synthesis of an EGFR-targeting peptide using solid-phase peptide synthesis

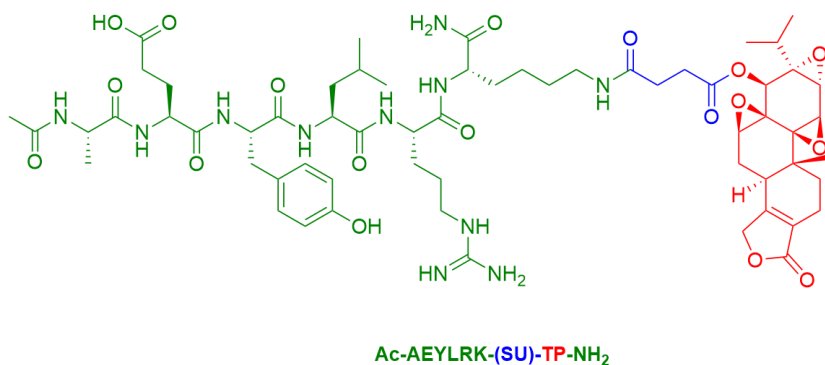
Previous research has identified a small peptide of the sequence 'AEYLR' that binds specifically to EGFR⁴⁸⁹ and possesses binding affinities of <10% and >90% in low and high EGFR-expressing non-small cell lung cancer (NSCLC) cell lines, respectively⁴⁸⁹. The application of this peptide sequence as an EGFR-targeting peptide in our cell culture conditions has been validated previously in collaboration with Prof. Mark Searcey's lab, wherein it was utilised to target gold nanoparticles to EGFR overexpressing cells⁴⁹⁴. As such, the AEYLR peptide was selected as the targeting moiety for the triptolide-peptide drug conjugate.

In the present study, an additional lysine (K) residue was attached at the C terminus of the peptide, which allowed site-selective conjugation of a chemical linker onto the amine of the lysine side chain, as previously established by Goddard *et al.*⁴⁹⁴. Additionally, a scrambled version of the AEYLRK peptide was synthesised (as shown in **Table 4.1** and **Figure 4.1**) to act as a control. This peptide was also validated by both Han *et al.*⁴⁸⁹ and Goddard *et al.*⁴⁹⁴ to not bind EGFR, however notably, the scrambled peptide contains a substitution of the tyrosine (Y) residue for an additional leucine (L), as tyrosine was found to be essential for EGFR binding⁴⁸⁹.

Table 4.1 Sequences of the EGFR targeting and scrambled peptide.

Peptide	Amino acid sequence
EGFR targeting peptide	AEYLRK
Scrambled control peptide	RALELK

(A)



(B)

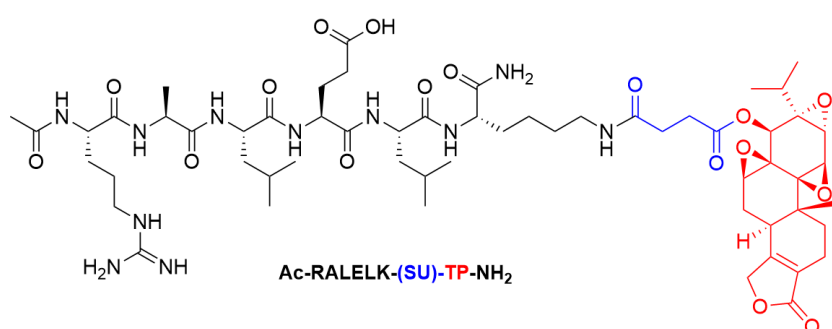


Figure 4.1 Structure of the EGFR and scrambled control triptolide-peptide conjugates. The chemical structures of the **(A)** EGFR-targeting (Ac-AEYLRK-(SU)-TP) and **(B)** scrambled control (Ac-RALELK-(SU)-TP) triptolide-peptide conjugates. Triptolide is highlighted in red, the SU linker in blue and the Ac-AEYLRK and Ac-RALELK peptides are shown in green and black, respectively. *Abbreviations: Ac; acetyl, SU; succinate, TP; triptolide.*

One benefit of using small peptides in drug conjugates is that they are relatively simple and cheap to synthesise compared to antibodies. A common way in which this is achieved is using solid-phase peptide synthesis (SPPS), which is summarised in **Figure 4.2**. This method utilises a solid support, such as a resin, onto which a peptide chain can be built. To create the peptide chain, amino acids are 'coupled' to each other via the formation of an amide bond between the carboxylic acid of one amino acid and the amine group of another⁴⁹⁵.

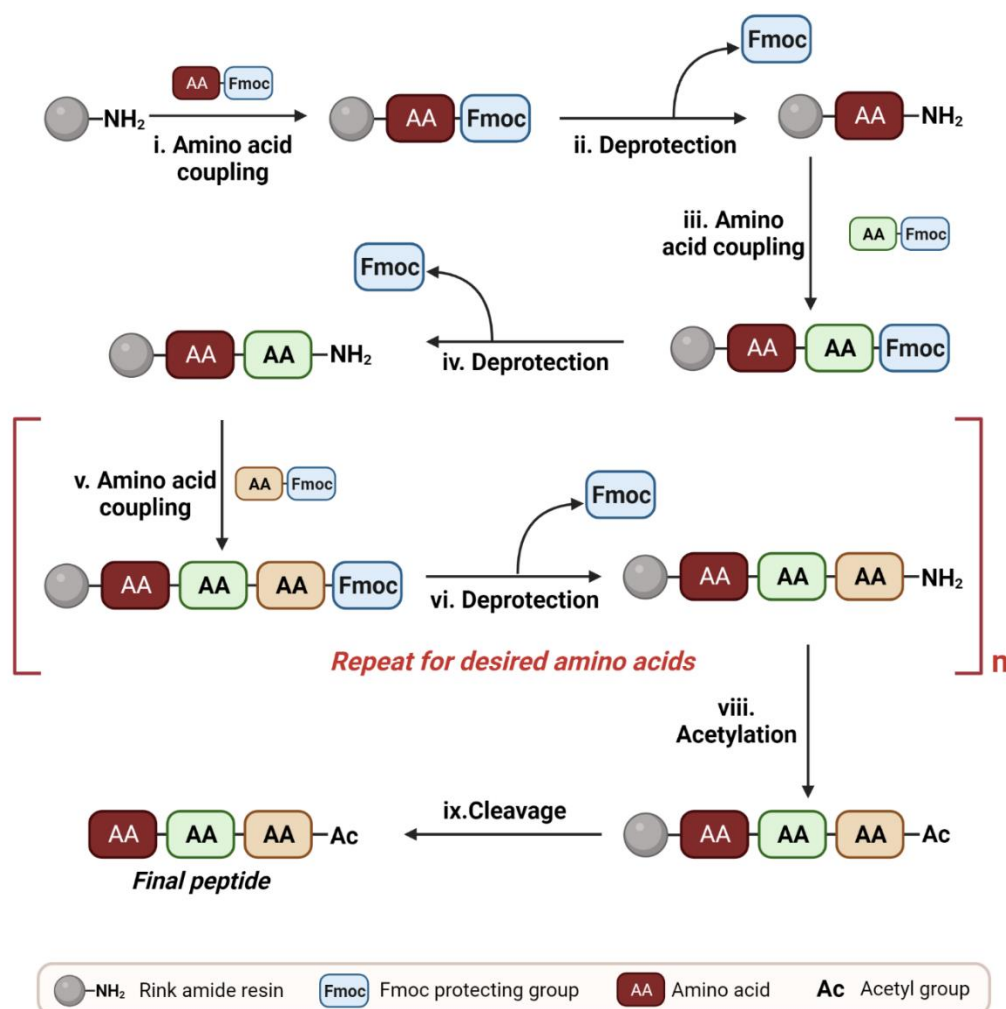


Figure 4.2 Schematic depicting Fmoc solid phase peptide synthesis.

In the present study, Fmoc (fluorenylmethyloxycarbonyl) SPPS was utilised, wherein amino acids are temporarily protected at their amine group via an Fmoc-moiety during coupling reactions⁴⁹⁶. This ensures that coupling only occurs between the amine of the already coupled amino acid and the carboxylic acid of the next amino acid. As the Fmoc group is base-labile, it can be easily removed via a deprotection reaction using a solution of 20% piperidine in DMF, prior to the next coupling step. Additionally, side chain protecting groups, such as tBut (*tert*-Butyl), Trt (trityl) and Pbf (2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl) are used to prevent unwanted reactions between acid and amine groups of amino acid side chains⁴⁹⁷.

As the peptide chain remains attached to the solid support, coupling reactions can be driven to completion with the addition of excess reagents, which are washed away once coupling is complete. This prevents the need for purification of reaction intermediates, and also produces a high yield of the peptide⁴⁹⁶. Once the desired sequence of amino acids has been coupled, the peptide can be detached from the resin via a cleavage

reaction under acidic conditions (**Figure 4.2**). This reaction not only allows for easy isolation of the peptide chain, but also removes the side chain protecting groups of the amino acid⁴⁹⁶. Following cleavage, the peptide is precipitated, purified via high-performance liquid chromatography (HPLC), and characterised via mass spectrometry.

To synthesise the AEYLRK and RALELK peptides (**Table 4.1**), Fmoc-SPPS was utilised, as shown in the reaction scheme displayed in **Figure 4.3**. Synthesis was performed using a Syro automated peptide synthesiser, and each peptide was acetylated at their N- termini to increase peptide stability. Upon successful synthesis and cleavage from the resin, peptides were purified and analysed via HPLC and MALDI-TOF mass spectrometry (matrix-assisted laser desorption/ionization- time of flight) to confirm that the correct peptide structure was obtained (see **Chapter 8.2.4**).

To conjugate the peptides to triptolide, a succinate linker was selected, as this has previously been shown to successfully link triptolide to Cetuximab, an anti-EGFR antibody⁴⁹⁸. Firstly, triptolide succinate (SU-TP) was synthesised as shown in **Figure 4.4**. The Ac-AEYLRK and Ac-RALELK peptides were then linked to SU-TP via amide bond formation between the amine of the lysine side chain, and the carboxylic acid of SU-TP (**Figure 4.4**) to produce Ac-AEYLRK-SU-TP and Ac-RALEK-SU-TP. Synthesis of both SU-TP and conjugation of SU-TP to the Ac-AEYLRK and Ac-RALELK peptides was performed by Dr Marco Cominetti. Identification of the correct products was conducted via MALDI-TOF mass spectrometry and purities of $\geq 95\%$ were confirmed via HPLC (see **Chapter 8.2.4**).

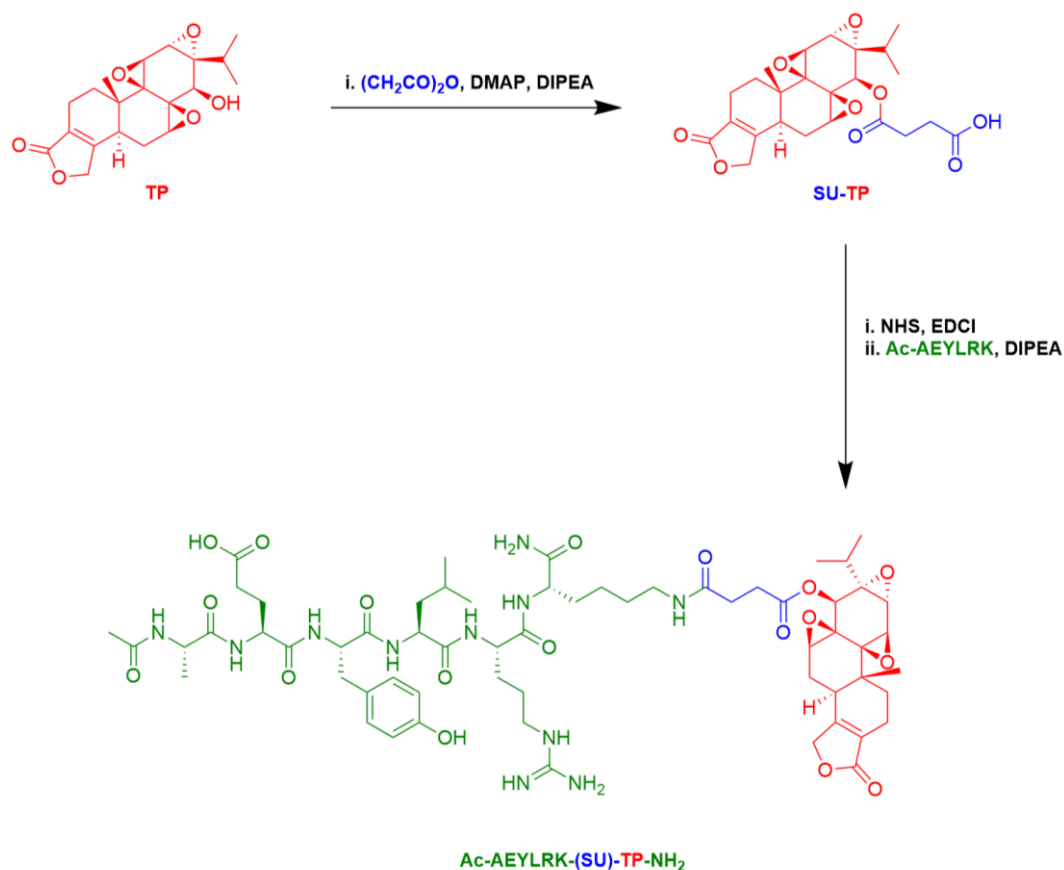


Figure 4.4 Synthesis of Ac-AEYLRK-(SU-TP)-NH₂ peptide conjugate. Conjugation of triptolide to the succinate linker was carried out as previously described⁴⁹⁸ and coupling of SU-TP to Ac-AEYLRK or Ac-RALELK. Abbreviations: DMAP; 4-dimethylaminopyridine, NHS; N-hydroxysuccinimide, EDCI; 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide.

4.3.2 Selection of EGFR^(low) and EGFR^(high) expressing cell lines

To test the specificity of the triptolide-peptide conjugate to target EGFR overexpressing cancer cells, cell lines with both high EGFR (EGFR^(high)) and low (EGFR^(low)) expression were used. To select suitable cell lines, EGFR expression was determined via flow cytometry, following staining with a mouse anti-EGFR antibody and secondary goat anti-mouse AlexaFluor488 antibody (**Figure 4.5**). Here, the A549 lung adenocarcinoma cell line, which displays high expression of Nrf2 in our study (**Figure 3.1**) and has been previously found to have high EGFR expression^{494,498,499} was selected. Although we have found high expression of Nrf2 in the M202 melanoma cells (**Figure 3.1**), to our knowledge, this cell line has not previously been characterised for EGFR expression.

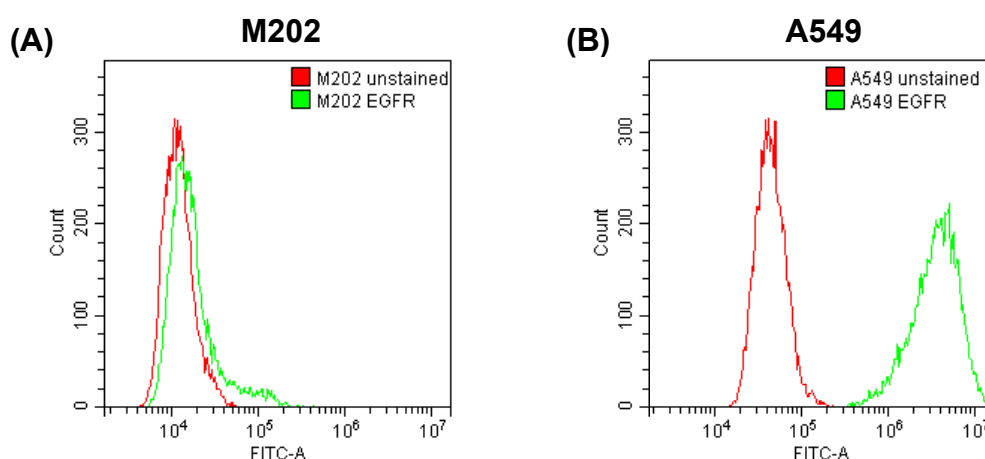


Figure 4.5 EGFR expression is higher in A549 compared to M202 cells. Overlay histograms of **(A)** A549 and **(B)** M202 cells incubated with PBS alone (unstained) or anti-EGFR antibody in PBS (EGFR). 1×10^6 cells/ condition were collected and incubated with PBS 0.05% BSA or primary mouse anti-EGFR antibody (1 $\mu\text{g/mL}$). All samples were then incubated with secondary goat anti-mouse AlexaFluor488 antibody (1 $\mu\text{g/mL}$). Fluorescence intensity was measured via flow cytometry. Unstained cells are represented in red, stained cells are represented in green. (N=3).

Table 4.2. Average fluorescence intensity of M202 and A549 cells incubated with PBS (unstained) or anti-EGFR antibody (EGFR stained). M202 and A549 cells were analysed for EGFR expression using flow cytometry and median fluorescence intensity for the FITC-A channel was calculated using CytExpert software. Data is representative of three individual experiments (N=3).

Cell line	Average fluorescence (FITC-A)		Fold increase in fluorescence
	Unstained	EGFR stained	
M202	12442.9	14716.7	1.2
A549	6988.45	452565.7	64.8

As shown in **Figure 4.5A**, EGFR expression was low in M202 cells, with a fold increase in fluorescence intensity of 1.2 of EGFR-stained compared to unstained cells (**Table 4.2**). On the other hand, A549 cells showed very high expression of EGFR via flow cytometry (**Figure 4.5B**), with a fold increase in fluorescence intensity of 64.8, compared to unstained cells (**Table 4.2**). Hence, the M202 cells were selected as the EGFR^(low) and the A549 cells selected as the EGFR^(high) cell lines for future studies.

4.3.3 Anti-proliferative effects of Ac-AEYLRK-SU-TP and Ac-RALELK-SU-TP in EGFR^(low) and EGFR^(high) expressing cell lines

Following the selection of EGFR^(low) and EGFR^(high) expressing cancer cell lines, we next investigated the effects of the triptolide-peptide conjugates (Ac-AEYLRK-SU-TP and Ac-RALELK-SU-TP) on cell proliferation. To achieve this, M202 and A549 cells were treated with serial dilutions of Ac-RALELK-SU-TP, Ac-AEYLRK-SU-TP and triptolide (TP) for 72 h and cell proliferation was measured via MTS assay (**Figure 4.6**).

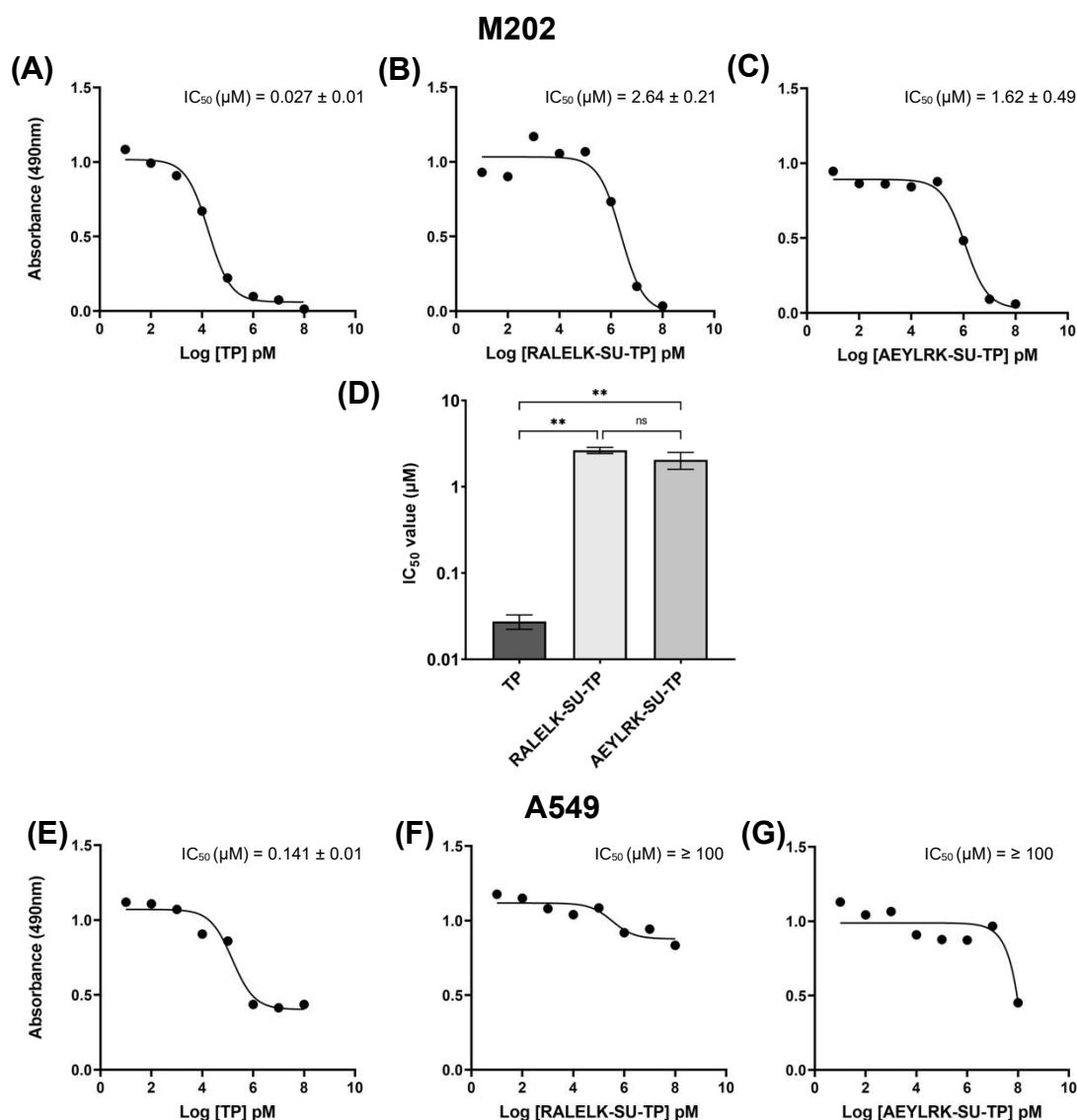


Figure 4.6 Effects of triptolide, scrambled and EGFR-targeting triptolide-peptide conjugates on cell proliferation of A549 and M202 cells. Representative concentration-response curves of (A,B,C) M202 and (E,F,G) A549 cells treated with triptolide alone (TP), scrambled (RALELK-SU-TP) and EGFR targeting (AEYLRK-SU-TP) triptolide-peptide conjugates for 72 h. Cell proliferation was measured via MTS assay and IC_{50} values were calculated using Graphpad Prism 9. (D) Bar chart of IC_{50} values of TP, AEYLRK-SU-TP and RALELK-SU-TP in M202 cells, presented as mean $\pm$ SEM (N=3). Statistical analysis was performed via one-way ANOVA and post-hoc Tukey test (* $P \leq 0.05$, ** $P \leq 0.01$, ns= not significant).

As displayed in **Figure 4.6A and 4.6E**, the M202 cells were more sensitive to triptolide alone than the A549 cells. The triptolide-peptide conjugates possessed greater IC₅₀ values than triptolide alone in both the M202 and A549 cells, denoted by the shift in the sigmoidal curve to the right, suggesting that addition of the peptide reduced triptolide's potency (**Figure 4.6**). Additionally, although in the M202 cells, a slight difference in IC₅₀ value was observed between the EGFR-targeting (Ac-AEYLRK-SU-TP) and scrambled (Ac-RALELK-SU-TP) peptides (1.62 μ M compared to 2.64 μ M, respectively), this difference was not significant (**Figure 4.6D**).

In the EGFR^(high) A549 cells, conjugation of the peptides onto triptolide drastically diminished triptolides potency, compared to triptolide alone (**Figures 4.6F-G**). However, it was not possible to calculate IC₅₀ values for either the AEYLRK-SU-TP or RALELK-SU-TP peptides as the concentration-response curves did not show a typical sigmoidal trend and did not reach plateaus $\leq 50\%$ inhibition (**Figures 4.6F and G**), signifying that their IC₅₀ values are ≥ 100 μ M. Despite this, differences in the trend of the curve suggest that the AEYLRK-SU-TP EGFR targeting peptide conjugate may have more potency than that of the RALELK-SU-TP scrambled peptide conjugate, as the former's concentration-response curve began to reach 50% inhibition at 100 μ M, while the RALELK-SU-TP curve remained above 50% inhibition at the same concentration (**Figure 4.6F and 4.6G**). Unfortunately, due to the small scale on which the triptolide-peptide conjugates were synthesised, higher concentrations were not tested, hence IC₅₀ values could not be fully characterised.

4.3.4 Targeting the Nrf2/MafG protein-protein interaction using a MafG peptide mimic

The majority of the Nrf2 inhibitors currently described in the literature are natural products, such as triptolide²⁵⁶, which work by either repressing Nrf2 protein expression, inducing its degradation or by enhancing its export from the nucleus. An alternative method of suppressing Nrf2 activity in Nrf2-addicted cancers is to disrupt Nrf2 from binding to the ARE by interrupting the Nrf2/MafG interaction. Disruption of the Nrf2/MafG PPI via mutation of the MafG ZIP domain has been found to abrogate Nrf2 signalling, demonstrating that this interaction plays a key role in Nrf2's transcriptional activity¹²⁶. Both Nrf2 and MafG are α -helical bZIP proteins, which can interact via key leucine residues that 'zip' together to form a coiled coil structure (as shown in **Figure 4.7**).

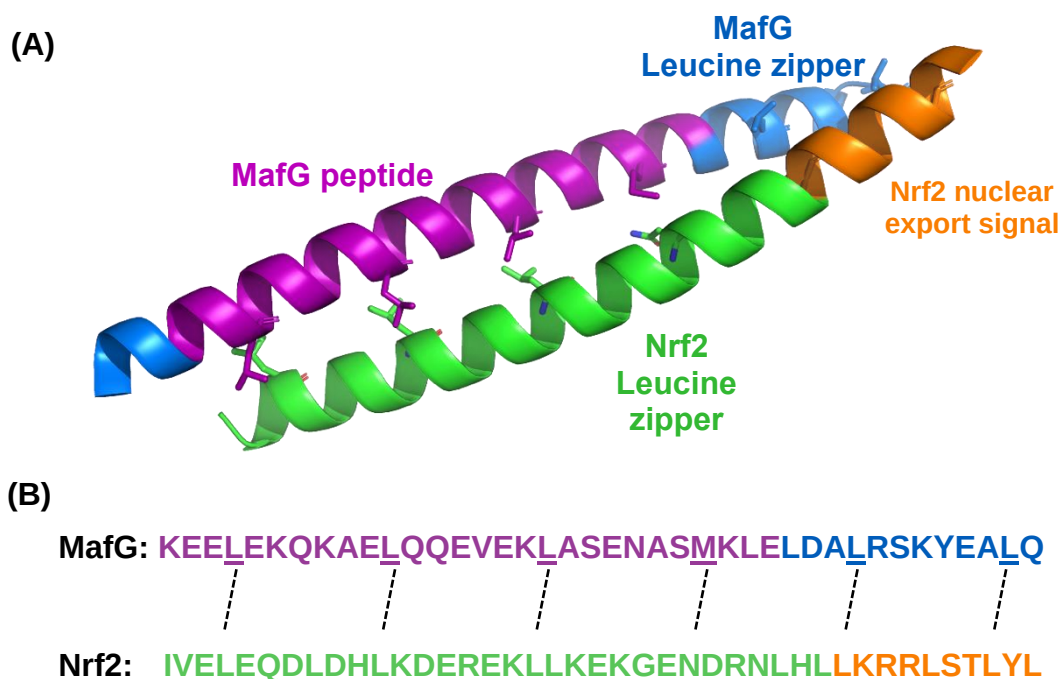


Figure 4.7 Schematic of the design of the MafG peptide mimic. (A) AlphaFold prediction⁴²⁸ of the MafG/Nrf2 coiled-coiled interaction, generated using ColabFold⁴²⁷ and modelled in PyMOL software. (B) The sequences of the MafG and Nrf2 ZIP domains that form the Nrf2/MafG leucine zipper. MafG is presented in blue, Nrf2 in green, and the Nrf2 nuclear export signal in orange, with the key leucine zipper residues displayed as (A) stick form or (B) underlined. Interactions forming the leucine zipper are shown in black dotted lines. The MafG peptide (purple) was designed from a section of the MafG leucine zipper sequence that does not overlap with the nuclear export signal of Nrf2.

The coiled coil PPI of Nrf2/MafG also masks a nuclear export signal (NES) present in the ZIP domain of Nrf2, thereby retaining Nrf2 within the nucleus to promote Nrf2 signalling¹²⁶, as displayed in **Figure 4.7**. Hence, we proposed that disruption of the Nrf2/MafG PPI could be useful as a novel way of inhibiting Nrf2 signalling in cancer.

To prevent the interaction between Nrf2/MafG, a peptide mimic of the MafG ZIP domain that retains 4 of the key leucine residues of the zipper was designed and synthesised by Dr Andrew Beekman, as displayed in magenta in **Figure 4.7**. In order to prevent masking of the Nrf2 NES domain by the MafG peptide, and thereby disrupt Nrf2 nuclear localisation, a truncated section of the MafG ZIP domain was selected, comprising of the sequence: KEELEKQKAELQQEVEKLASENASKLE, which lacks the residues required for interaction with the Nrf2 NES domain¹²⁶ (as highlighted in **Figure 4.7B**).

4.3.5 MafG peptide mimic suppresses binding of Nrf2 to the ARE

Firstly, to identify whether the MafG peptide mimic could interfere with Nrf2 binding to the ARE, a DNA-binding ELISA was conducted, as previously described (**Figure 3.6**). For these experiments, nuclear extracts of A549 cells were first prepared. These extracts were then added to a 96-well plate that contained the ARE consensus sequence (5'-GTCACAGTGAAGTCAGCAGAATCTG-3') coated on the bottom of each well. Concentrations of 25-100 μ M MafG peptide mimic were then added to the wells for 1 h, prior to quantification of DNA-bound Nrf2 via ELISA. In these experiments, recombinant Nrf2 is included as a positive control (**Figure 4.8A**).

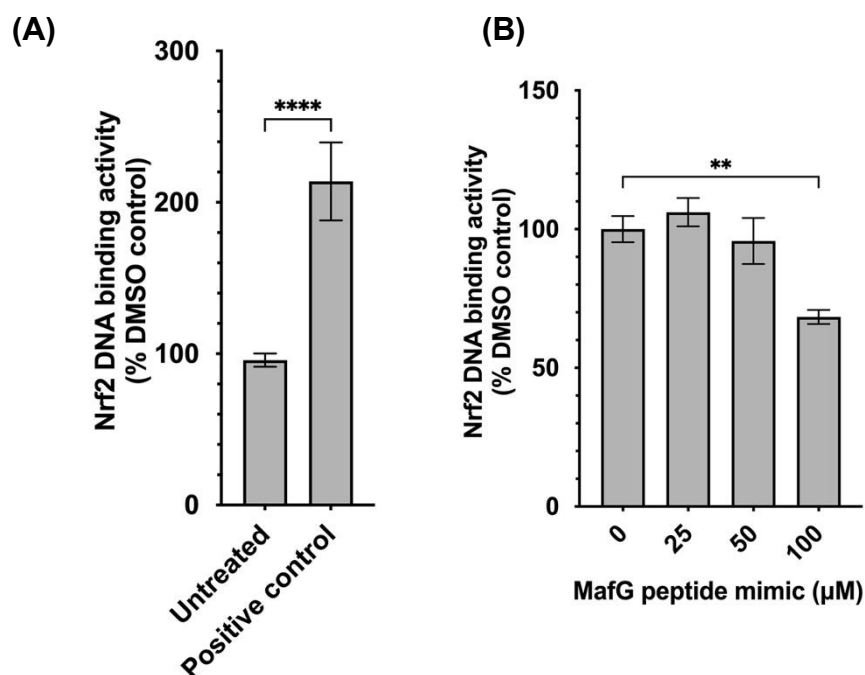


Figure 4.8 MafG peptide mimic represses Nrf2 DNA-binding activity in a concentration-dependent manner. Nuclear extracts of A549 cells were prepared from 3×10^5 cells/ sample and were added to 96-well plates coated with the ARE consensus sequence. Wells were (A) untreated or subjected to positive control (recombinant Nrf2) or were incubated with (B) 25-100 μ M MafG peptide mimic for 1 h. Nrf2 DNA-binding activity was then determined via ELISA. Data is presented as percent change with respect to DMSO vehicle control $\pm$ SEM (N=3). Statistical analysis was conducted via one-way ANOVA and post-hoc Dunnett's test (**P $\leq$ 0.01).

As shown in **Figure 4.8B**, compared to vehicle control, the MafG peptide mimic inhibited Nrf2-DNA binding activity in nuclear extracts of A549 cells in a concentration-dependent manner. Here, the greatest inhibitory effects were observed with 100 μ M MafG peptide mimic, wherein inhibition to 68% DMSO vehicle control was observed (**Figure 4.8B**). At 50 μ M, a slight reduction in Nrf2-DNA binding activity was observed, but 25 μ M did not decrease activity (**Figure 4.8B**).

4.3.6 The MafG peptide mimic does not potently affect A549 cell proliferation

As the previous experiments were conducted on nuclear Nrf2 derived from cell lysates, we next explored the effect of the MafG peptide mimic in live cells. For this, we selected A549 cells as these possess particularly high Nrf2 expression (**Figure 3.3**). Firstly, we characterised the effects of the MafG peptide mimic on A549 cell proliferation via treatment of A549 cells with serial dilutions of the MafG peptide mimic for 72 h. Cell proliferation was then measured via MTS assay, and an IC_{50} value was calculated using the resulting concentration-response curve, as displayed in **Figure 4.9**.

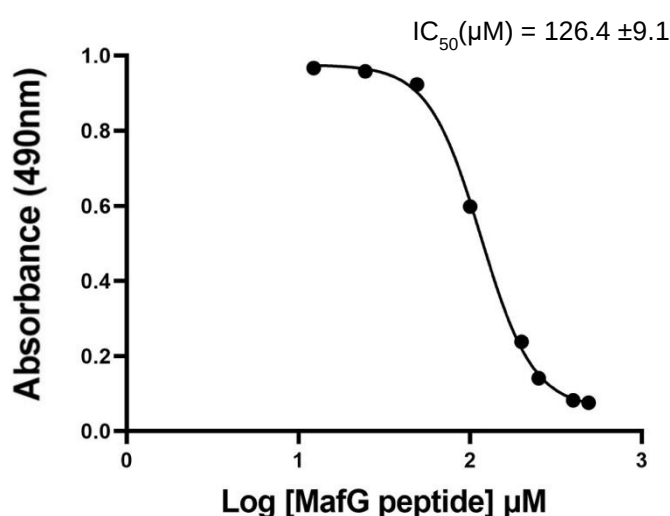


Figure 4.9 Cell viability of A549 cells following treatment with MafG peptide mimic.

Representative concentration-response curve of cell viability of A549 cells (5×10^3 cells/well) treated with serial dilutions of MafG peptide mimic for 72 h. Cell viability was measured via MTS assay and the IC_{50} value was calculated using Graphpad Prism 9 software. Data is representative of 3 individual experiments (N=3) and the IC_{50} value is presented as mean $IC_{50} \pm SEM$.

From these experiments, the mean IC_{50} value was identified to be 126.4 μ M, suggesting that the MafG peptide mimic does not have potent anti-proliferative effects in A549 compared to other Nrf2 inhibitors such as brusatol and triptolide (**Figure 3.2**).

4.3.7 The MafG peptide mimic significantly represses Nrf2 downstream gene expression in A549 cells

Next, to study the effects of the MafG peptide on Nrf2 signalling, we first explored its ability to repress Nrf2 target gene expression, such as NQO1 and HO-1. To select the best treatment time, a time-response experiment was conducted using 100 μ M of MafG peptide, as this concentration demonstrated the best inhibitory activity in the Nrf2-DNA binding ELISA previously conducted (**Figure 4.8**) and was below the IC₅₀ for cell viability (**Figure 4.9**). A549 cells were then treated with MafG peptide mimic for 2-48 h and NQO1 and HO-1 mRNA expression was measured via RT-qPCR (**Figure 4.10**).

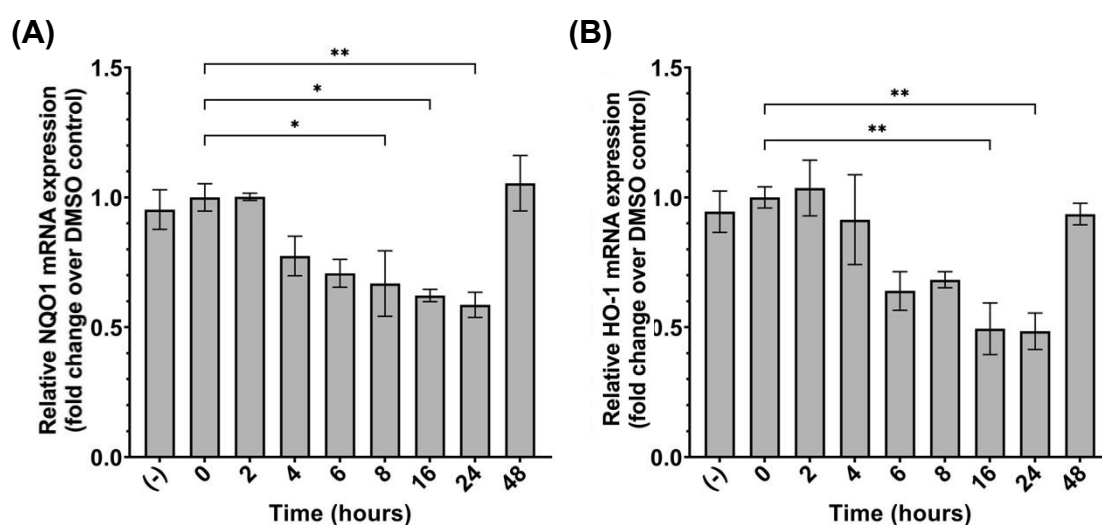


Figure 4.10 MafG peptide mimic represses Nrf2-target gene expression in A549 cells in a time-dependent manner. A549 cells (5×10^5 cells/ well) were treated with 100 μ M MafG peptide mimic for 0-48 h or media alone (-). Relative mRNA expression of **(A)** NQO1 and **(B)** HO-1 was then analysed via RT-qPCR, following RNA isolation and cDNA synthesis. Expression was normalised to GAPDH and is presented as fold change in expression compared to vehicle control. Data is presented as mean $\pm$ SEM (N=3) and statistical analysis was performed using one-way ANOVA and post-hoc Dunnett's test (*P $\leq$ 0.05, **P $\leq$ 0.01).

As shown in **Figure 4.10A**, 100 μ M of the MafG peptide mimic repressed NQO1 mRNA expression in a time-dependent manner from 2-24 h, with significant effects observed between 8-24 h treatment. The greatest inhibition of NQO1 mRNA was observed with 24 h treatment (**Figure 4.10A**). Additionally, HO-1 mRNA expression was also inhibited in a time-dependent manner, compared to vehicle control, with treatment times of 16 and 24 h showing the best inhibitory effects (**Figure 4.10B**). Interestingly, both NQO1 and HO-1 mRNA expression returned to levels observed with vehicle control by 48 h (**Figure 4.10**), suggesting that inhibition is transient. Furthermore, inhibition of HO-1 mRNA

expression only appeared from 4 h onwards, compared to 2 h onwards observed for NQO1 mRNA expression (**Figure 4.10**).

From the data in **Figure 4.10**, a treatment time-point of 24 h was selected for further experiments, as it showed the best inhibitory effects on both NQO1 and HO-1 mRNA expression. To further characterise the effects of the MafG peptide mimic on Nrf2-target gene expression, a concentration-response experiment was conducted for effects on both NQO1 and HO-1 mRNA expression (**Figure 4.11**).

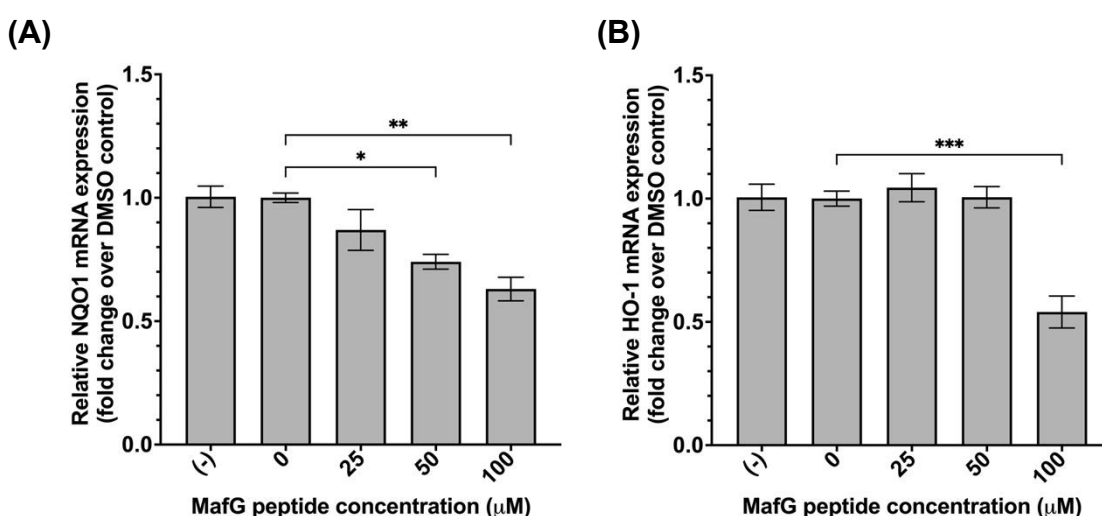


Figure 4.11 MafG peptide inhibits Nrf2-target gene expression in A549 cells in a concentration-dependent manner. Total RNA of A549 cells (5×10^5 cells/ well) was isolated following cell treatment with 0-100 µM MafG peptide mimic for 24 h or media alone (-). Relative mRNA expression of **(A)** NQO1 and **(B)** HO-1 was analysed via RT-qPCR. Expression was normalised to GAPDH and is presented as fold change in expression compared to DMSO vehicle control. Data is presented as mean $\pm$ SEM (N=3) and statistical analysis was performed using one-way ANOVA and post-hoc Dunnett's test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

For these experiments, A549 cells were treated with 0-100 µM MafG peptide mimic for 24 h prior to RNA extraction and NQO1 and HO-1 mRNA expression was measured via RT-qPCR. **Figure 4.11A** shows that the MafG peptide mimic displayed concentration-dependent inhibitory effects on NQO1 mRNA expression in A549 cells, with the largest reduction observed with 100 µM, which significantly reduced NQO1 mRNA expression to that of 63% of DMSO control (**Figure 4.11A**). Additionally, the MafG peptide also significantly inhibited HO-1 mRNA expression at 100 µM to that of 54% of DMSO control, although no effects were observed with 25 µM or 50 µM treatment (**Figure 4.11B**).

4.3.8 The MafG peptide mimic inhibits NQO1 enzymatic activity in A549 cells in a concentration-dependent manner

As we observed that the MafG peptide mimic could inhibit NQO1 mRNA expression in A549 cells, we next explored effects on NQO1 enzymatic activity, using a previously described assay⁴¹⁶. Here, A549 cells were treated with 0-100 μ M MafG peptide mimic for 24 h and NQO1 activity was determined via enzymatic assay. In these studies, 0.1 μ M brusatol was included as a positive control (as previously validated in **Figure 3.8**) and reaction buffer without substrate (-Menadione) was included as a negative control (**Figure 4.12A**).

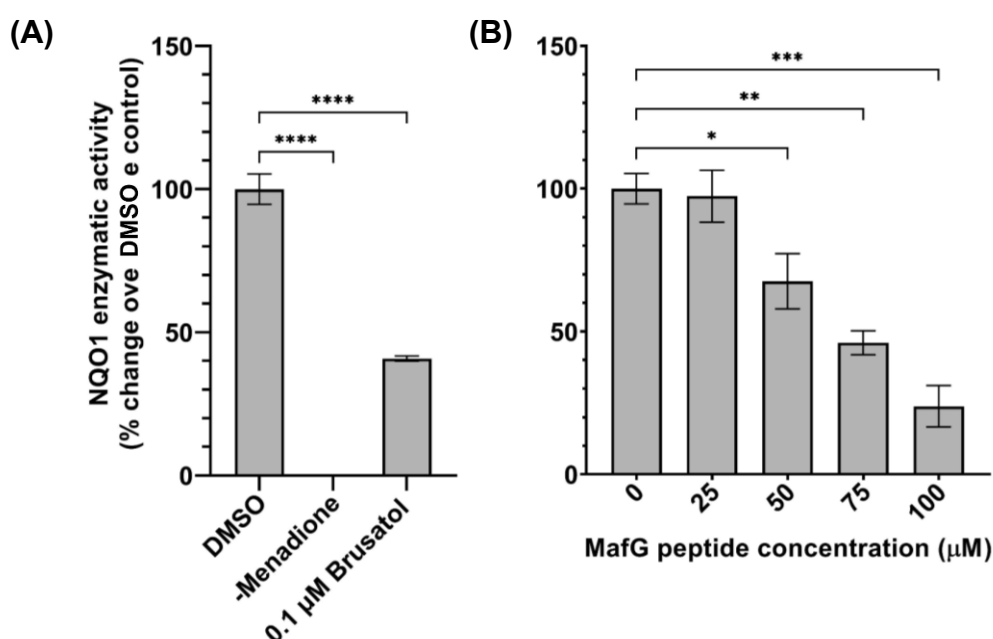


Figure 4.12 MafG peptide mimic inhibits NQO1 enzymatic activity in A549 cells in a concentration-dependent manner. A549 cells (8×10^3) were incubated with (A) 0.1 μ M brusatol or (B) 25-100 μ M MafG peptide mimic for 24 h and NQO1 activity was quantified via enzymatic assay. Reaction buffer without substrate (-Menadione) was included as a negative control. Data is presented as mean NQO1 activity (% change over DMSO control) $\pm$ SEM (N=3). Statistical analysis was conducted via one-way ANOVA and post-hoc Dunnett's test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

As displayed in **Figure 4.12B**, the MafG peptide mimic significantly repressed NQO1 enzymatic activity in a concentration-dependent manner. The greatest inhibition of NQO1 activity was observed with 75 μ M and 100 μ M, with activity reduced to that of 48.4% and 23.8% of DMSO control, respectively (**Figure 4.12B**). On the other hand, no inhibition of NQO1 activity was observed with 25 μ M treatment (**Figure 4.12B**). As 100 μ M showed the best inhibitory activity on Nrf2-DNA binding activity, NQO1 and HO-1 mRNA expression and NQO1 activity, this concentration was selected for future experiments.

4.3.9 The MafG peptide mimic sensitises A549 cells to gemcitabine

As we found that the novel MafG peptide mimic was able to repress Nrf2-DNA binding, Nrf2-target gene expression and gene activity, we next investigated the potential of this peptide as a chemo-sensitising agent in A549 cells. Here, gemcitabine was used (as previously described in **Chapter 3**), as this is a commonly used chemotherapeutic agent in the treatment of NSCLC that often loses efficacy once tumours become resistant^{500,501}. For these experiments, A549 cells were incubated with 100 μ M MafG peptide or 0.01-1 μ M gemcitabine, either alone or in combination for 72 h. Cell viability was then determined via MTS assay (**Figure 4.13**).

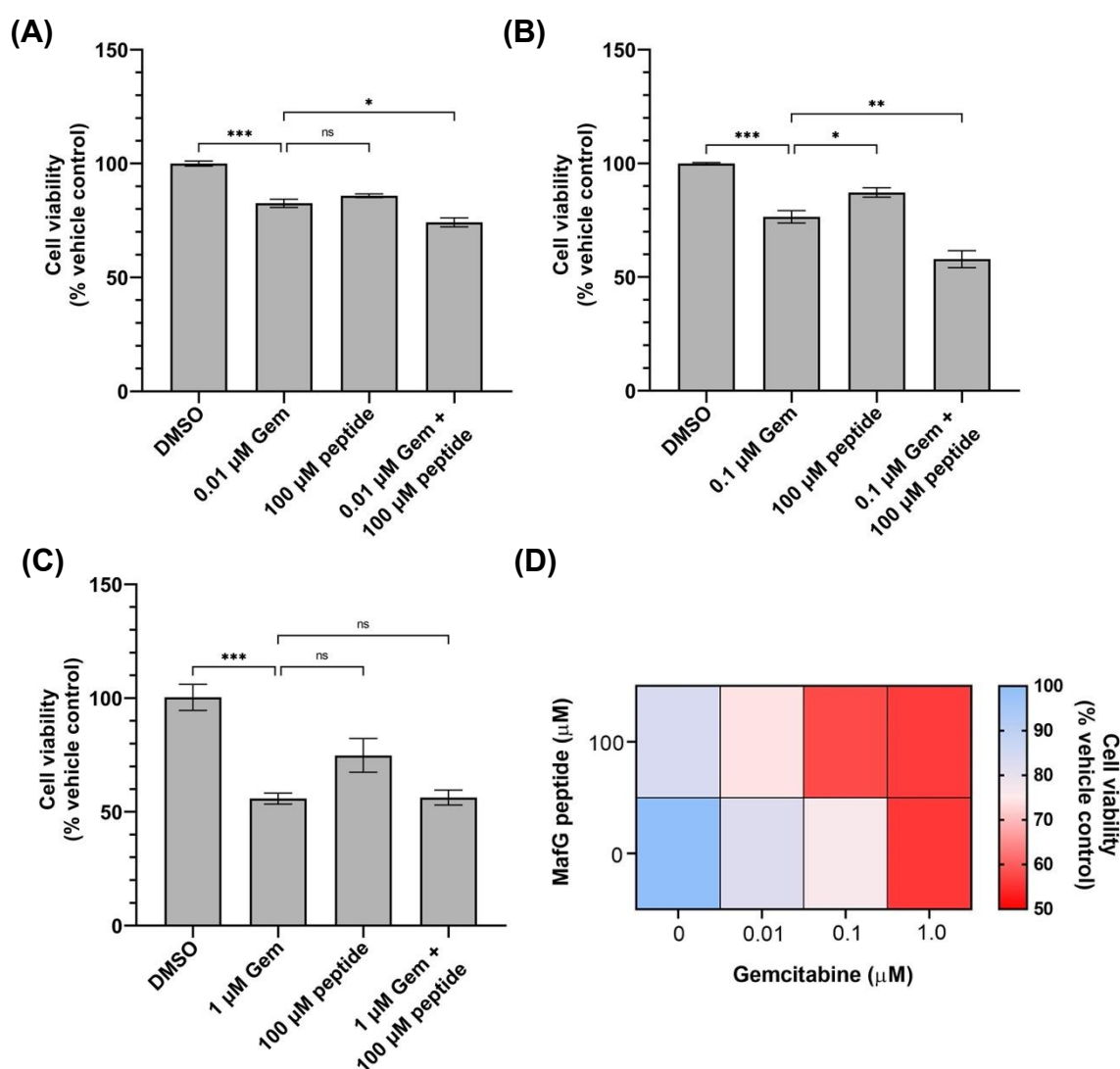


Figure 4.13 MafG peptide mimic sensitises A549 cells to gemcitabine. A549 cells (5×10^3 cells/ well) were treated with **(A)** 0.01 μ M, **(B)** 0.1 μ M or **(C)** 1 μ M gemcitabine (Gem) or 100 μ M MafG peptide (peptide) alone, or in combination for 72 h and cell proliferation was determined via MTS assay. Data is presented as mean cell viability (% vehicle control) $\pm$ SEM (N=3) as both **(A-C)** bar charts **(D)** heat map. Statistical analysis was performed via one-way ANOVA and post-hoc Tukey test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

As displayed in **Figure 4.13**, no significant difference was observed between co-incubation of 100 μ M MafG peptide mimic with 1 μ M gemcitabine, compared to gemcitabine alone (**Figure 4.13A and C**). However, combination of 100 μ M and 0.1 μ M significantly reduced cell proliferation from 67.5% of DMSO control for gemcitabine alone to 57.9% in cells treated with the MafG peptide mimic and gemcitabine co-incubation (**Figure 4.13B**). Furthermore, while cell treatment with the MafG peptide mimic does not greatly affect cell proliferation (87.2% of DMSO control), the co-incubation of 100 μ M MafG peptide and 0.1 μ M gemcitabine reduced cell proliferation to a similar level achieved with the highest concentration of gemcitabine alone (1 μ M; a 10-fold increase in concentration), suggesting that the MafG peptide mimic may sensitise A549 cells to gemcitabine.

4.4 Discussion

Inhibitors of the Nrf2 pathway have been explored as potential anti-cancer treatments in many cancer types⁴³², however, the majority of these inhibitors are small molecules and natural products, many of which possess drawbacks such as off-target effects or contradicting effects on Nrf2 signalling. Alternative methods of specifically targeting Nrf2 in cancer may therefore prove valuable as both anti-cancer agents and to better understand Nrf2 signalling in Nrf2-addicted cancers. One method in which this could be addressed is via the use of targeted therapies, however at present, no targeted therapies aimed to inhibit Nrf2 have been reported. In this study, we describe two novel targeted methods of inhibiting Nrf2 in NSCLC cancer, including a peptide-drug conjugate (PDC) incorporating the Nrf2 inhibitor triptolide, and a novel MafG peptide mimic, designed to repress Nrf2 signalling via disrupting the Nrf2/MafG protein-protein interaction (PPI).

As discussed in **Chapter 3**, we characterised triptolide as a potent inhibitor of cell proliferation in several cancer cell lines, as well as a robust inhibitor of Nrf2 signalling in A549 NSCLC cells. One of the drawbacks that triptolide has faced however, is that it induces several off-target effects, likely due to its ability to repress mRNA synthesis of additional proteins besides Nrf2^{480,481}. In relation to this, triptolide has also been shown to possess cardiotoxicity and hepatotoxicity, which has limited its progression into clinical trials⁴⁷⁷. Despite these drawbacks, the potency of triptolide as a cytotoxic agent, both observed in the present study (**Figure 3.2**) and in the literature⁴⁷⁵, makes it an ideal candidate for use in a targeted therapy to limit triptolide delivery to the tumour site.

Recently, Zhang *et al.*⁴⁹⁸ synthesised an anti-EGFR antibody-triptolide conjugate, for potential use in NSCLC and head and neck cancers, via conjugation of triptolide to cetuximab, an anti-EGFR monoclonal antibody used in the clinic. Results from this research found that the cetuximab-triptolide conjugate was not only successfully internalised into EGFR overexpressing cells, such as A549, but also induced apoptosis, repressed cell proliferation and RNA polymerase II expression in EGFR^{high} but not EGFR^{low} tumour models using *in vitro* cancer cell cultures and *in vivo* murine tumour xenograft models⁴⁹⁸. Interestingly however, the effect of the cetuximab-triptolide conjugate on Nrf2 signalling and ability to sensitise cells to chemotherapy was not explored. While this approach supports the use of EGFR-targeting moieties as a delivery system for triptolide in NSCLC, as discussed in **Chapter 4.1**, ADCs are both difficult and costly to synthesise compared to PDCs⁴⁵. Hence, in the present study, an EGFR-targeting peptide was selected for use in a novel triptolide-PDC, to specifically deliver the cytotoxic effects of triptolide to cancer cells with high EGFR expression.

Several EGFR-binding peptides have been reported in the literature^{487,488,490}, including a peptide of the sequence AEYLR, which displayed the greatest binding affinity for EGFR among several peptides in a study by Han *et al.*⁴⁸⁹. Previous studies in our lab in collaboration with the Searcey lab have also validated AEYLR as an EGFR-targeting peptide and have examined its suitability for use in nanoparticle directed photodynamic targeted therapies in NSCLC⁴⁹⁴. Hence, the AEYLR peptide was selected for use in a triptolide-PDC in the present study. To produce the triptolide-PDC, the EGFR-targeting peptide AEYRLK and a scrambled control (of the sequence RALELK) was synthesised by Fmoc-SPPS synthesis.

Following this, triptolide was then conjugated to a succinate linker, which is commonly used in PDCs as a non-cleavable linker⁴⁵ and has been previously used to conjugate triptolide to a targeting moiety in the cetuximab-triptolide ADC⁴⁹⁸. The triptolide-succinate compound (SU-TP) was then linked to the lysine residues of Ac-AEYLRK and Ac-RALELK to produce the final PDCs; Ac-AEYLRK-SU-TP and Ac-RALELK-SU-TP (**Figure 4.4**).

To characterise whether the EGFR targeting triptolide-PDC could specifically deliver triptolide to EGFR overexpressing cells, firstly cancer cell lines with low (EGFR^(low)) and high EGFR (EGFR^(high)) expression were selected. While the A549 NSCLC cell line has been previously shown to have high expression of EGFR⁴⁹⁹, the M202 melanoma cells were previously not characterised for their EGFR expression. From an initial screen of several cancer cell lines using flow cytometry (data not shown), the A549 and M202 cells,

both of which show high expression of Nrf2 (**Figure 3.1**), were further explored for EGFR expression via flow cytometry. As the A549 cells possessed high EGFR expression and the M202 cells showed low EGFR expression, as indicated by the fold change in median fluorescence intensity of unstained vs EGFR-stained cells (**Table 4.2**), the M202 and A549 cells were selected as the EGFR^(low) and EGFR^(high) cell lines used in this study.

Using these cell lines, the anti-proliferative effects of the EGFR-targeting and scrambled triptolide-PDCs were explored via IC₅₀ calculations from MTS assays. Interestingly, the anti-proliferative effects of triptolide were much less potent when conjugated to the AEYLRK and RALELK peptides for both EGFR^(low) M202 and EGFR^(high) A549 cells, compared to triptolide alone (**Figure 4.6**). This is not unusual however, as conjugation to targeting moieties often decreases the overall potency of anti-cancer drugs. For example, although IC₅₀ values were not calculated for the cetuximab-triptolide conjugate described by Zhang *et al.*⁴⁹⁸, the cetuximab-triptolide conjugate was approximately 250 times less potent than triptolide alone at inhibiting cell proliferation via MTS assay in A549 cells. In the M202 cells studied herein, while there was a significant increase in IC₅₀ value between triptolide alone and the two PDCs, no significant difference between the IC₅₀s of the EGFR-targeting and scrambled peptides was observed (**Figure 4.6D**). This suggests that although the PDC reduced sensitivity to triptolide's anti-proliferative effects in EGFR^(low) cells, triptolide was still able to induce some anti-proliferative effects, regardless of the peptide that was conjugated.

In EGFR^(high) A549 cells however, a decrease in potency between triptolide alone and the EGFR-targeting and scrambled PDCs was observed, with IC₅₀ values increasing from 0.140 μ M to >100 μ M for triptolide and PDCs, respectively (**Figure 4.6E-G**). Additionally, while triptolide alone displayed a typical sigmoidal concentration-response curve, the two PDCs did not show the same trend, meaning that IC₅₀ values could not be calculated for either of these conjugates (**Figure 4.6E-G**). In the case of the scrambled PDC, the sigmoidal curve shape appeared to be present, however inhibition of cell viability was minimal and did not reach 50% inhibition (**Figure 4.6F**). On the other hand, while the trend for the EGFR-targeting PDC initially showed a similar trend to that of the scrambled PDC, the cell viability dramatically decreased at 100 μ M to approx. 50% (**Figure 4.6G**). Although this may suggest that higher concentrations of the EGFR-targeting PDC could reach and sustain 50% inhibition, as observed with triptolide (**Figure 4.6E**), as the curve did not reach a plateau it was not possible to calculate an IC₅₀ value. Despite this, the difference in trendlines between the scrambled and EGFR-targeting PDCs in the A549 cells suggests that the EGFR-targeting PDC may induce some selective delivery of triptolide to EGFR overexpressing cells.

Importantly however, as the PDCs showed a much lower potency than unconjugated triptolide and would likely need to be administered at concentrations $>100\ \mu\text{M}$, at present they may not be optimised for use as a targeted chemotherapy agent. Although potencies within the micromolar range is not unexpected when cytotoxic agents are conjugated to targeting moieties, such as in ADCs⁴⁹⁸, possible further steps to improve the potency of the PDC could be to utilise a different chemical linker, as the current succinate linker may be interfering with triptolide's ability to induce cytotoxic effects. This could be due to a number of reasons; for example, the linker may interfere with the ability of the PDC to bind to EGFR and become internalised into the cell. To address this, other linkers with different cleavage mechanisms or longer chain lengths could be studied for use in our PDC.

Additionally, another limiting factor in these experiments was the small quantity of the PDCs that could be synthesised. As commercially available triptolide is expensive, synthesis was initially performed on a small scale, with a yield of only $\sim 4\text{mg}$ for the triptolide-PDCs. While this limited the ability to characterise the IC_{50} s of the PDCs, such an approach was necessary while the synthesis was being optimised. Additionally, we also planned to explore the uptake of the PDC's into cells via fluorescence microscopy using fluorescently tagged peptides, produced via conjugation of a fluorescein isothiocyanate (FITC) fluorescent tag on the peptides C-termini. However, although the FITC-AEYLRK and FITC-RALELK could be successfully synthesised, the conjugation of these peptides onto SU-TP proved difficult as the FITC tag quickly degraded during purification and handling. Further research could therefore study the effects of different linkers and different fluorescent tags, in order to characterise and assess the efficacy of these triptolide-PDCs in both cancerous and non-cancerous cell lines.

Despite this, increasing evidence suggests that targeted delivery of triptolide is a promising strategy in a number of cancer types, with several studies having explored the conjugation of triptolide to other targeting moieties and delivery systems such as nanoparticles⁵⁰²⁻⁵⁰⁴, sugar molecules^{505,506} and oligonucleotides⁵⁰⁷. Although to date, none of these studies have explored their effects on Nrf2 signalling, they have proven that triptolide-conjugates could reduce the systemic toxicity of triptolide and increase its therapeutic window in *in vivo* models of several tumour types, including NSCLC, hepatocellular carcinoma and head and neck squamous cell carcinomas⁴⁸². While an EGFR-targeting moiety was selected in the present study, the potential of triptolide as a targeted therapy is broad, especially in the case of a triptolide-PDC, which could utilise simple and cheap synthesis of small targeting peptides to target triptolide towards a

variety of tumour antigens. Furthermore, as triptolide also has potent anti-inflammatory activity⁵⁰⁸, these targeted delivery systems could also be adapted for use in chronic inflammatory disease, such as rheumatoid arthritis and inflammatory bowel disease.

While the majority of Nrf2 inhibitors that have been reported in the literature thus far are natural products, interest into small molecule and peptide inhibitors of Nrf2 signalling is increasing. This would address the issue of availability and affordability, as some natural products, such as triptolide, are both complex to synthesise synthetically and are difficult to isolate⁵⁰⁹. One novel way of targeting Nrf2 is to disrupt its interaction with sMaf proteins, which is required in order to induce Nrf2 target gene transcription¹⁵⁰. To date, only one small molecule, ML385, has been reported to inhibit the Nrf2/MafG interaction, however conflicting evidence in the literature^{358,359} and in our own studies (see **Chapter 3**) suggests that alternative methods of disrupting the Nrf2/MafG PPI could be explored. Previously, the main hurdle in the development of such inhibitors is the absence of a fully resolved crystal structure of Nrf2/MafG, however advances in protein structure predictions via the use of artificial intelligence systems, such as AlphaFold⁴²⁸, have assisted in better understanding and modelling PPIs, such as Nrf2/MafG. Furthermore, while PPIs were previously believed to be “undruggable” targets, several PPI inhibitors have shown great promise in disrupting PPIs involved in cancer progression³⁹¹, including one p53 peptide mimic that is currently being investigated in Phase I clinical trials of leukemic patients³⁹². Although PPIs can be targeted with both small molecules and peptides, compared to small molecules, peptides are both larger and can better mimic the secondary structures of native proteins, allowing them to bind to hot spots within the hydrophobic core of PPIs⁴⁹¹. Additionally, ‘peptide mimics’ derived from the native protein sequence can bind with high affinity to the PPI interface and therefore block binding of the native protein⁵¹⁰.

To study the viability of this approach to repress Nrf2 signalling in cancer, an initial proof of concept study was conducted based upon disrupting the Nrf2/MafG heterodimer with a MafG peptide mimic. At present, only 3 studies have explored peptide-based PPI inhibitors of the Nrf2/MafG interaction, all of which are based upon the structure of Nrf2, with only 1 demonstrating inhibitory effects on Nrf2 signalling *in vitro*³⁹⁵. As the bZIP domain of MafG is important for the formation of the Nrf2/MafG coiled coil interaction⁴⁹³, a 28 amino acid peptide was selected derived from the KEELEKQKAELQQEVEKLAENASMKLE sequence located in the MafG ZIP domain⁴⁹³ (referred to herein as the MafG peptide mimic). At commencement of this work, no crystal structure for the Nrf2/MafG interaction was reported hence, an AlphaFold⁴²⁸ predicted model of the Nrf2 and MafG leucine zipper interaction was generated (**Figure 4.7**), which

displays the key interactions between the ZIP domains of Nrf2 and MafG (highlighted in stick form in **Figure 4.7A**) that we aimed to recreate with the peptide mimic. Importantly, as shown in purple in **Figure 4.7**, the peptide sequence selected for synthesis was specifically chosen as it does not overlap with the NES domain of the Nrf2 leucine zipper, as shown in orange, which is typically masked by native MafG (blue) to promote Nrf2 localisation within the nucleus⁷⁹. This, we hypothesised, would allow for the NES domain to remain available to promote nuclear export of Nrf2, thereby further inhibiting Nrf2-driven gene transcription.

As shown in **Figure 4.8**, presence of 100 μ M MafG peptide significantly repressed Nrf2-DNA binding, suggesting that it may interrupt the Nrf2/MafG-ARE interaction. Although these results were initially promising, as the results were not conducted in live cells, further experiments were conducted to test whether effects could be observed *in vitro*. In Nrf2-overexpressing A549 cells, the MafG peptide mimic was found to have an IC₅₀ 126.4 μ M in cell proliferation assays, suggesting that while it is not a potent inhibitor of cell viability, it could inhibit cell proliferation (**Figure 4.9**). Additionally, the MafG peptide mimic significantly repressed both NQO1 and HO-1 mRNA expression in a time-dependent and concentration-dependent manner (**Figures 4.10-4.11**). The MafG peptide mimic also significantly inhibited NQO1 enzymatic activity in a concentration-dependent manner at concentrations of 50-100 μ M (**Figure 4.12B**).

As Nrf2 potentiates chemoresistance in NSCLC⁴³⁶, one of the main benefits of inhibiting Nrf2 is that it may sensitise tumours to already existing chemotherapy, through the induction of genes involved in drug efflux, resistance to apoptosis and DNA-damage repair mechanisms^{149,268,511}. To study whether the MafG peptide mimic could act as a chemotherapy sensitising agent, A549 cells were treated with gemcitabine (a clinically used chemotherapy that inhibits DNA replication⁵⁰¹) either alone or in combination with 100 μ M MafG peptide and analysed cell proliferation via MTS assay. When combined, both the MafG peptide potentiated the anti-proliferative effects of gemcitabine (**Figures 4.13A, B and D**), suggesting that the MafG peptide mimic may act to sensitise A549 cells to chemotherapy. On the other hand, while the 1 μ M gemcitabine and 100 μ M peptide combination significantly repressed cell proliferation, little difference was observed between gemcitabine alone and the combination (**Figure 4.13C**). Additionally, the combination of 0.1 μ M gemcitabine and 100 μ M MafG peptide significantly reduced cell proliferation to a similar level than that observed with the higher concentration of 1 μ M gemcitabine alone (**Figure 4.13B and C**). This suggests that the MafG peptide mimic, in combination with lower concentrations of gemcitabine (e.g. 0.1 μ M) may be as efficacious as higher concentrations of gemcitabine alone (e.g. 1 μ M). Together, these

results suggest that the MafG peptide mimic utilised here can both repress Nrf2 downstream gene expression and activity, and also sensitises NSCLC cells to chemotherapy.

To date, only 3 peptide-based inhibitors of the Nrf2/sMaf interaction have been reported in the literature. Based upon the crystal structure of the Skn-1 Nrf2-homolog in *C.elegans*, Wiedemann *et al.*³⁹³ developed a series of stapled peptides derived from the DNA binding domain of Nrf2. Although many of these peptides were able to bind the Nrf2/MafG ARE, a key issue of this study is that the peptides also show unspecific binding for randomised DNA sequences³⁹³. Additionally, all but one of the peptides investigated were not cell permeable, which limits their potential as anti-cancer agents in Nrf2-addicted cancers. Despite this, Wiedemann *et al.* did show that the cell-permeable peptide showed better nuclear localisation when stapled, compared to its linear (unstapled) counterpart³⁹³, thereby supporting the use of stapled α -helical peptides as inhibitors of the Nrf2/sMaf PPI. Another approach by Simov *et al.*³⁹⁴ found that a heterodimeric peptide inhibitor of Nrf2/MafG produced from linkage of sections of the Nrf2 and MafG ZIP domains could promote dissociation of native Nrf2/MafG from the ARE. Despite this initial promising result, the peptide developed by Simov *et al.* was also not cell permeable, possibly due to the large size of the heterodimeric peptide, and therefore did not inhibit Nrf2 target gene transcription, even when electroporated into NSCLC cells³⁹⁴. As such, efforts to produce smaller, cell penetrating peptides, such as the peptide described in the present study, may be more applicable for use in further studies.

Another application of a smaller, cell penetrating peptide to inhibit the Nrf2/sMaf PPI was described in a recent publication by Modi *et al.*³⁹⁵. This research studied the synthesis and application of stapled peptides derived from the Nrf2 ZIP sequence that could bind MafG and repress both Nrf2 transcriptional activity via luciferase assay and inhibit NQO1, HO-1, TXRND1 (thioredoxin reductase 1) and GCLC (glutamate-cysteine ligase catalytic subunit) mRNA expression in A549 NSCLC cells³⁹⁵. Additionally, the peptide was also investigated for its potential to sensitise A549 cells to cisplatin, where it showed synergistic effects in reducing cell viability, compared to cisplatin alone³⁹⁵. As the α -helicity of the Nrf2 ZIP domain is necessary for coiled coil interactions with other ZIP proteins, such as MafG, Modi *et al.* introduced peptide staples to their design, which is a useful tool in constraining the conformation of a peptide to maintain α -helicity⁵¹⁰. Although not applied in the MafG peptide mimic studied in this thesis, ongoing research by the Beekman laboratory is exploring the inclusion of staples onto the MafG peptide mimic, in conjunction with experiments to measure the α -helicity of the peptide, via

circular dichroism (data not shown). Although Modi *et al.*'s research further supports the use of peptides as inhibitors of the Nrf2/MafG complex, the ability of their Nrf2-derived stapled peptide to inhibit Nrf2 signalling has not been fully characterised and requires further study to investigate any concentration and time-dependent effects on Nrf2 target gene expression. Additionally, as these experiments were only conducted in A549 NSCLC cells, the application to other Nrf2-addicted cancer types, such as ovarian or breast cancer, could be studied.

One limitation of the present study is that, while the peptide could repress mRNA expression of Nrf2 target genes, suggesting it can enter cells, full characterisation of its ability to pass through the cell membrane and enter the nucleus has not yet been conducted. Although these peptides are relatively small, at only 28 amino acids long, further studies using a fluorescently tagged MafG peptide mimic could be used in order to understand their cellular localisation, which could be conducted via fluorescent microscopy. Inclusion of a scrambled peptide control could also assist in demonstrating the specificity of the MafG peptide mimic sequence to disrupt Nrf2 signalling. Additionally, the binding affinity of the MafG peptide mimic to bind Nrf2 was not studied in this thesis, however further studies by the Beekman lab are underway to quantify the binding affinity of both the peptide studied here, and a number of stapled and truncated MafG peptide mimics via surface plasmon resonance (SPR).

In conclusion, the data shown here however presents unique approaches to both targeting the Nrf2 inhibitor triptolide to EGFR-overexpressing cells and also describes the activity of a novel Nrf2/MafG PPI inhibitor in NSCLC, which could further be explored in numerous cancer types for application as a chemotherapy sensitising agent.

Chapter 5. Pharmacological inhibition of Nrf2 as a potential novel therapeutic intervention in melanoma

5.1 Introduction

Melanoma is the 5th most common form of skin cancer in the UK and accounts for 80% of skin cancer related deaths⁵¹². Despite increased public awareness of risk factors, such as high UV exposure, and improved methods of early detection⁵¹³, incidence rates are predicted to rise by 9% between 2023 and 2025⁵¹². Treatment of early-stage (stage 1 and 2) melanoma is primarily in the form of surgical excision of the tumour, and is typically successful, with 5-year survival rates of early-stage melanoma patients being >90%⁵¹⁴. However, late-stage (stage 3 and 4) metastatic melanoma cannot be treated solely by surgical excision and is often resistant to traditional chemotherapy, such as dacarbazine⁵¹⁵. As such, late-stage metastatic melanoma patients face limited treatment options, and 5-year survival rates remain below 30%⁵¹⁶. Although traditional chemotherapies (such as dacarbazine) may be used in melanoma, low response rates have led to exploration of alternatives resulting in advances in treatment options including immunotherapy and targeted therapies⁵¹⁴.

Several immunotherapies are licenced for use in melanoma including the immune checkpoint inhibitors ipilimumab and pembrolizumab, which target the CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and PD-1 (programmed cell death protein 1) receptors, respectively, to reactive immune destruction of cancer cells⁵¹⁷. Targeted therapies on the other hand, are designed to take advantage of common mutations that drive tumorigenesis and disease progression, such as mutations in the BRAF gene, which are present in approximately 50% of melanoma patient tumours^{38,518}. As oncogenic BRAF mutations potentiate uncontrolled proliferative signalling, inhibition of this pathway via small molecule inhibitors, such as vemurafenib, can abrogate cell proliferation and disease progression in mutant BRAF tumours³⁹. While such therapies have shown increased overall survival rates compared to traditional chemotherapy regimes³⁸, resistance to both BRAF inhibitors and immunotherapy is common and 5-year survival rates are typically below 50% for both immunotherapy and BRAF-targeted therapy in stage 4 melanoma patients^{65,519}. With incidence rates ever on the increase, novel methods of targeting melanoma are therefore essential in providing better patient outcomes.

Excessive oxidative stress, denoted by high levels of reactive oxygen species (ROS), promotes DNA damage, genetic instability and is a driving force in tumourigenesis^{260,520}. Due to their frequent exposure to DNA-damaging agents such as UV radiation, melanocytes are subjected to particularly high levels of oxidative stress, creating a heavy reliance on mechanisms of redox regulation⁵²¹⁻⁵²³. Interestingly, while melanocytes can

produce melanin to protect against UV-induced damage, excessive melanin synthesis (melanogenesis) can itself produce high levels of ROS, which can further exacerbate DNA damage in response to chronic UV exposure²⁵⁹. Once neoplastic transformation has occurred, melanoma cells are very resistant to oxidative stress⁵²⁴, which greatly hampers the action of several types of therapy, including immunotherapies, BRAF inhibitors and classical cytotoxic drugs^{522,525}.

Nrf2 is a key regulator of oxidative stress that is known to influence redox balance in a number of chronic diseases, including cancer¹¹⁹, through its regulation of antioxidant and cytoprotective genes⁵²⁶. It has been proposed therefore that Nrf2 may also have a role in the development and progression of melanoma⁵²⁷. Currently however, the involvement of Nrf2 in melanoma is not well understood, with some reports suggesting that it is upregulated and promotes chemoresistance and metastasis⁵²⁸, and others hypothesising that Nrf2 is downregulated in melanoma, which acts as an initiating factor in carcinogenesis^{529,530}. On the one hand, Nrf2 not only plays an important role in protecting non-malignant melanocytes against potentially oncogenic mutations⁵²⁶, but also defends against ROS generation as a result of excessive melanogenesis in response to UVA radiation²⁵⁹. Nrf2 downregulation has also been identified in several patient melanoma tumours^{529,530}, correlated with increased levels of ROS⁵²⁹. As such, phenolic antioxidants that activate Nrf2 have been proposed as potential chemopreventive agents in melanoma²⁵⁹.

Alternatively, Nrf2 is also known to possess a 'dark side' in cancer and several studies have observed upregulation of Nrf2 in melanoma^{444,524}, correlated with increased metastatic lesions^{68,528,531}, poorer patient prognosis^{68,528}, and enhanced resistance to BRAF-targeted therapies (e.g. vemurafenib)⁵³² and chemotherapies such as dacarbazine⁵³³ and temozolomide⁵³⁴. A number of Nrf2 target genes are also commonly overexpressed in melanoma, including HO-1⁵³⁵ and NQO1⁵³⁶, which assist in promoting cell proliferation, migration and angiogenesis of melanoma tumours^{536,537}. Despite these associations, the mechanisms through which Nrf2 is upregulated in melanoma are not currently known. Recent studies of Nrf2 knockdown via siRNA treatment can induce anti-proliferative and anti-migratory effects in *in vitro* and *in vivo* models of melanoma, and sensitised melanoma cells to BRAF inhibition and DNA-damaging agents, such as cisplatin, dacarbazine and temozolomide^{282,538}. Hence, although Nrf2's overall role in melanoma remains under contention, melanoma patients with high levels of Nrf2 may benefit from adjuvant treatment with Nrf2 inhibitors to prevent further disease progression and treatment resistance⁵²⁷.

The present study therefore explored the expression of Nrf2 in melanoma, both in patient samples accessed via public cancer databases and in melanocyte and melanoma cell lines. Furthermore, the effects of a panel of Nrf2 inhibitors (brusatol, luteolin, ML385, retinoic acid, triptolide, trigonelline and clobetasol) were compared for the first time in melanoma to assess their potential therapeutic benefit and to sensitise BRAF-mutant melanoma cells to the anti-proliferative and anti-migratory effects of the BRAF inhibitor vemurafenib.

5.2 Aims

1. Investigate the mRNA and protein expression of Nrf2 and related genes in melanoma versus melanocytes and normal skin samples using public databases and human cell lines.
2. Compare Nrf2 inhibitors in high Nrf2-expressing melanoma cells for their effects on Nrf2 protein expression, Nrf2-ARE binding and Nrf2 downstream signalling.
3. Explore the potential of pharmacological inhibition of Nrf2 to suppress melanoma cell migration and sensitise cells to standard of care targeted therapies, such as BRAF inhibitors.

5.3 Results

5.3.1 Expression of NFE2L2 and NFE2L2-related genes in melanoma

Firstly, to explore whether Nrf2 may be a good target in melanoma, we investigated Nrf2 expression in public databases of patient melanoma tumours. Using the GEO (Gene Expression Omnibus) database, a microarray dataset (GSE15605) containing patient samples of normal skin, primary melanoma and metastatic melanoma biopsies was selected (**Figure 5.1**, see **Chapter 2.2.12** for further details).

Currently, the number of open-access studies containing normal skin samples and melanoma tumour biopsies of melanoma patients is limited, with studies often containing very few samples of normal skin (often of <10 samples). Additionally, no other studies using patient-matched normal and tumour tissue could be found. Due to these limitations, the GSE15605 dataset was selected due to the larger number of samples for each

sample type (e.g. normal skin, primary melanoma) compared to other datasets, and inclusion of patient-matched normal and tumour samples (available for 11 patients). Data from the GSE15605 dataset is derived from patients ranging between the ages of 20-92 years, with a median age 59.4 years, 72% of whom were male and 28% were female⁴²⁰.

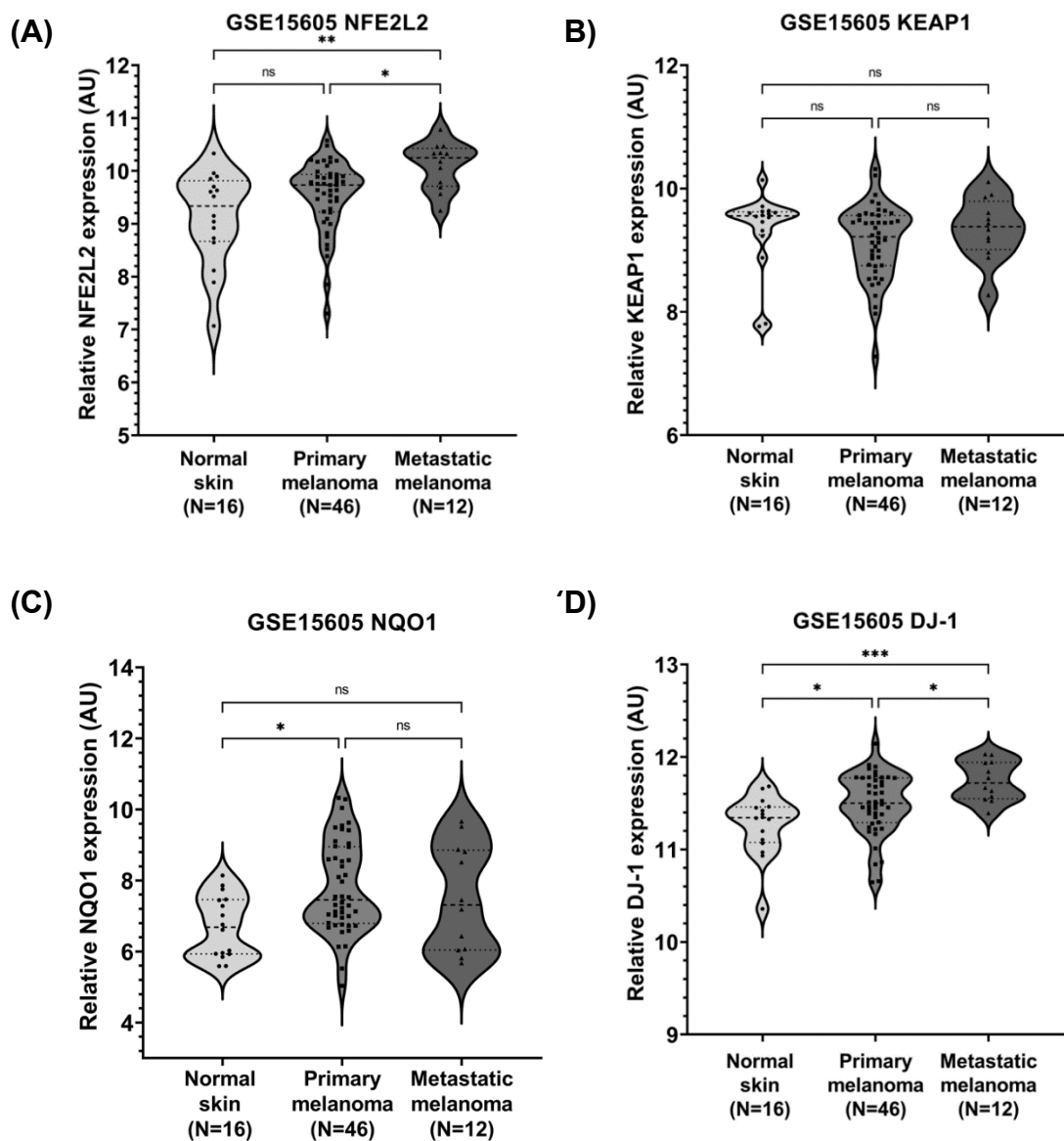


Figure 5.1 Expression of NFE2L2, DJ-1, KEAP1 and NQO1 in patient samples of normal skin, primary melanoma and metastatic melanoma. Relative mRNA expression of (A) NFE2L2, (B) KEAP1, (C) NQO1 and (D) DJ-1 in microarray samples of normal (non-cancerous) skin, primary melanoma and metastatic melanoma from the GSE15605 GEO dataset. Expression is presented as relative expression (AU; arbitrary units) and statistical analysis was conducted via Kruskal-Wallis and post-hoc Dunns test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns= not significant).

The GSE15605 dataset was first queried for mRNA expression counts of NFE2L2 (Nrf2), and Nrf2-related genes such as KEAP1 and NQO1 as displayed in **Figure 5.1**. In the GEO dataset, NFE2L2 mRNA was significantly increased in metastatic melanoma compared to normal skin samples, although no significant difference was observed between normal skin and primary melanoma (**Figure 5.1A**). As KEAP1 is one of the key mechanisms that regulates Nrf2 turnover, we also investigated whether aberrations in KEAP1 expression were observed. However, no significant change in KEAP1 expression was found between normal skin, primary melanoma and metastatic tumours (**Figure 5.1B**).

As an increase in NFE2L2 expression was observed however, we next explored the expression of NQO1, a downstream gene of Nrf2 which has previously shown to be upregulated in melanoma⁵³⁶. As shown in **Figure 5.1C**, NQO1 expression was significantly increased in primary melanoma vs normal skin, however, no significant difference was observed between normal skin and metastatic melanoma. As the mechanism of Nrf2 regulation in melanoma is under-characterised, DJ-1 (also known as PARK7) was also studied, as it has been reported to be upregulated in melanoma⁵³⁹ and assists in stabilising Nrf2 through disrupting its interaction with Keap1⁴⁵⁸. In the GSE15605 dataset, DJ-1 was significantly increased in both primary melanoma and metastatic melanoma patient samples compared to normal skin (**Figure 5.1D**), suggesting that it may have a role in melanoma pathogenesis.

To validate the results observed in **Figure 5.1**, NFE2L2, KEAP1, NQO1 and DJ-1 expression was then further studied using RNAseq data accessed via The Cancer Genome Atlas (TCGA) database (**Figure 5.2**, see **Chapter 2.2.12** for details of study selection). In this study, patient age ranged between 15-90 years (median 58 years), 62% of whom were male and 38% were female. Interestingly, in contrast to the GSE15605 GEO dataset, NFE2L2 was found to be decreased in melanoma tissue vs normal skin in the TCGA melanoma database (**Figure 5.2A**), suggesting that Nrf2 expression may vary across patient cohorts.

Expression of KEAP1 was observed to be increased in melanoma tumours compared to normal skin (**Figure 5.2B**), which also contradicted the data presented in **Figure 5.1B**. The expression of NQO1 and DJ-1 however, was much more consistent with the results observed in **Figure 5.1**, as both were significantly increased in melanoma patient samples compared to normal skin (**Figure 5.2C** and **5.2D**).

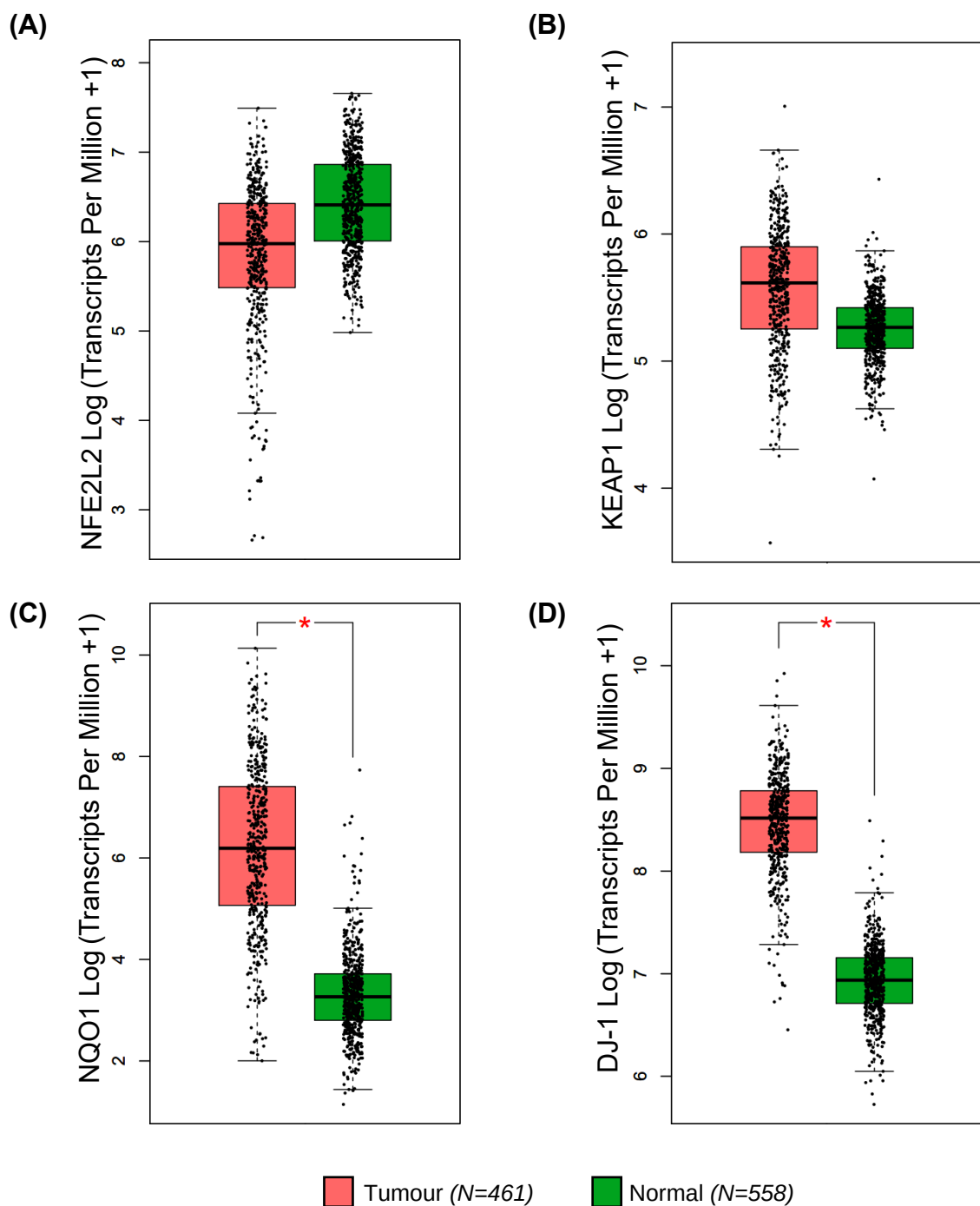


Figure 5.2 Differential gene expression of NFE2L2, KEAP1, NQO1 and DJ-1 in patient samples of normal skin and melanoma. Differential gene expression of (A) NFE2L2, (B) KEAP1, (C) NQO1 and (D) DJ-1 in RNAseq data from The Cancer Genome Atlas database of normal (non-cancerous) skin and skin cutaneous melanoma patient samples (N=461 and 558, respectively). Box and violin plots were generated using the GEPIA webserver and expression is presented Log (Transcripts Per Million +1). Statistical analysis was conducted via one-way ANOVA (* $P \leq 0.05$).

5.3.2 Nrf2 and DJ-1 expression in melanoma cell lines

To further explore the expression of Nrf2 and Nrf2-related genes in melanoma, we next investigated Nrf2 and DJ-1 mRNA (**Figure 5.3**) in primary human HEMA-LP (human epidermal melanocytes, lightly pigmented) melanocytes and 9 melanoma cell lines (M202, A375, MEL501, M308, UACC1273, SKMEL-5, M263, SKMEL-28, A2058), as shown in **Figure 5.3**.

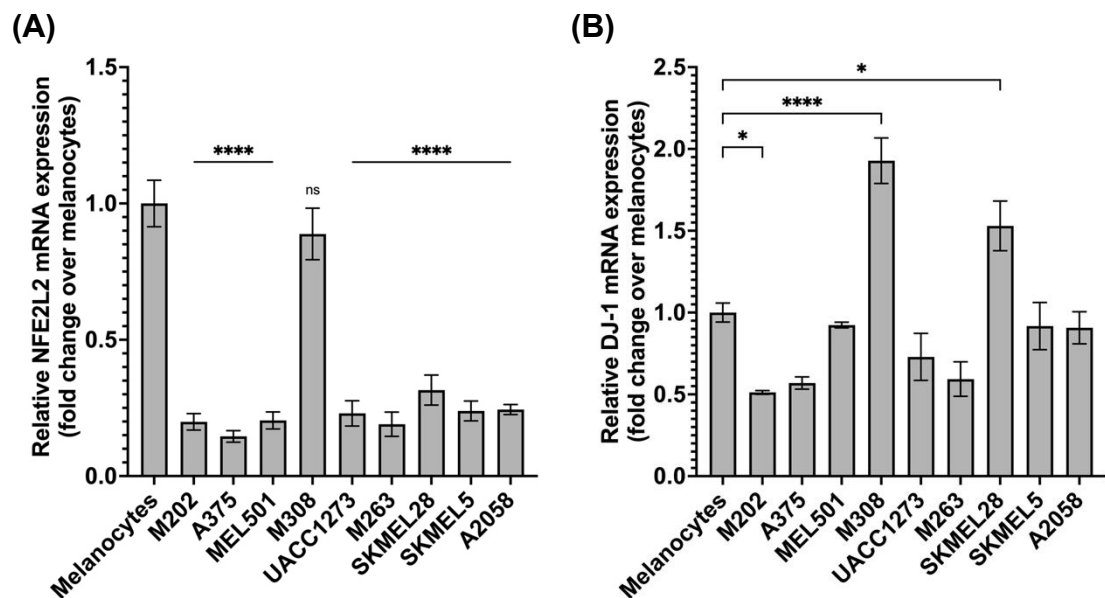


Figure 5.3 Expression of NFE2L2 and DJ-1 mRNA in primary melanocytes and melanoma cell lines. Relative mRNA expression of **(A)** NFE2L2 and **(B)** DJ-1 in primary HEMA-LP melanocytes and melanoma cell lines. RNA was prepared from 5×10^5 cells and mRNA expression was measured via RT-qPCR. Gene expression was normalised to GAPDH and is presented as fold change over melanocytes. Data is presented as mean $\pm$ SEM (N=3). Statistical analysis was conducted via one-way ANOVA and post-hoc Dunnett's test in comparison to melanocytes (* $P \leq 0.05$, **** $P \leq 0.0001$, ns= not significant).

Although all 9 of the melanoma cell lines showed lower expression of NFE2L2 compared to melanocytes, the highest expression among the melanoma cells was found in the M308 cells (**Figure 5.3A**). On the other hand, DJ-1 mRNA expression was much more variable across the melanocytes and melanoma cell lines than that observed with NFE2L2, with the highest expression observed in the M308 and SKMEL-28 cells (**Figure 5.3B**). SKMEL-5, A2058 and MEL501 melanoma cells all showed a similar expression of DJ-1 mRNA to that observed in melanocytes, although other melanoma cell lines such as M202, A375, UACC1273 and M263 had significantly lower expression than melanocytes (**Figure 5.3B**).

Next, to explore whether the mRNA expression of NFE2L2 and DJ-1 matches with the protein expression of these genes, we investigated Nrf2 and DJ-1 protein expression of melanocytes and melanoma cells via western blot (**Figure 5.4**).

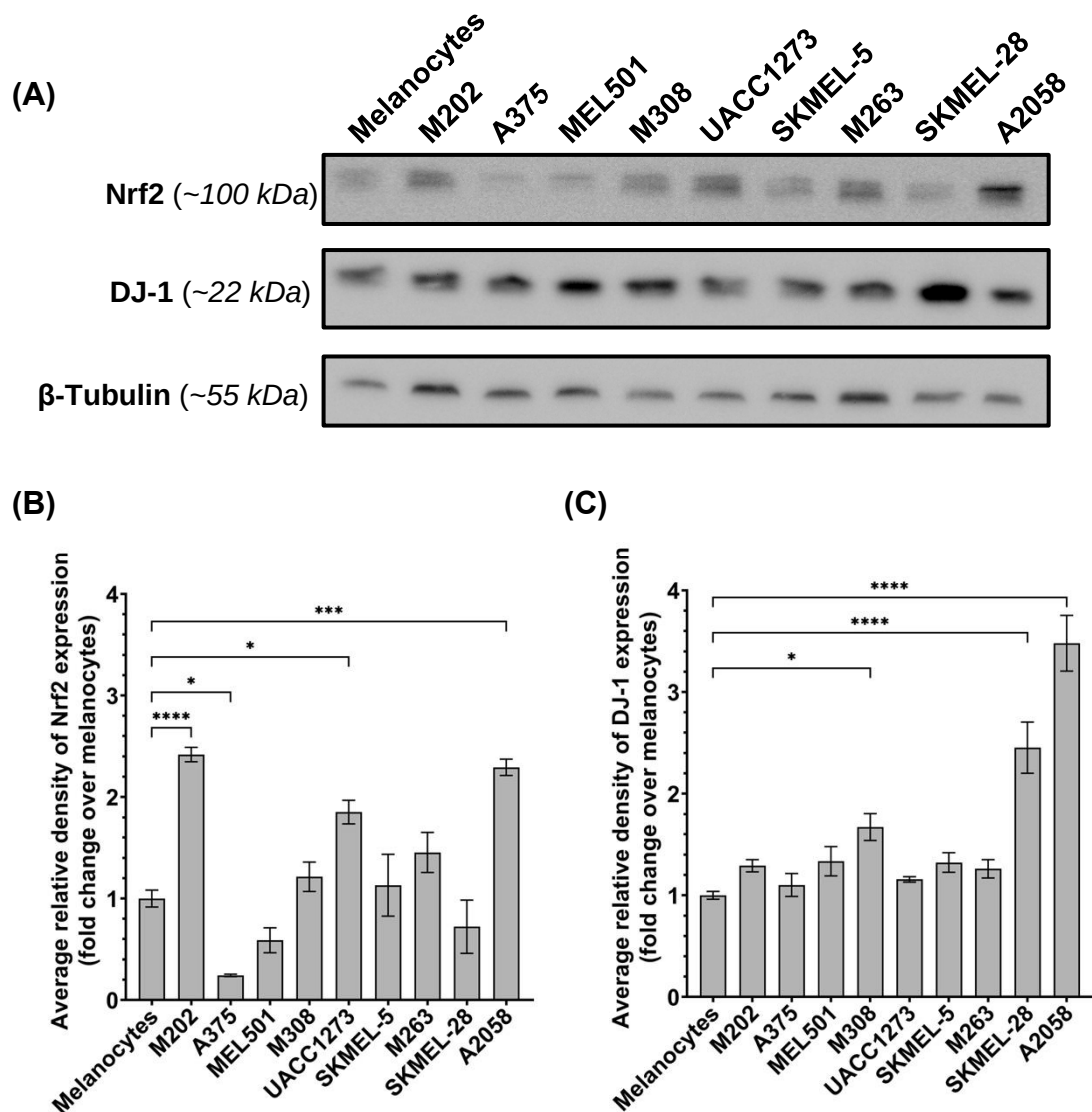


Figure 5.4 Expression of Nrf2 and DJ-1 in primary melanocytes and melanoma cell lines. (A) Representative western blot images of Nrf2 and DJ-1 expression in primary melanocytes and melanoma cell lines. Protein extracts were prepared from 1×10^6 HEMA-LP melanocyte or melanoma cells per sample and protein expression was measured via SDS-PAGE and western blot analysis. β -Tubulin was included as a loading control. Densitometry of western blot images was conducted to quantify (B) Nrf2 and (C) DJ-1 protein expression. Densitometry was normalised to β -Tubulin and is presented as mean fold change over melanocytes $\pm$ SEM. Western blot images and densitometry are representative of three individual experiments (N=3). Statistical analysis was conducted via one-way ANOVA and post-hoc Dunnett's test (* $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

As shown in **Figure 5.4A** and **Figure 5.4B**, 5/8 of the melanoma cell lines possessed higher Nrf2 protein expression than that observed in melanocytes, the highest of which was observed in M202, UACC1273, M263 and A2058 cells (**Figure 5.4B**). These results therefore suggest that the mRNA levels of Nrf2 (**Figure 5.3A**) do not directly correlate with protein expression (**Figure 5.3B**), potentially due to high mRNA turnover or aberrations in protein regulation (such as loss of Keap1 function)⁵⁴⁰. Although most melanoma cell lines displayed elevated DJ-1 expression than that seen in melanocytes, the SKMEL-28 and A2058 cells exhibited significantly higher DJ-1 expression (**Figure 5.4C**). Additionally, **Figure 4.4C** shows that DJ-1 protein expression did not directly align with its expression at the mRNA level (**Figure 5.3B**), however does occasionally correlate with high Nrf2 protein expression, such as in the A2058 cells, potentially suggesting that high DJ-1 expression may influence Nrf2 stability in some melanoma cell lines.

From this cell line panel, we next investigated the mechanisms of Nrf2 in melanoma and its potential as a therapeutic target in a high Nrf2 expressing melanoma cell line. For this we selected the M202 cells, as these had a 2.5-fold higher expression of Nrf2 than melanocytes (**Figure 5.4B**).

5.3.3 Nrf2 inhibitors repress Nrf2 expression in melanoma cells

Although genetic inhibition of Nrf2 via siRNA is reported to repress cell proliferation of melanoma cells and can sensitise melanoma to chemotherapeutic agents^{282,533,541}, to date, only two studies have emerged exploring the use of Nrf2 inhibitors to repress Nrf2 signalling in melanoma, which only focussed on brusatol³³⁸ and DMF⁴⁶⁰. As Nrf2 is upregulated in some melanoma patients tumours and promotes cancer cell proliferation, chemoresistance and resistance to apoptosis, pharmacological inhibition of Nrf2 may have therapeutic potential in metastatic melanoma, which is traditionally very resistant to chemotherapy⁵¹⁵. To explore this, the Nrf2 inhibitors brusatol, luteolin, ML385, retinoic acid, triptolide, trigonelline and clobetasol and the Nrf2 modulator DMF were compared for their effects on Nrf2 signalling in melanoma and their ability to repress migration and sensitise melanoma cells to standard of care chemotherapy. Firstly however, Nrf2 inhibitors were investigated for their effects on Nrf2 protein expression in M202 melanoma cells via western blot analysis (**Figure 5.5**). As we previously have shown DMF to both inhibit and induce Nrf2 signalling, for these experiments, we selected a concentration of 30 μ M treatment for 4 h, as we have shown this to inhibit Nrf2 in A549 cells (**Figure 8.2**).

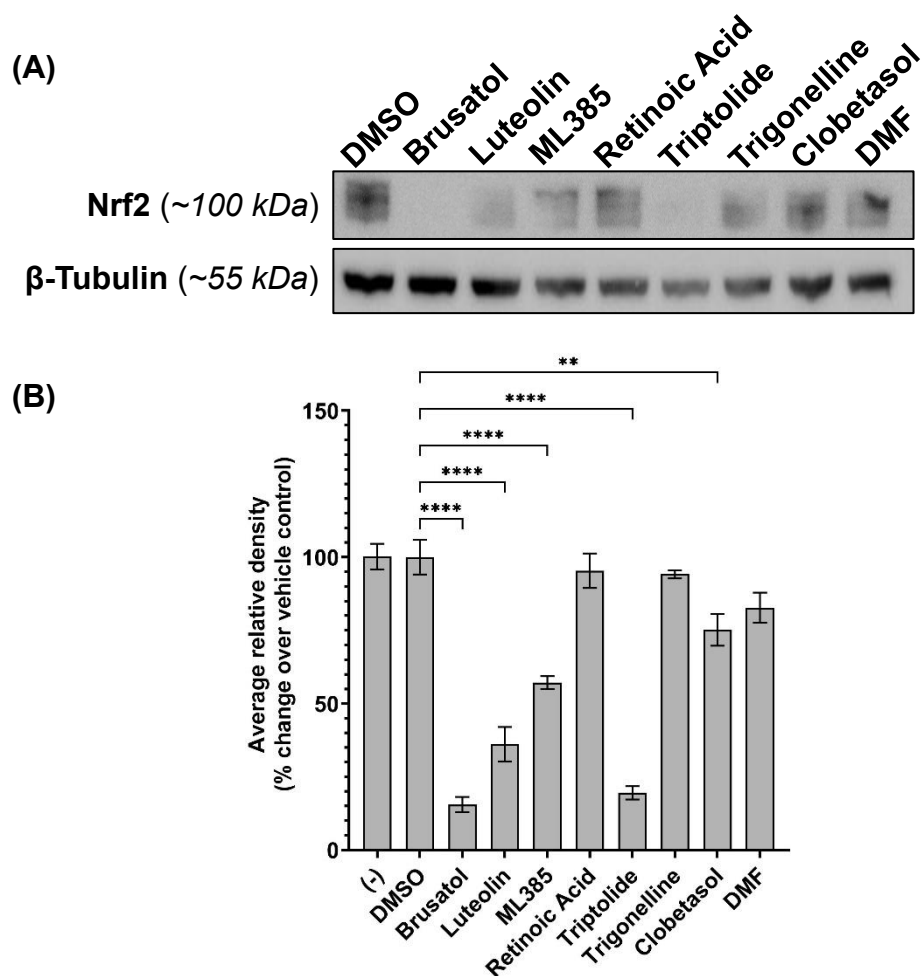


Figure 5.5 The effects of Nrf2 inhibitors on Nrf2 protein expression in M202 melanoma cells. (A) Representative western blot images of Nrf2 protein expression in M202 cells following treatment with Nrf2 inhibitors. 1×10^6 M202 cells were incubated with media (-), DMSO, 30 μ M DMF or 20 μ M Nrf2 inhibitors for 4 h prior to SDS-PAGE and western blot analysis of Nrf2 protein expression. β -Tubulin was included as a loading control and images are representative of three individual experiments (N=3). (B) Densitometry analysis of Nrf2 expression from western blot images was measured and normalised to β -Tubulin. Data is presented as average relative density (percent change over vehicle (DMSO) control) $\pm$ SEM. Statistical analysis was carried out by one-way ANOVA and post-hoc Dunnett's test (**P $\leq$ 0.01, ***P $\leq$ 0.001, ****P $\leq$ 0.0001).

As displayed in **Figure 5.5A**, following incubation of M202 cells with 20 μ M Nrf2 inhibitors for 4 h (as optimised in **Figure 3.3**), both brusatol and triptolide significantly repressed Nrf2 protein expression compared to DMSO vehicle control, with both of these inhibitors displaying the greatest inhibitory effects among the Nrf2 inhibitors included in the present study. Interestingly, luteolin and ML385 also significantly repressed Nrf2 protein expression in M202 cells, while retinoic acid and trigonelline exerted little effects on Nrf2 protein expression (**Figure 5.5B**). Furthermore, although it is more commonly reported as an Nrf2 activator, DMF also slightly repressed Nrf2 protein expression (**Figure 5.5**), similarly to that observed in **Figure 3.3**. As brusatol and triptolide both repressed Nrf2 protein expression to the greatest extent among the Nrf2 inhibitors studied here, we next

explored whether these repressive effects were concentration and time dependent (**Figure 5.6** and **Figure 5.7**, respectively).

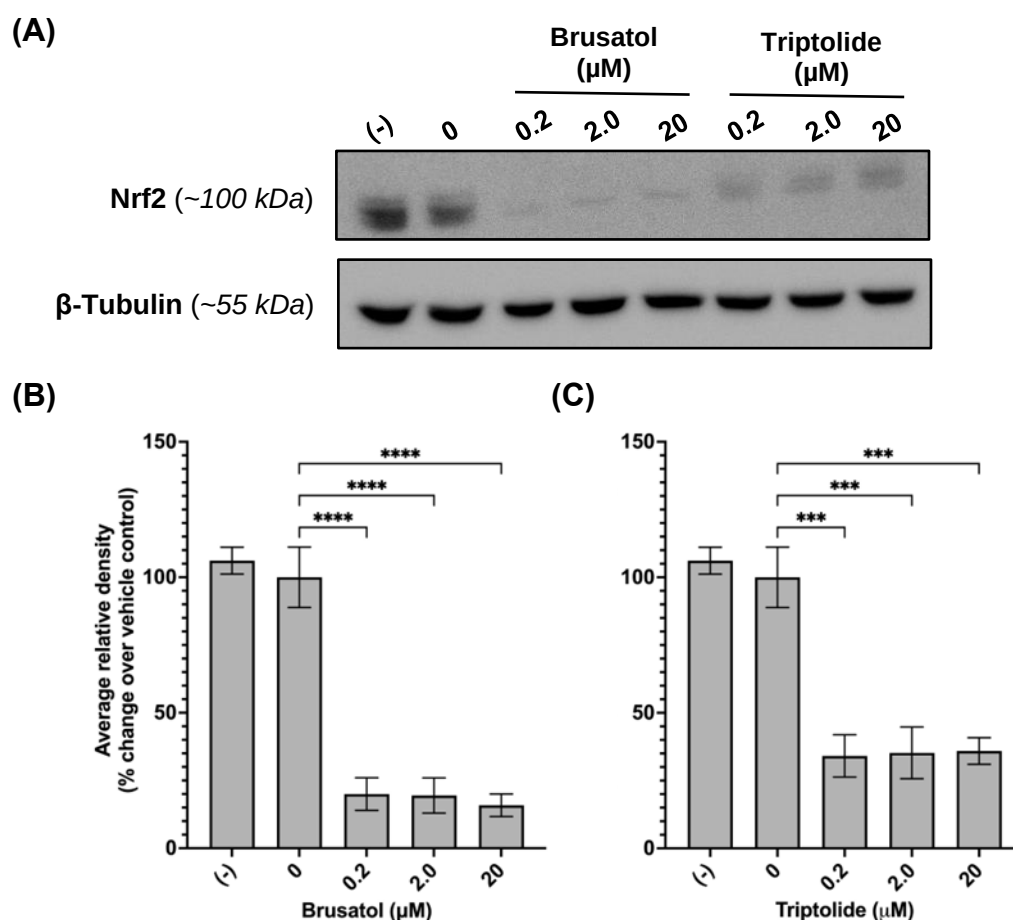


Figure 5.6 Brusatol and triptolide potently repress Nrf2 protein expression in M202 cells. (A) Representative western blot images of M202 cells treated with 0-20 μM brusatol or triptolide. 1×10^6 M202 cells were incubated with media (-) or 0-20 μM brusatol or triptolide for 4 h prior to cell lysis and protein quantification via SDS-PAGE and western blot analysis. β -Tubulin was included as a loading control. Images are representative of 3 individual experiments (N=3). Densitometry analysis of Nrf2 protein expression in western blot images was normalised to β -Tubulin for cells treated with (B) brusatol and (C) triptolide. Data is presented as average relative density with respect to vehicle (DMSO) control $\pm$ SEM. Statistical analysis was carried out via one-way ANOVA and post-hoc Dunnett's test (***P $\leq$ 0.0001, ****P $\leq$ 0.0001).

As displayed in **Figure 5.6**, brusatol potently repressed Nrf2 protein expression at concentrations of 0.2-20 μM , however little difference was observed between these concentrations, suggesting that lower concentrations could be selected while still possessing strong inhibitory effects. On the other hand, while triptolide did not repress Nrf2 protein expression to the same degree as brusatol, it did inhibit Nrf2 at concentrations as low as 0.2 μM (**Figure 5.6A**).

Next, the effect of treatment time of brusatol and triptolide on Nrf2 expression in M202 cells was investigated. To explore this, M202 cells were treated with 2 μ M brusatol or triptolide for 2-8 h and protein expression was measured via western blot (**Figure 5.7A**).

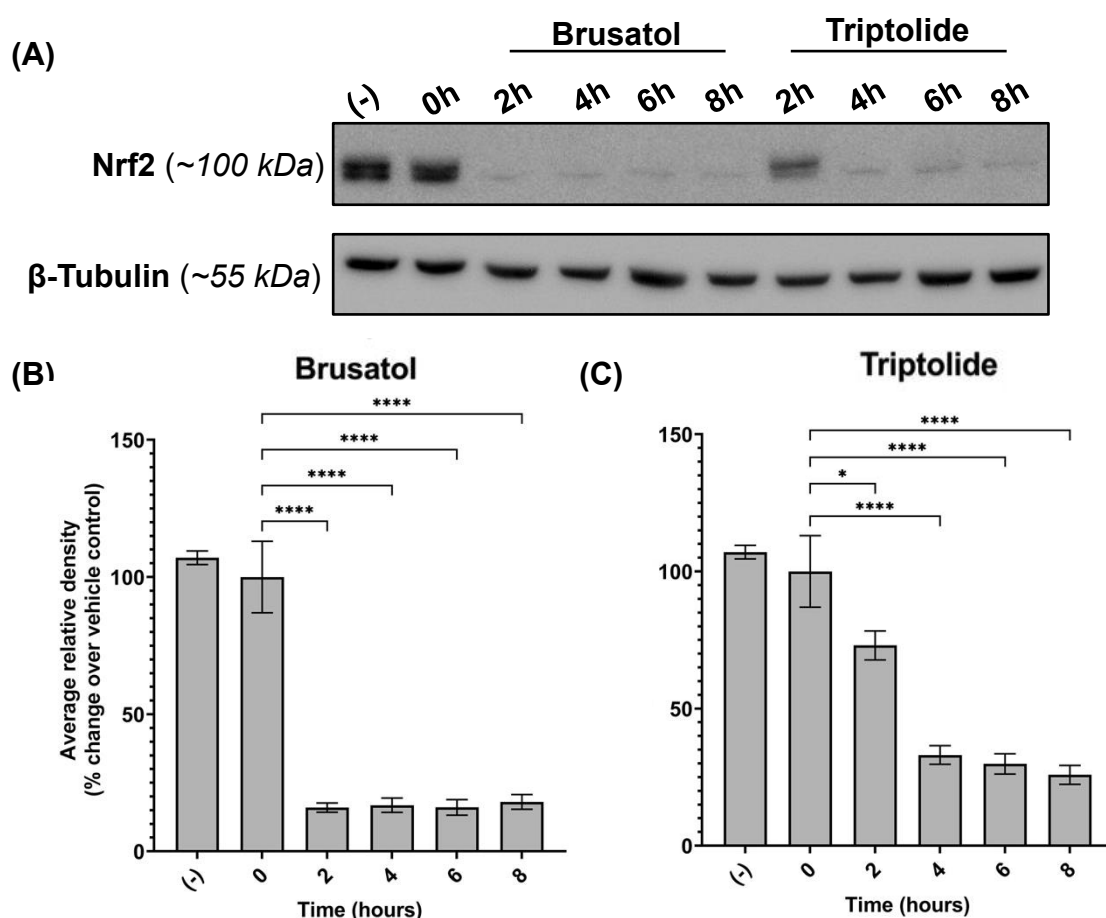


Figure 5.7 Brusatol and triptolide rapidly repress Nrf2 protein expression in melanoma cells. (A) Representative western blot images of Nrf2 protein expression in 1×10^6 M202 cells treated with 2 μ M brusatol and triptolide for 2-8 h prior. Cell lysates are separated by SDS-PAGE prior to western blot analysis with β -Tubulin included as a loading control. Images are representative of three individual experiments (N=3). Densitometry analysis of Nrf2 protein expression for M202 cells treated with (B) brusatol and (C) triptolide. Data is normalised to β -Tubulin and is presented as average relative density with respect to vehicle (DMSO) control $\pm$ SEM. Statistical analysis was carried out via one-way ANOVA and post-hoc Dunnett's test (*** $P \leq 0.0001$, **** $P \leq 0.0001$).

As shown in **Figure 5.7B**, brusatol significantly repressed Nrf2 protein expression at 2-8 h, with expression maintained $<20\%$ of DMSO control. On the other hand, triptolide's inhibitory effects on Nrf2 protein expression were only observed at a later treatment time point of 4 h, wherein protein expression continued to decline further until the longest treatment time point of 8 h (**Figure 5.7C**).

5.3.4 Nrf2 inhibitors inhibit Nrf2 DNA binding in M202 melanoma cells

As Nrf2's cytoprotective effects are dependent on nuclear Nrf2 binding to the ARE, to further explore the effects of Nrf2 inhibition in melanoma, we next investigated the ability of the Nrf2 inhibitors to repress Nrf2/ARE binding. To study this, we utilised an ELISA-based assay that included the ARE consensus sequence immobilised on the bottom on 96 well plates (as previously described in **Chapters 3 and 4**). Nuclear extracts of M202 cells treated with Nrf2 inhibitors for 4 h were added to the plate and Nrf2-ARE binding was quantified via ELISA (**Figure 5.8**).

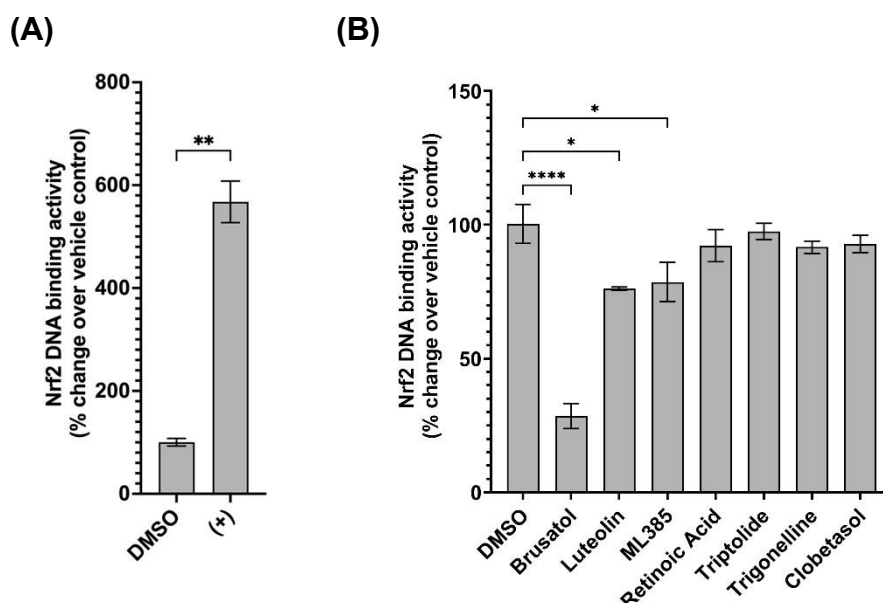


Figure 5.8 Inhibitory effects of Nrf2 inhibitors on Nrf2 DNA binding activity in melanoma cells. M202 cells (3×10^5 cells/well) were incubated with DMSO or 20 μ M Nrf2 inhibitors for 4 h prior to preparation of nuclear protein extracts. **(A)** Positive control (recombinant Nrf2; (+)) or **(B)** nuclear extracts of treated cells were added to a 96-well plate coated with the ARE consensus sequence and Nrf2-DNA binding activity was measured via ELISA. Data is presented as percent change in Nrf2-DNA binding activity with respect to vehicle (DMSO) control $\pm$ SEM (N=3). Statistical analysis was conducted via one-way ANOVA and post-hoc Dunnett's test (* $P \leq 0.05$, ** $P \leq 0.01$ **** $P \leq 0.0001$).

As presented in **Figure 5.8B**, among the Nrf2 inhibitors, brusatol showed the greatest effects at repressing nuclear Nrf2 binding to the ARE, wherein it significantly reduced binding to 28% of DMSO control. Luteolin and ML385 also decreased Nrf2-ARE binding in M202 cells to 75% and 78% of DMSO control, respectively (**Figure 5.8B**). Surprisingly, despite having potent repressive effects on Nrf2 protein expression in whole cell lysates of M202 cells (**Figure 5.6C**), triptolide did not show any effects in the DNA ELISA (**Figure 5.8B**). Additionally, retinoic acid, trigonelline and clobetasol did not have any effects on Nrf2 ARE binding (**Figure 5.8B**).

5.3.5 Nrf2 inhibitors repress NQO1 enzymatic activity in M202 melanoma cells

NQO1, which is one of the canonical Nrf2 downstream target genes, is commonly overexpressed in numerous cancer types⁵⁴², including melanoma⁵³⁶, and promotes cell proliferation, and chemoresistance by assisting in detoxification reactions⁵⁴². NQO1 knockdown has been shown to repress melanoma cell proliferation⁵³⁶, hence Nrf2 inhibition may therefore also be useful in reducing the activity of NQO1. As such, we investigated the effects of the Nrf2 inhibitors to repress NQO1 activity via enzymatic assay following M202 cell treatment with Nrf2 inhibitors (20 μ M) for 20 h (**Figure 5.9**). For these experiments, a concentration of 10 μ M DMF was selected as a positive control, as this concentration has been previously found to induce NQO1 activity in A549 cells after 20 h treatment (**Figure 3.7**).

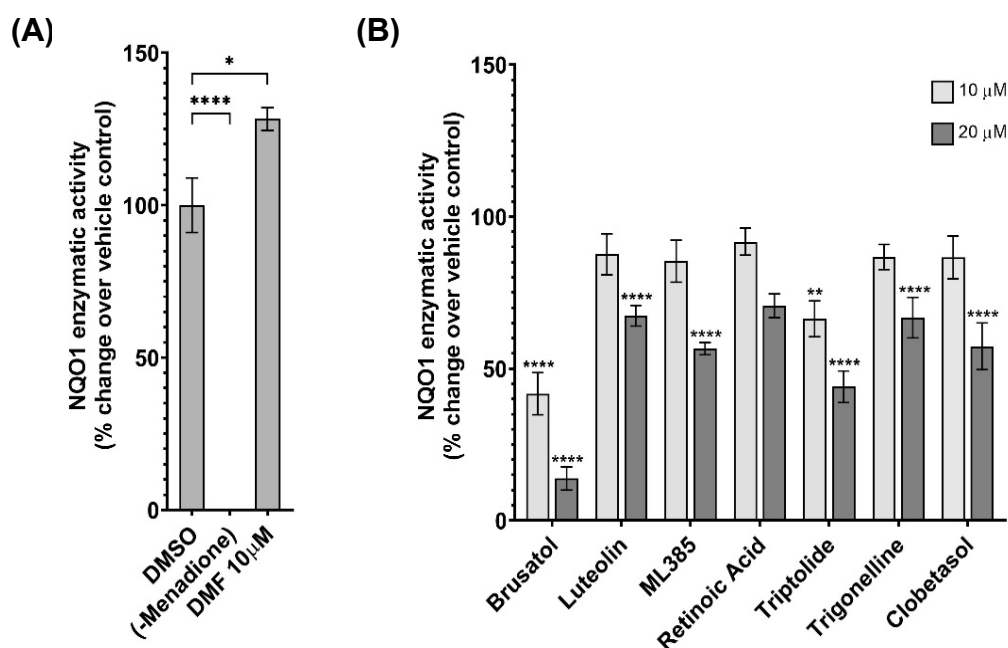


Figure 5.9 Inhibitory effects of Nrf2 inhibitors on NQO1 enzymatic activity in M202 melanoma cells. M202 cells (4×10^4 cells/well) were treated with **(A)** DMSO, 10 μ M DMF or **(B)** 20 μ M Nrf2 inhibitors for 20 h and NQO1 activity was determined via enzymatic assay. Reaction buffer without substrate was included as a negative control (-Menadione). Data is presented as average percent NQO1 enzymatic activity with respect to vehicle (DMSO) control $\pm$ SEM (N=3). Statistical analysis was conducted via two-way ANOVA and post-hoc Dunnett's test with comparisons made to DMSO (* $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$).

As shown in **Figure 5.9A**, 10 μ M DMF significantly increased NQO1 enzymatic activity, while reaction buffer without substrate (-Menadione) abrogated any activity observed via this assay. Interestingly, all of the Nrf2 inhibitors showed some activity in decreasing

NQO1 activity at a concentration of 20 μ M, however, only brusatol and triptolide induced significant inhibitory effects at a concentration of 10 μ M (**Figure 5.9B**). Furthermore, although brusatol and triptolide displayed the best inhibitory effects on NQO1 enzymatic activity (to that of 13.7% and 43.5% of DMSO control, respectively), ML385 and luteolin (20 μ M) also significantly repressed NQO1 activity (**Figure 5.9B**). Despite showing no effects on Nrf2-DNA binding activity (**Figure 5.8B**), trigonelline and clobetasol also significantly reduced NQO1 activity at 20 μ M to that of 66.7% and 57.2% of DMSO control, respectively (**Figure 5.9B**).

5.3.6 Nrf2 inhibitors inhibit migration of BRAF wild-type and mutant melanoma cell lines

Previous studies have found that Nrf2 promotes cell invasion and metastasis of cancer cell lines by regulating the expression of epithelial to mesenchymal transition (EMT) markers such as N-cadherin and E-cadherin²⁵⁸. As melanoma becomes increasingly harder to treat once it has metastasised and formed secondary tumours, we next explored whether inhibition of Nrf2 using a selection of the Nrf2 inhibitors represses the migration of M202 cells. To achieve this, a wound scratch assay was performed, wherein scratches are introduced across the cell monolayer. Inhibitors are then added and the area of the scratch is measured at 0 h and 24 h via ImageJ analysis of bright light microscopy images⁴¹⁷. The migration rate (μ m/h) is then calculated using the change in scratch area (μ m²) between 24 h and 0 h divided by the hours of treatment (**Figure 5.10**).

As displayed in **Figure 5.10B**, brusatol and luteolin significantly decreased M202 cell migration by $\geq 50\%$ to 2.2 μ m/h and 2.1 μ m/h, respectively, compared to DMSO treated cells, which migrated at a rate of 4.2 μ m/h. ML385, triptolide and trigonelline also reduced the migration rate of M202 cells to 2.8 μ m/h, 2.9 μ m/h and 2.8 μ m/h, respectively (**Figure 5.10B**). Out of the Nrf2 inhibitors explored, clobetasol had the lowest effects on cell migration, although did reduce migration to 3.2 μ m/h (**Figure 5.10B**).

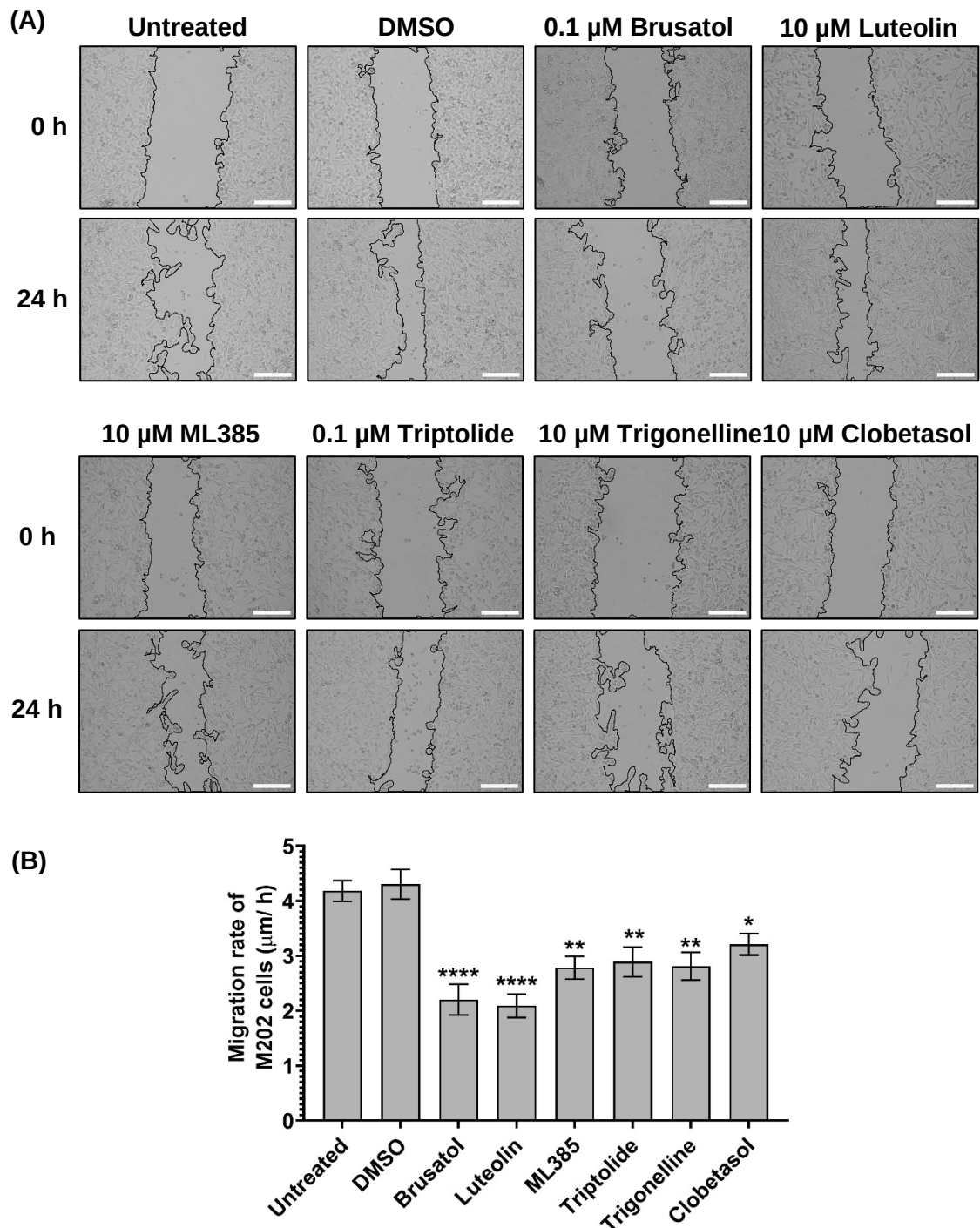


Figure 5.10 Nrf2 inhibitors repress cell migration of M202 melanoma cells. (A) Representative bright light images of scratch areas of M202 cells (2×10^5 cells/well) at 0 h and 24 h post-treatment with 0.1 or 10 μM Nrf2 inhibitors. The scratch area is outlined in black. Scale bar 100 μm . **(B)** Migration rate was calculated from the scratch area (μm^2) at 0 h and 24 h using ImageJ. Data is presented as average migration rate ($\mu\text{m/h}$) $\pm$ SEM (N=3) and statistical analysis was carried out via one-way ANOVA and post-hoc Dunnett's test, with comparisons made to DMSO (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.0001$, **** $P \leq 0.0001$).

We also explored the ability of the Nrf2 inhibitors to repress cell migration in BRAF^{V600E} mutant melanoma cells (**Figure 5.11**), which is the most common BRAF mutation observed in melanoma⁵¹⁸. For this we used A375 cells, which migrate at a faster rate of 6 $\mu\text{m/h}$ (**Figure 5.11B**) compared to 4.2 $\mu\text{m/h}$ observed in M202 cells (**Figure 5.10B**).

As shown in **Figure 5.11**, the Nrf2 inhibitors displayed a similar trend in reducing cell migration of A375 cells as was seen in the M202 cells (**Figure 5.10**). Furthermore, in A375 cells, luteolin showed the best inhibitory effects on migration (**Figure 5.11B**), reducing migration to 1.8 $\mu\text{m/h}$ (70% decrease compared to DMSO control). Brusatol, also had significant effects on A375 cell migration compared to untreated cells, decreasing the rate to 2.3 $\mu\text{m/h}$ (**Figure 5.11B**). Although not significant, ML385 and clobetasol also showed inhibitory effects on A375 cell migration, reducing migration rate to 2.5 $\mu\text{m/h}$ and 2.6 $\mu\text{m/h}$, respectively (**Figure 5.11B**). While triptolide and trigonelline did not reduce migration to the same extent as the other Nrf2 inhibitors screened here, both inhibitors reduced migration to 3.9 $\mu\text{m/h}$ (**Figure 5.11B**).

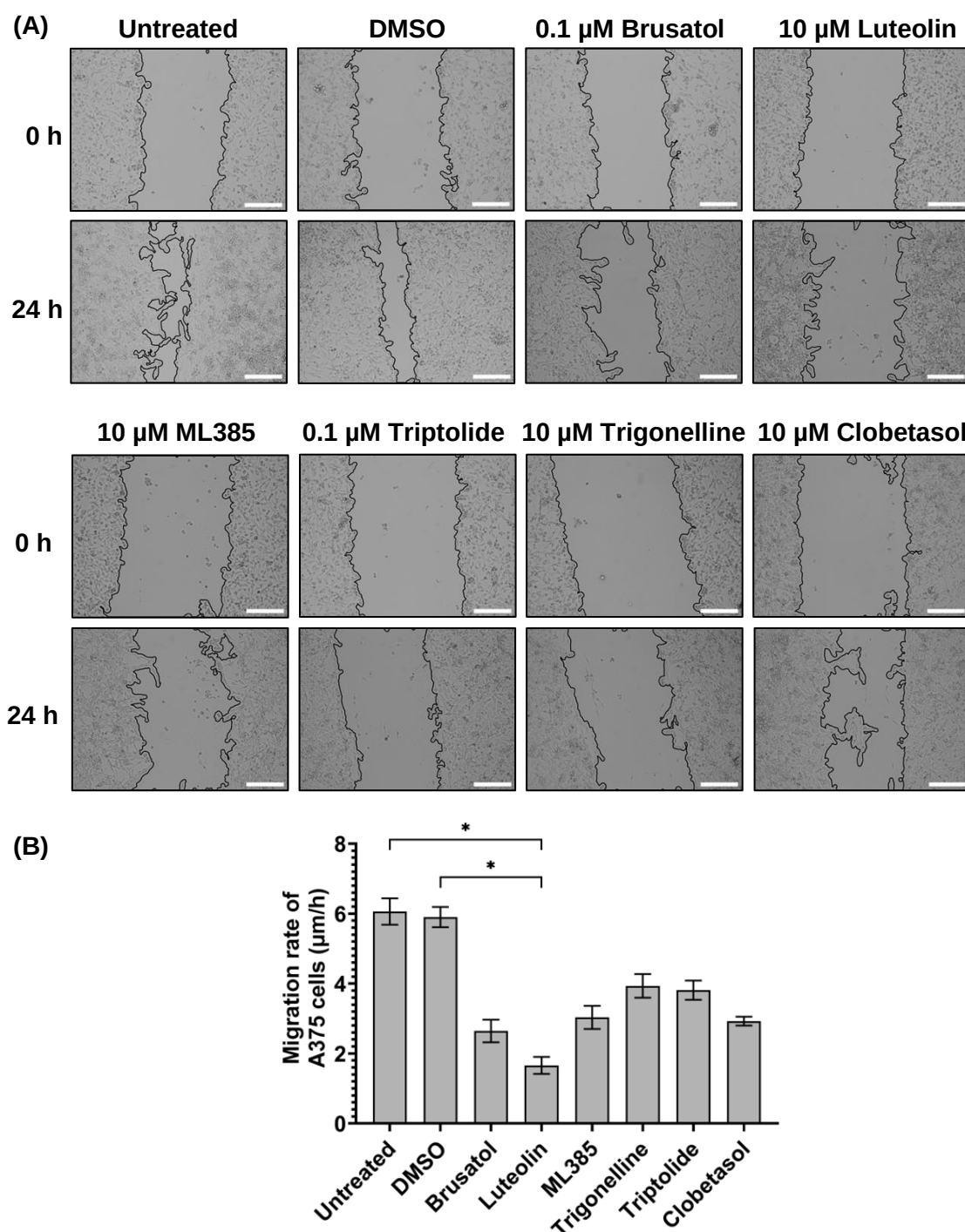


Figure 5.11 Nrf2 inhibitors inhibit cell migration of BRAF mutant A375 melanoma cells. (A) Representative bright light images depicting scratch areas of A375 cells (2×10^5 cells/well) at 0 h and 24 h post-treatment with 0.1 (brusatol, triptolide) or 10 μ M Nrf2 inhibitors. The scratch area is outlined in black and the scale bar represents 100 μ m. **(B)** A375 cell migration rate was calculated using ImageJ from the scratch area (μm^2) in microscopy images at 0 h and 24 h. Data is presented as average migration rate ($\mu\text{m}/\text{h}$) $\pm$ SEM. Statistical analysis was carried out via Kruskal-Wallis and post-hoc Dunn test (** $P \leq 0.0001$, **** $P \leq 0.0001$).

5.3.7 Brusatol, triptolide and luteolin sensitise BRAF mutant A375 cells to the BRAF inhibitor vemurafenib

As over 50% of melanomas possess mutations in BRAF that potentiate cell proliferation⁵¹⁸, BRAF inhibitors have become one of the leading forms of chemotherapy utilised in melanoma treatment, including vemurafenib³⁸. Although vemurafenib has shown to improve overall survival of metastatic melanoma patients compared to dacarbazine, many patients do not respond or develop resistance⁶⁵. Previous reports have found that genetic Nrf2 inhibition can sensitise BRAF mutant melanoma cells to BRAF inhibitors⁵³², hence pharmacological Nrf2 inhibition may also be useful in reversing resistance to BRAF-targeted therapies, such as vemurafenib.

In the present study, as we found that brusatol, luteolin and triptolide showed the best inhibitory effects on Nrf2 protein expression (**Figure 5.4**), Nrf2-DNA binding (**Figure 5.8**), NQO1 activity (**Figure 5.9**) and cell migration (**Figures 5.10** and **5.11**), these inhibitors were selected for further experiments. However, as melanomas with wild-type BRAF expression do not respond to BRAF inhibitors, as they are designed to target mutant BRAF⁶⁵, M202 cells could not be studied; therefore the BRAF^{V600E} mutant A375 cells were utilised. Firstly, cell viability of A375 cells when incubated with vemurafenib alone (concentration selected using previous MTS assay data, see **Figure 8.8**), or in combination with brusatol, luteolin or triptolide was investigated via MTS assay (**Figure 5.12**).

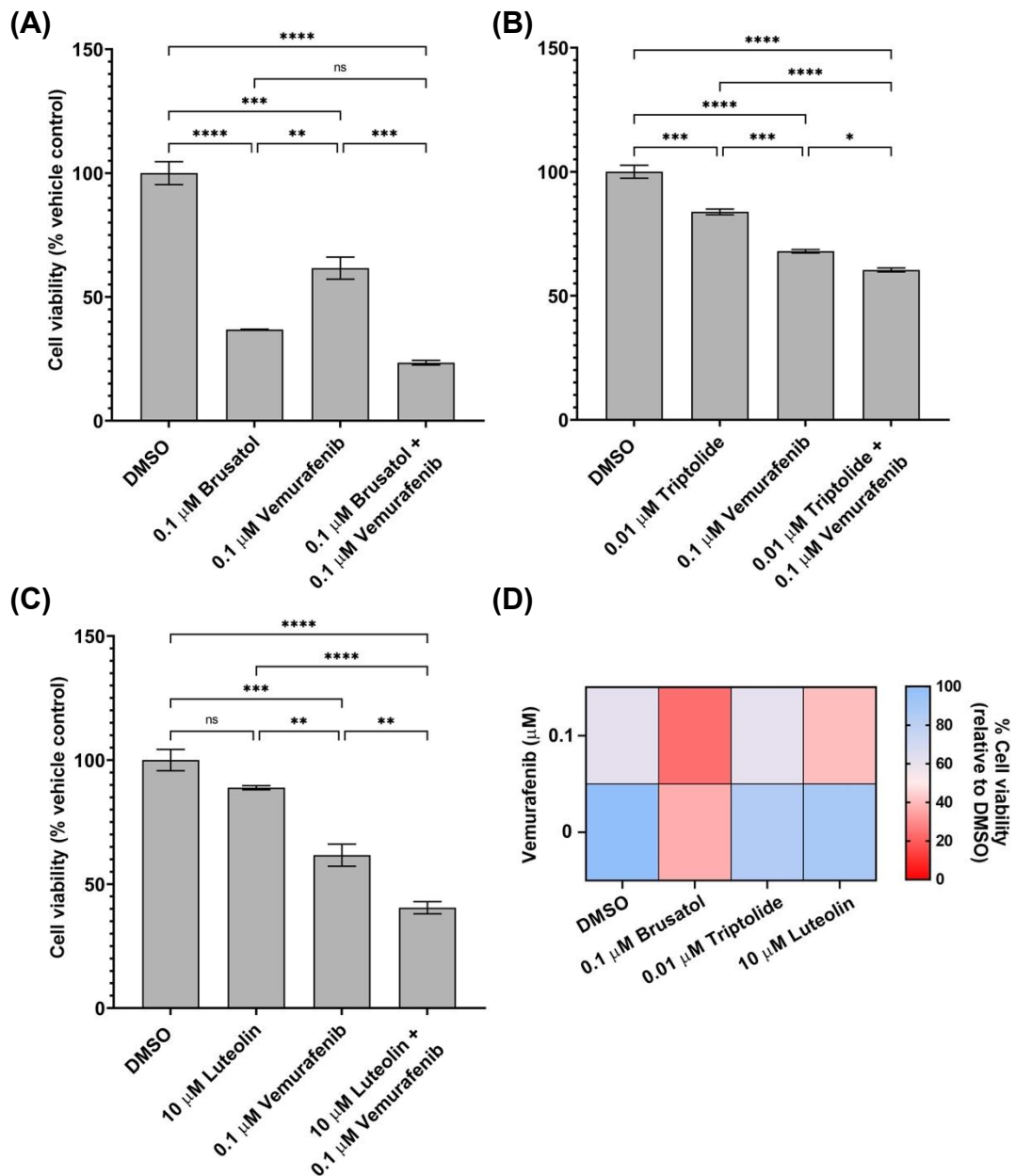


Figure 5.12 Nrf2 inhibitors sensitise BRAF mutant A375 melanoma cells to the BRAF inhibitor vemurafenib. A375 cells (5×10^3 cells/well) were treated for 72 h with 0.1 μ M vemurafenib alone or in combination with either **(A)** 0.1 μ M brusatol, **(B)** 0.01 μ M triptolide or **(C)** 10 μ M luteolin and cell viability was measured via MTS assay. Data is presented as percent cell viability with respect to vehicle (DMSO) control $\pm$ SEM (N=3) alongside **(D)** a heat map depicting percent cell viability. Statistical analysis was carried out via one-way ANOVA and post-hoc Tukey test (*P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001, ****P $\leq$ 0.0001, ns= not significant).

Figure 5.12A shows that 0.1 μ M brusatol and 0.1 μ M vemurafenib alone significantly reduced A375 cell viability to that of 35% and 60% of DMSO control, respectively. When combined with vemurafenib however, cell viability was further reduced to 22.5% of DMSO control, suggesting that brusatol has an additive inhibitory effect on the anti-proliferative effects of vemurafenib in melanoma (**Figure 5.12A**). A similar trend was observed with 0.01 μ M triptolide, wherein triptolide treatment in combination with 0.1 μ M

vemurafenib significantly reduced cell viability to 60% compared to 67.5% and 88% of DMSO, for vemurafenib and triptolide alone (**Figure 5.12B**). Interestingly, luteolin also sensitised A375 cells to vemurafenib, reducing cell viability from 89% and 61% of DMSO for 10 μ M luteolin and 0.1 μ M vemurafenib alone, respectively, to 40% for luteolin and vemurafenib in combination (**Figure 5.12C**).

5.3.8 Brusatol and luteolin potentiate the effects of vemurafenib on A375 cell migration

As melanoma metastasises, novel therapeutic interventions that prevent migration could be useful to administer alongside therapies such as BRAF inhibitors to potentially slow disease progression and improve patient outcomes⁵¹⁴. To further explore whether Nrf2 inhibitors in combination with current standard of care chemotherapy, such as vemurafenib, could be useful in melanoma, we next explored the effects of co-incubation of Nrf2 inhibitors in with vemurafenib on cell migration in A375 cells via wound scratch assay (**Figure 5.13A**). For these experiments, only brusatol and luteolin were selected due to their promising effects at repressing both Nrf2 signalling (**Figures 5.5-5.9**), inhibiting M202 and A375 cell migration (**Figure 5.10 and 5.11**) and in sensitising A375 cells to vemurafenib (**Figure 5.12**).

As shown in **Figure 5.13B**, brusatol significantly potentiated the anti-migratory effects of vemurafenib in A375 cells, with migration reduced by 66% to 1.2 μ m/h compared to 3.5 μ m/h of cells incubated with vemurafenib alone. As visualised in **Figure 5.13C**, however the combination of luteolin and vemurafenib decreased migration to a greater extent than observed with brusatol in combination with vemurafenib. Here, luteolin inhibited the migration rate by 71.4% from 3.5 μ m/h to 1 μ m/h when co-incubated with vemurafenib, compared to vemurafenib alone (**Figure 5.13B**).

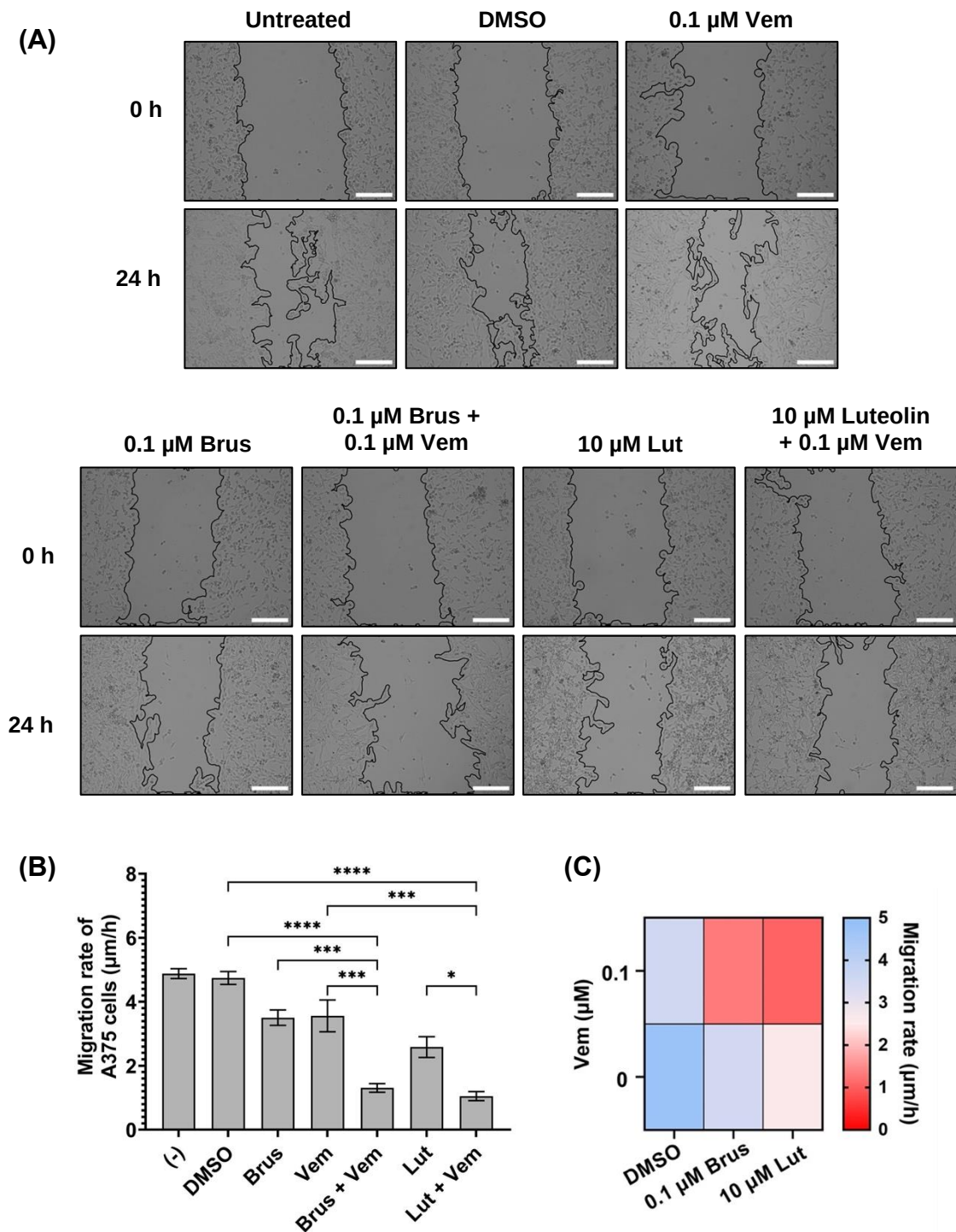


Figure 5.13 Brusatol and luteolin further potentiate the effect of the BRAF inhibitor vemurafenib on A375 cell migration. (A) Representative bright light images of the scratch area of A375 cells (2×10^5 cells/well) when treated with 0.1 μ M brusatol (Brus) or 10 μ M luteolin (Lut), alone or in combination with 0.1 μ M vemurafenib (Vem), at 0 h and 24 h post-treatment (N=3). The scratch area is outlined in black and the scale bar represents 100 μ m. The migration rate of A375 cell was calculated from bright light images using ImageJ analysis of the scratch area (μ m²). Data is presented as average migration rate (μ m/h) $\pm$ SEM as a (B) bar chart and (C) heat map. Statistical analysis was carried out via one-way ANOVA and post-hoc Tukey test, with comparisons made to DMSO (***P $\leq$ 0.0001, ****P $\leq$ 0.0001).

5.4 Discussion

Melanoma possesses the highest mortality rate of all skin cancers in the UK, with 5-year survival rates of below 30% for patients with late-stage disease⁵¹². Although advances in melanoma treatment, such as immunotherapies and targeted therapies, have greatly improved patient outcomes in the past decade, overall response rates in patients with metastatic melanoma remain low, typically ranging between 40-60%⁵¹⁴. One of the challenges in treating metastatic melanoma is that cells can quickly develop resistance to the current standard of care immunotherapy and BRAF-targeted therapies^{38,517}, leaving limited other treatment options for patients. Melanocytes, from which melanoma originates, experience especially high levels of oxidative stress and ROS, leading to a heavy reliance on redox regulating pathways⁵²⁶.

The major redox regulator Nrf2 may therefore serve as a useful therapeutic target to sensitise melanoma to already approved therapies, that otherwise show poor overall response rates⁵¹⁴. Current evidence from Nrf2 knockdown studies *in vitro* suggests that Nrf2 inhibition may repress cell proliferation, migration and chemoresistance of melanoma cells^{282,532,541}. Despite this, to date, very little research has been conducted on the use of pharmacological modulators of Nrf2 in melanoma, with only two studies currently published on Nrf2 inhibition in melanoma, focussed solely on brusatol and DMF^{338,460}. The present study therefore aimed to not only elucidate Nrf2's involvement in melanoma but also explore Nrf2 as a therapeutic target using natural product and small molecule inhibitors of Nrf2. Here, Nrf2 expression was investigated across the largest panel of melanoma cell lines to date, and the effects of a suite of Nrf2 inhibitors were compared for their effects on Nrf2 signalling, cell migration and ability to sensitise cells to BRAF inhibition in melanoma, for the first time.

Perturbations in redox homeostasis, for example through alterations in the Nrf2 pathway, may predispose melanocytes to neoplastic transformation, suggesting that initial loss of Nrf2 function may be an initiating event in melanoma development⁵⁴³. Despite this, melanoma cells appear to possess even higher levels of ROS than melanocytes, indicating that not only do they rely heavily on redox mechanisms to prevent apoptosis and DNA damage²⁵⁹, but that they may be more resistant to ROS-induced damage than healthy cells⁵²⁶. The role of Nrf2 expression in melanoma is also widely contended, with conflicting reports suggesting that, as is seen in other cancer types, Nrf2 may possess a dual role in melanoma to both prevent and promote tumorigenesis.

Firstly, to better understand the involvement of Nrf2 in melanoma, we explored mRNA expression of NFE2L2 (Nrf2) and Nrf2-related genes; KEAP1, PARK7 (DJ-1) and NQO1 in databases from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA). In a GEO dataset containing microarray data from patient samples of normal skin and primary melanoma and metastatic melanoma tumours, we observed that NFE2L2 expression was significantly elevated in metastatic but not primary melanoma, compared to melanocytes (**Figure 5.1A**). However, this was contradicted in **Figure 5.2A** and **Figure 5.3A**, wherein NFE2L2 was downregulated in RNA-Seq data of patient tumours in the TCGA dataset and in cell lines of melanoma versus primary melanocyte cells, respectively. Despite this, the Nrf2-downstream target gene NQO1 was consistently increased in melanoma compared to melanocytes (**Figure 5.1C** and **Figure 5.2C**), which is supported by a study by Garate *et al.*⁵³⁶.

As mRNA expression does not always correlate with protein expression, often due to epigenetic changes, reduced mRNA stability or increased protein degradation⁵⁴⁰, we also explored Nrf2 protein expression in primary melanocyte and melanoma cell lines (**Figure 5.4A**). Using western blot analysis, we observed that Nrf2 expression was higher in 6/9 of the melanoma cell lines studied compared to primary melanocytes, with M202 and A2058 melanoma cells demonstrating the highest expression (**Figure 5.4B**). Together, **Figures 5.1-5.4** suggest that while NFE2L2 may be elevated in melanoma, its expression pattern varies between patient cohorts and disease stage, further evidencing that Nrf2 may have a dual role in melanoma⁵²⁷. As the expression of Nrf2 mRNA did not correlate with Nrf2 protein expression, it is also possible that Nrf2 expression is predominantly regulated by post-translational mechanisms in melanoma, resulting in Nrf2 accumulation. In support of this hypothesis, Hintsala *et al.*⁵²⁸ observed that Nrf2 is especially enriched in the nucleus of patient melanoma tumours, associated with a more proliferative and invasive phenotype, implying that decreased cytosolic localisation and increased nuclear import of Nrf2 may be a key factor in increased expression of Nrf2 target genes, such as NQO1, in melanoma.

In healthy cells, Nrf2 is kept under tight regulation within the cytosol via a number of pathways, including Keap1-mediated proteasomal degradation¹³⁹. Disruption of the Nrf2/Keap1 interaction has been observed in numerous cancers, resulting in increased Nrf2 nuclear accumulation^{269,432}. Such perturbations can occur through a number of mechanisms, including loss of function mutations in KEAP1²⁶⁹, and increased expression of proteins that disrupt the Keap1/Nrf2 interaction, such as DJ-1⁴⁵⁸. However, as shown in **Figure 5.1B**, no difference in KEAP1 mRNA expression was observed between melanocytes and melanoma samples in the GEO melanoma dataset, however KEAP1

expression was slightly elevated in the TCGA melanoma samples (**Figure 5.2B**). This contrasts previous research that found that Keap1 expression was decreased in melanoma, associated with increased nuclear Nrf2^{528,530}. However, as KEAP1 expression did not appear to correlate with Nrf2 expression and increased malignancy, the accumulation of Nrf2 in some melanomas may be via a Keap1-independent mechanism.

DJ-1 on the other hand, which interrupts the Nrf2/Keap1 interaction⁴⁵⁸, was consistently increased at the mRNA level in patient melanomas versus adjacent healthy skin tissue (**Figure 5.1D** and **Figure 5.2D**), suggesting that it may be involved in melanoma pathogenesis. To further investigate this, we explored DJ-1 protein expression in melanoma and melanocyte cell lines and found that DJ-1 protein was increased in all 9 of the melanoma cell lines studied, compared to primary melanocytes (**Figure 5.4A**). Furthermore, some of the cell lines that expressed significantly elevated DJ-1 also showed significantly high Nrf2 expression, such as A2058 (**Figure 5.4**). Interestingly, increasing evidence suggests that DJ-1 may be a novel oncogene and is upregulated and implicated in the pathogenesis of many cancers, including glioma, breast, lung, prostate, colorectal and liver cancers^{544,545}. Emerging evidence also suggests that DJ-1 is involved in melanoma, where it acts to promote cell proliferation and metastasis via the PTEN/AKT pathway⁵³⁹. As DJ-1 is commonly upregulated in melanoma and is linked with increased Nrf2 expression, we propose that DJ-1 may assist in promoting nuclear accumulation of Nrf2 in melanoma by disrupting the binding of Nrf2 to its negative regulator, Keap1, which in turn promotes melanoma malignancy. While further investigations into DJ-1's role in melanoma were beyond the scope of the present study, ongoing research within the O'Connell lab aims to elucidate DJ-1's involvement in melanoma and evaluate whether it would be a suitable therapeutic target (data not shown).

Despite the observation in the present study that Nrf2 overexpression does not appear to occur in all melanoma patient tumours, increased expression is often linked with increased malignancy^{68,444,528,532,534,543}, suggesting that Nrf2 may be a useful therapeutic target in melanoma. Previously, studies of Nrf2 inhibition in melanoma have focussed on using siRNA to induce Nrf2 knockdown, which has been shown to increase ROS levels⁵³³, inhibit cell proliferation⁴⁴⁴, cell migration⁵³⁸ and sensitise cells to vemurafenib⁵³², cisplatin⁵³³, temozolomide⁵³⁴ and dacarbazine⁵³³. In a study by Argenziano *et al.*²⁸², Nrf2 siRNA-loaded nanobubbles were studied in M1 melanoma cells, which not only reduced M1 cell viability but also potentiated the anti-tumour effects of cisplatin. Currently, the use of pharmacological inhibitors of Nrf2 has not been well studied in melanoma, with

only two studies published, to date^{338,460}. In a study by Wang *et al.*³³⁸, combination of the Nrf2 inhibitor brusatol with UVA radiation significantly decreased Nrf2 and Nrf2 downstream gene expression, inhibited melanoma cell proliferation and induced apoptosis in A375 cells both *in vitro* and in a murine xenograft model *in vivo*. While Wang *et al.*'s findings support the use of Nrf2 inhibitors as therapeutic agents in melanoma, brusatol is not a good clinical candidate due to its off-target effects³⁸⁴.

Another study by Li *et al.*⁴⁶⁰ found that the small molecule DMF, which is currently marketed as an Nrf2 activator, sensitised A375 melanoma cells to the BRAF inhibitor vemurafenib, both *in vitro* and *in vivo*. Interestingly, although Li *et al.* observed that 50 μ M DMF induced Nrf2 cytosolic localisation in A375 cells, associated with increased cell sensitivity to vemurafenib, which appears to contradict the mechanism of action of Nrf2 in cancers, wherein activation of Nrf2 would further potentiate chemoresistance as opposed to inhibiting it. Furthermore, we have observed dual effects of DMF in melanoma, wherein high concentrations (e.g., 30 μ M) can inhibit Nrf2 protein expression in M202 melanoma cells (**Figure 5.5**). Due to these conflicting findings, also observed in other cancer types such as NSCLC (see **Chapter 3**) and in other cancers^{449,546}, DMF does not consistently act as an Nrf2 inhibitor in melanoma, thus further research is required to understand its suitability as an anti-cancer agent.

Other Nrf2 inhibitors, may however have use in melanoma treatment, hence we next compared the therapeutic potential of Nrf2 inhibitors in the high Nrf2-expressing M202 melanoma cell line (as displayed in **Figure 5.4A**). Several natural product and small molecules that have been reported to inhibit Nrf2 in the literature²⁵⁶ were investigated, including brusatol, luteolin, ML385, retinoic acid, triptolide, trigonelline and clobetasol. Interestingly, 5/7 of these inhibitors significantly repressed Nrf2 protein expression in M202 cells, with brusatol, luteolin and triptolide showing the greatest inhibitory effects (**Figure 5.5B**). ML385 and trigonelline did not show any effects on Nrf2 protein expression (**Figure 5.5**). Although these results could possibly be expected for ML385, which acts to inhibit the Nrf2/ARE interaction³⁵⁹, this was surprising for trigonelline, which is reported to repress Nrf2 nuclear translocation and therefore increase degradation of cytosolic Nrf2³⁶⁶.

As brusatol and triptolide are potent inhibitors of cell proliferation, with IC₅₀ values in the nanomolar range in M202 cells (**Figure 3.1**), we further explored these inhibitors at lower concentrations of 0.2-20 μ M, which demonstrated that the decrease in Nrf2 protein expression was maintained at concentrations as low as 0.2 μ M for both inhibitors (**Figure 5.6**). Additionally, we also found that while brusatol rapidly repressed Nrf2 protein

expression in as little as 2 h treatment, triptolide required a longer treatment time to inhibit Nrf2 expression, with the best effects observed at 8 h treatment (**Figure 5.7**). This demonstrates for the first time that such inhibitors show similar activity in melanoma cells to that observed in other cell types, such as A549 NSCLC cells (**Figure 3.4** and **Figure 3.5**), both in our own studies and in the literature^{334,336,368,370,373,440}.

Next, to further expand on the inhibitory effects of Nrf2 modulators on Nrf2 signalling in melanoma, we compared their ability to repress Nrf2 downstream signalling in melanoma. Among the Nrf2 inhibitors studied, only brusatol, luteolin and ML385 repressed Nrf2/ARE binding via a DNA-binding activity ELISA in M202 cells, with brusatol once again showing the greatest inhibitory effects (**Figure 5.8**). To better understand how these results relate to gene expression, we also explored the effects of the inhibitors on the enzymatic activity of NQO1, which is a Nrf2-target gene that is often upregulated and known to promote cell proliferation and migration of melanoma cells⁵³⁶. When compared in an assay that measured NQO1 enzymatic activity, although brusatol showed the best repressive effects on NQO1 activity, all of the Nrf2 inhibitors showed significant inhibition of NQO1 activity at 20 μ M (**Figure 5.9B**).

As genetic inhibition of Nrf2 via siRNA can repress melanoma cell migration⁵³⁸, we hypothesised that pharmacological Nrf2 inhibition could also be useful in reducing melanoma tumour progression via inhibiting cell migration. As shown in **Figure 5.10**, brusatol and luteolin demonstrated the greatest repressive effects on M202 cell migration, although ML385, triptolide, trigonelline and clobetasol also significantly repressed cell migration (**Figure 5.10**). While many of the inhibitors have not been previously studied for their ability to repress melanoma cell migration previously, reports by Wang *et al.*⁵⁴⁷ and Yao *et al.*⁵⁴⁸ support our findings that triptolide and luteolin can suppress melanoma cell migration. To further validate these effects and to demonstrate the use of Nrf2 inhibitors in melanoma cells with lower Nrf2 expression than M202 cells, we also explored the anti-migratory effects of the Nrf2 inhibitors in the BRAF^{V600E} mutant A375 melanoma cells, which have been previously studied in experiments of siRNA-mediated Nrf2 knockdown^{444,532,541}. While these cells possess lower Nrf2 expression, Nrf2 inhibitors could still find use in BRAF mutant cells with lower Nrf2 expression, wherein they could act as chemosensitisers to standard of care chemotherapies such as BRAF inhibitors. As the BRAF^{V600E} is the most common form of BRAF mutation, present in 90% of BRAF mutated melanomas, these cells are a useful *in vitro* model in melanoma⁵¹⁸. In the A375 cells, a similar trend was observed to that seen in the M202 cells, with all of the inhibitors significantly repressing A375 cell migration (**Figure 5.11**). Once again however, brusatol and luteolin demonstrated the best inhibitory effects

(**Figure 5.11**). Interestingly, this suggests that pharmacological inhibition of Nrf2 may also induce anti-migratory effects in both BRAF wild-type and mutant melanoma cells, suggesting that their use could be applied to a variety of melanoma patients.

Lastly, we explored the co-treatment of Nrf2 inhibitors with a standard of care melanoma therapy, as chemoresistance is a common road block in melanoma treatment. In particular, patients with BRAF mutant melanomas frequently develop resistance to BRAF inhibitors, such as vemurafenib, and response rates are often below 50%⁶⁵. Although several mechanisms underpin melanoma resistance, Nrf2 has been reported to promote resistance to vemurafenib and several classical cytotoxic chemotherapies in melanoma^{282,533}. Hence, we next explored whether pharmacological inhibition of Nrf2 could also reverse therapy resistance in BRAF mutant cells. As brusatol, triptolide and luteolin all showed the best inhibitory effects on Nrf2 protein expression, Nrf2/ARE binding, NQO1 enzymatic activity and cell migration (**Figures 5.5 -5.11**), we explored these inhibitors for their ability to sensitise A375 cells to the anti-proliferative and anti-migratory effects of vemurafenib using MTS and wound scratch assays, respectively.

As displayed in **Figure 5.12B** and **5.12C**, both luteolin and triptolide in combination with vemurafenib significantly decreased the proliferation of A375 cells, compared to either luteolin, triptolide or vemurafenib alone. The chemosensitising effects of triptolide in melanoma are supported by a previous report by Wang *et al.*⁵⁴⁷, which demonstrated that triptolide could sensitise A375 cells to the anti-proliferative and anti-migratory effects of carboplatin. However, as carboplatin is not clinically used in melanoma treatment, our results demonstrate that triptolide could sensitise melanoma tumours to a clinically relevant standard of care treatment. Furthermore, we also observed that luteolin, which possesses limited off-target effects compared to brusatol and triptolide^{384,481}, significantly sensitised A375 cells to the anti-migratory effects of vemurafenib (**Figure 5.13B**). This suggests that not only does luteolin treatment add to the anti-proliferative effects of vemurafenib, but that it can also potentiate the anti-migratory effects of BRAF inhibition in BRAF mutant cells. This is further supported by Yao *et al.*⁵⁴⁸, who observed that luteolin treatment in A375 cells could repress cell migration, associated with a decrease in matrix metalloproteases MMP-2 and MMP-9. While the study by Yao *et al.* did not explore luteolin's effects on Nrf2 signalling directly, the inhibitory effects of luteolin were related to decreased activation of PI3K/AKT signalling⁵⁴⁸. This may suggest that luteolin may not solely act to induce Nrf2 mRNA turnover, but may also regulate Nrf2 via suppression of PI3K/AKT-induced Nrf2 degradation.

One limitation of the present study was that all experiments were conducted in a 2D cell culture model. Hence in future studies, we could further explore the Nrf2 inhibitors in a 3D culture model, such as with the use of tumouroids comprised of melanoma cells, fibroblasts and macrophages, to better recapitulate the tumour microenvironment⁵⁴⁹. Additionally, as this work was only conducted *in vitro*, studies could also be conducted to explore the efficacy of Nrf2 inhibitors in an *in vivo* xenograft model of melanoma to better understand how these therapies could translate into the clinic. Finally, more research could also be conducted on the mechanisms of Nrf2 overexpression in melanoma to better understand patient-patient variability in clinical trials. While ongoing research within the O'Connell laboratory is investigating the role of Nrf2-stabilising proteins that are upregulated in melanoma, such as DJ-1, future studies could also explore other mechanisms of regulation such as proteins involved in Nrf2 turnover and nuclear translocation, such as Keap1 and GSK-3 β .

In conclusion, the present study found that while Nrf2 appears to play a dual role in melanoma, as is the case in many other cancers⁵¹¹, it may be a useful therapeutic target in Nrf2-overexpressing melanoma tumours and in chemoresistant melanoma tumours. Additionally, several Nrf2 inhibitors were compared for the first time in melanoma, with brusatol, triptolide and luteolin demonstrating consistent repressive effects on both Nrf2 signalling, cell migration and proliferation of melanoma cells *in vitro*. Here we also identified that the Nrf2 inhibitors brusatol, luteolin and triptolide could sensitise BRAF^{V600E} melanoma cells to vemurafenib, a standard of care BRAF inhibitor. Although both brusatol and triptolide are known to have off-target effects, targeted delivery of these inhibitors in melanoma, for example through nanoparticle delivery or use in an antibody or peptide-drug conjugate^{475,482}, could reduce their off-target effects and serve as a useful therapeutic intervention in resistant melanoma cells. One mechanism through which this could be achieved is via conjugation of a peptide that targets receptors overexpressed on melanoma cells, such as the melanocortin 1 receptor (MC1R), using a similar design as that described in **Chapter 4**.

On the other hand, the flavonoid luteolin, which induces Nrf2 mRNA turnover^{349,350}, sensitises breast, liver and ovarian cancers to several chemotherapies^{349,351,352} and possesses minimal off-target effects, also showed promise as an anti-tumour agent in melanoma in the present study. Although more research is warranted, for example using *in vivo* models of melanoma, luteolin could also be used as a potential sensitising agent in therapy resistant melanomas.

Chapter 6. Nrf3 as a potential therapeutic target in cancer

Acknowledgments:

Data for **Figures 6.4** and **6.5** was provided by Dr Mark Williams and data analysis was carried out by Sean Tattan. Prof Akira Kobayashi donated the anti-Nrf3 antibody for the experiments conducted in **Figure 6.7**.

6.1 Introduction

Nrf3 (also known as NFE2L3) was first identified in 1999¹⁹⁵ as a member of the CNC family of transcription factors. While the function of Nrf3's sister protein, Nrf2, is widely researched¹¹⁹, the physiological function of Nrf3 is unclear. However, emerging evidence suggests a potential involvement of Nrf3 in tumour promotion in cancer¹⁹⁷. Although Nrf3 is primarily expressed in the placenta¹⁹⁶, it is also expressed in numerous cell types, such as keratinocytes and smooth muscle cells^{196,200,550}. In cells, Nrf3 is mainly localised within the cytosol, where it is anchored to the endoplasmic reticulum (ER) via its N-terminal domains NHB1 and NHB2 (N-terminal homology box 1/2)^{101,199} (**Figure 1.5**). Upon stress stimuli, such as stimulation with TNF- α (tumour necrosis factor alpha) and the ER stress inducers tunicamycin and thapsigargin, Nrf3 is cleaved via the aspartyl protease DDI2 (DNA damage inducible 1 homolog 2), causing release of Nrf3 from the ER¹⁹⁹ (**Figure 1.9**). Nrf3 is then also deglycosylated at its NST (asparagine/serine/threonine) domain by NGLY1 (N-glycanase 1), which produces a cleaved, de-glycosylated form, of approximately 76 kDa, which can heterodimerise with sMaf proteins¹⁹⁸ (as shown in **Figure 1.9**). Like other CNC family members, Nrf3 can then translocate into the cell nucleus¹⁹⁸, where it regulates gene transcription via binding to ARE sequences on its target genes.

Once in the nucleus, Nrf3 has been shown to act as both an activator and repressor of gene transcription¹¹⁷. To date, only a small number of Nrf3 target genes have been validated (as detailed in **Table 1.4**), which assist in mediating cell proliferation, proteasome assembly, glucose transport and redox signalling, such as DUX4 (double homeobox factor 4)²¹², POMP (proteasome maturation protein)¹⁹⁷, NQO1 (NAD(P)H dehydrogenase [quinone] 1)¹⁹⁸, UHMK1 (U2AF homology motif kinase 1)²⁰², GLUT1 (glucose transporter 1)²¹⁰, CPEB3 (cytoplasmic polyadenylation element binding protein 3)⁴¹³ and VEGFA (vascular endothelial growth factor alpha)²⁰⁹. Owing to the lack of understanding surrounding Nrf3's transcriptional role, its physiological function is also poorly characterised. However, recent studies have suggested a potential role of Nrf3 in cancer, as Nrf3 upregulation has been noted in numerous cancer types including colorectal^{1210,212,400,402,551}, kidney^{405,406,552}, pancreatic²⁰⁹, liver⁴⁰³, bladder⁴⁰⁹, Hodgkin lymphoma³⁹⁸, gastric^{408,553}, thyroid³⁹⁹ and cervical^{551,554} cancers. Additionally, high Nrf3 expression in patient tumours is often associated with poorer prognosis^{401,403,406,408,409,552} and occurrence of resistance to both chemotherapy agents⁴⁰⁰ and radiotherapy⁴¹⁰.

To date, limited studies have explored the mechanisms underpinning the upregulation and downstream effects of Nrf3 signalling in cancer. Initial studies have suggested that

Nrf3 promotes the proteasomal degradation of tumour suppressor proteins, such as Rb (retinoblastoma protein) and p53 (tumour protein p53), through inducing expression of genes associated with 20S proteasome assembly, such as POMP^{206,413}. In colorectal adenocarcinoma and hepatocellular carcinoma, Nrf3 has also been identified as a downstream target of the Wnt/ β -catenin pathway^{210,404}, suggesting that it may crosstalk with other key pathways involved in tumorigenesis. Such evidence is corroborated by studies both *in vitro* and *in vivo*, in which Nrf3 knockdown increases apoptosis and represses cancer cell proliferation, metastasis and growth of xenograft tumours^{400,409,410}. Although this research field is still in its early stages, this evidence suggests that Nrf3 may serve as a useful therapeutic target in cancers with high Nrf3 expression.

To further investigate the role of Nrf3 in cancer, and evaluate its potential as a therapeutic target, this study explored the expression of Nrf3 in a variety of cancers, using patient data from The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO). Additionally, Nrf3 and Nrf3 target gene expression was also studied in both patient-derived tissue of colorectal cancer patients and in a panel of cancer cell lines, which may serve as useful screening platforms for further research in this field. Finally, knockdown of Nrf3 was explored in the high Nrf3-expressing MIAPACA2 pancreatic cell line, to study the effects of Nrf3 repression on cancer cell proliferation and migration.

6.2 Aims

1. Explore Nrf3 expression in patient matched tumour and healthy tissue across a variety of common cancer types, using cancer databases, such as the TCGA and GEO.
2. Study the differential expression of Nrf3 and Nrf3-target genes in patient matched colonic organoids and tumouroids.
3. Characterise the expression of Nrf3 mRNA and protein in a panel of cancer cell lines.
4. Investigate the effects of Nrf3 knockdown on cancer cell proliferation and migration in a high Nrf3 expressing cancer cell line, via the use of siRNA.

6.3 Results

6.3.1 Nrf3 is upregulated in patient tumours across a variety of cancer types

Firstly, differential expression of NFE2L3 mRNA in normal and tumour tissue was explored in a panel of cancer types using datasets from The Cancer Genome Atlas (TCGA), as shown in **Figure 6.1**.

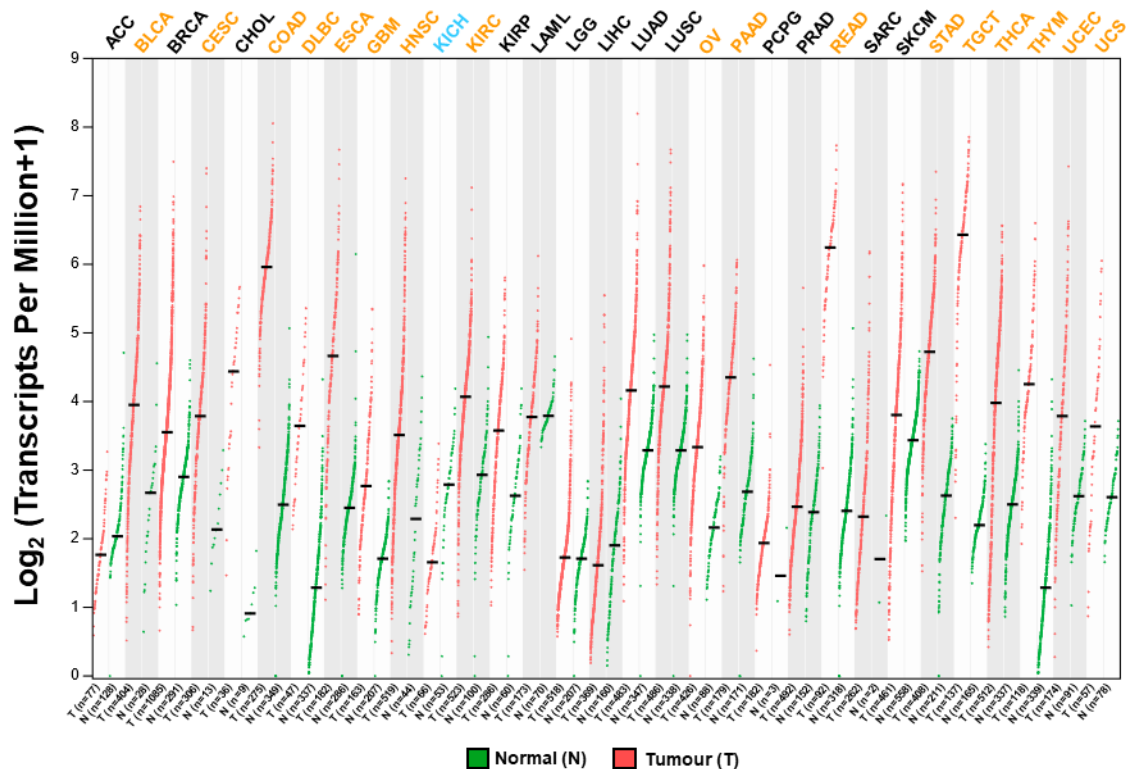


Figure 6.1 NFE2L3 mRNA is upregulated in patient tumours compared to normal tissue across a variety of cancer types. Expression of NFE2L3 mRNA was explored using RNA-Seq data from the TCGA, using the GEPIA (Gene Expression Profiling Interactive Analysis) web server. Expression is presented as Log_2 (Transcripts Per Million+ 1) for both normal (N) and tumour (T) tissue from a panel of cancer types. Cancer types displaying upregulation or downregulation of NFE2L3 in tumour vs normal tissue are denoted by orange and blue text, respectively. Cancer types listed in black text show no significant difference in NFE2L3 expression among normal and tumour samples. Statistical analysis was conducted via two-way ANOVA and Benjamini & Hochberg adjustment using the GEPIA web server ($\text{Padj} \leq 0.05$ were considered statistically significant). *Cancer abbreviations: ACC, Adrenocortical carcinoma; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangial carcinoma; COAD, Colon adenocarcinoma; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LAML, Acute Myeloid Leukaemia; LGG, Brain Lower Grade Glioma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; TGCT, Testicular Germ Cell Tumours, THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma; UCS, Uterine Carcinosarcoma.*

As shown in **Figure 6.1**, NFE2L3 is significantly upregulated in tumour tissue compared to patient-matched normal tissue in 17/31 cancer types included in the TCGA database, including in bladder, colon, ovarian, pancreatic, rectal, testicular and thyroid carcinomas. Of these, the highest NFE2L3 transcripts per million were recorded in colon adenocarcinoma (COAD), rectal adenocarcinoma (READ) and testicular germ cell tumours (TGCT), (**Figure 6.1**). Interestingly, **Figure 6.1** also shows that NFE2L3 was only significantly downregulated in one cancer type: kidney renal clear cell carcinoma (KICH).

To further corroborate the results observed in the TCGA, we next investigated whether NFE2L3 expression is elevated in other cancer databases, such as the Gene Expression Omnibus (GEO), as displayed in **Figure 6.2**. For this analysis, datasets of patient-matched normal and tumour tissue samples of colorectal and pancreatic cancers were selected as these were shown to have significant upregulation of NFE2L3 in **Figure 6.1**. Additionally, datasets of breast and hepatocellular carcinoma that did not show upregulation of NFE2L3 expression (**Figure 6.1**) were selected to study whether they also matched the data from the TCGA. Differential gene expression analysis was then conducted via the GEO2R server for the following GEO datasets: GSE44076, GSE15471, GSE70947 GSE14520. These datasets were selected on the basis that they contained patient-matched tumour and non-tumour samples and contained the highest sample numbers compared to other open-access GEO datasets that contained patient-matched tissue for these cancer types (see **Chapter 2.2.12**, **Table 2.7** for further details of these datasets).

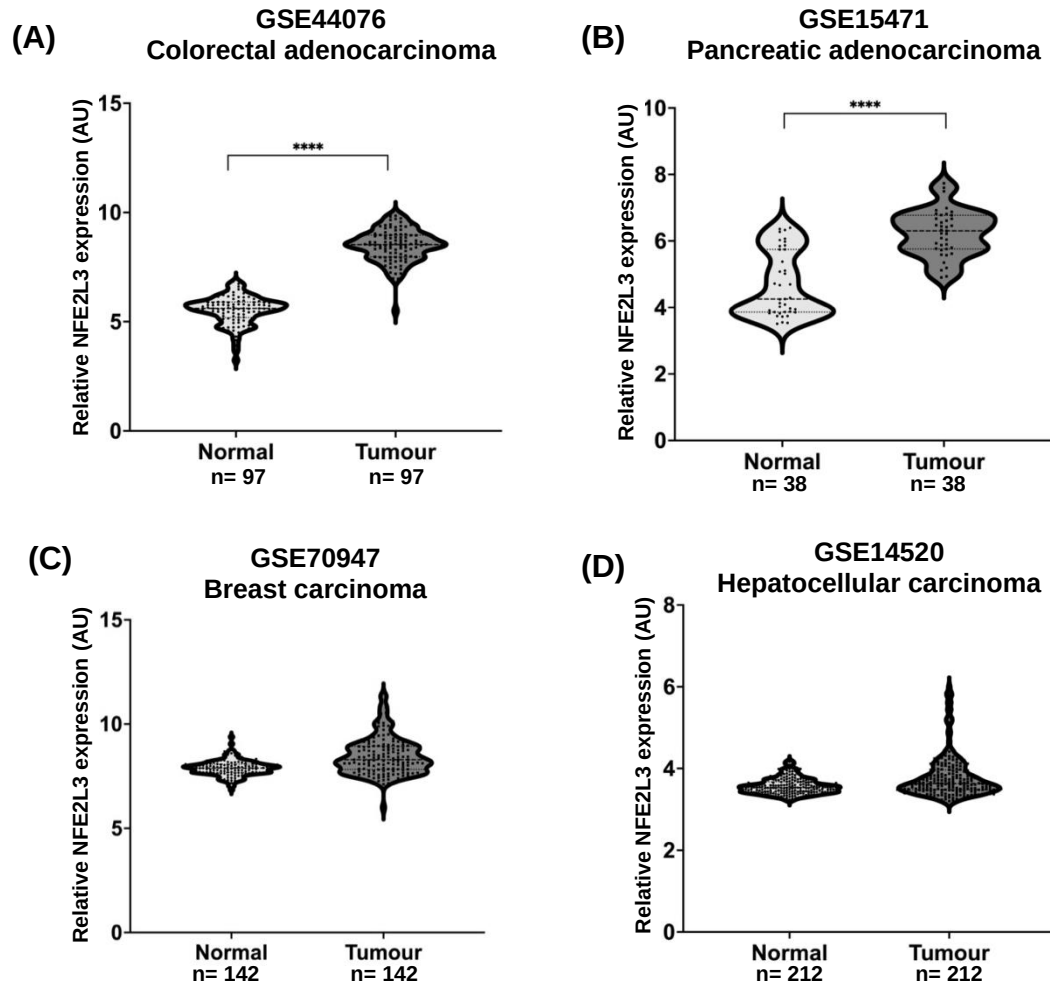


Figure 6.2 NFE2L3 expression in patient tumours of colorectal, pancreatic, breast and liver cancers. NFE2L3 mRNA expression in patient matched normal and tumour tissue was analysed from microarray data accessed via GEO datasets of **(A)** colorectal adenocarcinoma (GSE44076), **(B)** pancreatic ductal adenocarcinoma (GSE15471), **(C)** breast carcinoma (GSE70947) and **(D)** hepatocellular (liver) carcinoma (GSE14520). Expression is presented as relative NFE2L3 expression (AU; arbitrary units). Differential expression analysis of microarray data was performed using Limma and the Benjamini & Hochberg adjustment via the GEO2R server (**** $P_{adj} \leq 0.0001$).

As shown in **Figure 6.2**, NFE2L3 is significantly upregulated in tumour tissues of both colorectal adenocarcinoma and pancreatic ductal adenocarcinoma patient tumours, compared to patient-matched normal adjacent tissue. In contrast, no significant difference in NFE2L3 expression was observed in healthy and tumour tissue of breast carcinoma or hepatocellular carcinoma patients (**Figure 6.2**).

Next, using the GEPIA webserver and data accessible from TCGA, the association between NFE2L3 expression and overall survival of patients with colorectal, pancreatic, breast and liver carcinomas was investigated (**Figure 6.3**). As displayed in **Figure 6.3B**, pancreatic adenocarcinoma patients with high NFE2L3 expression displayed

significantly poorer overall survival than those with low NFE2L3 expression. Hepatocellular carcinoma patients also showed a similar trend, with high NFE2L3 expression correlating with significantly poorer percent overall survival (**Figure 6.3D**). While colorectal adenocarcinoma patients with high NFE2L3 expression showed lower percent survival compared to the low expression cohort, this difference was not significant (**Figure 6.3A**). On the contrary, the association between NFE2L3 expression and overall survival in breast cancer patients was not clear, with no significant difference between overall survival of the two expression cohorts (**Figure 6.3C**).

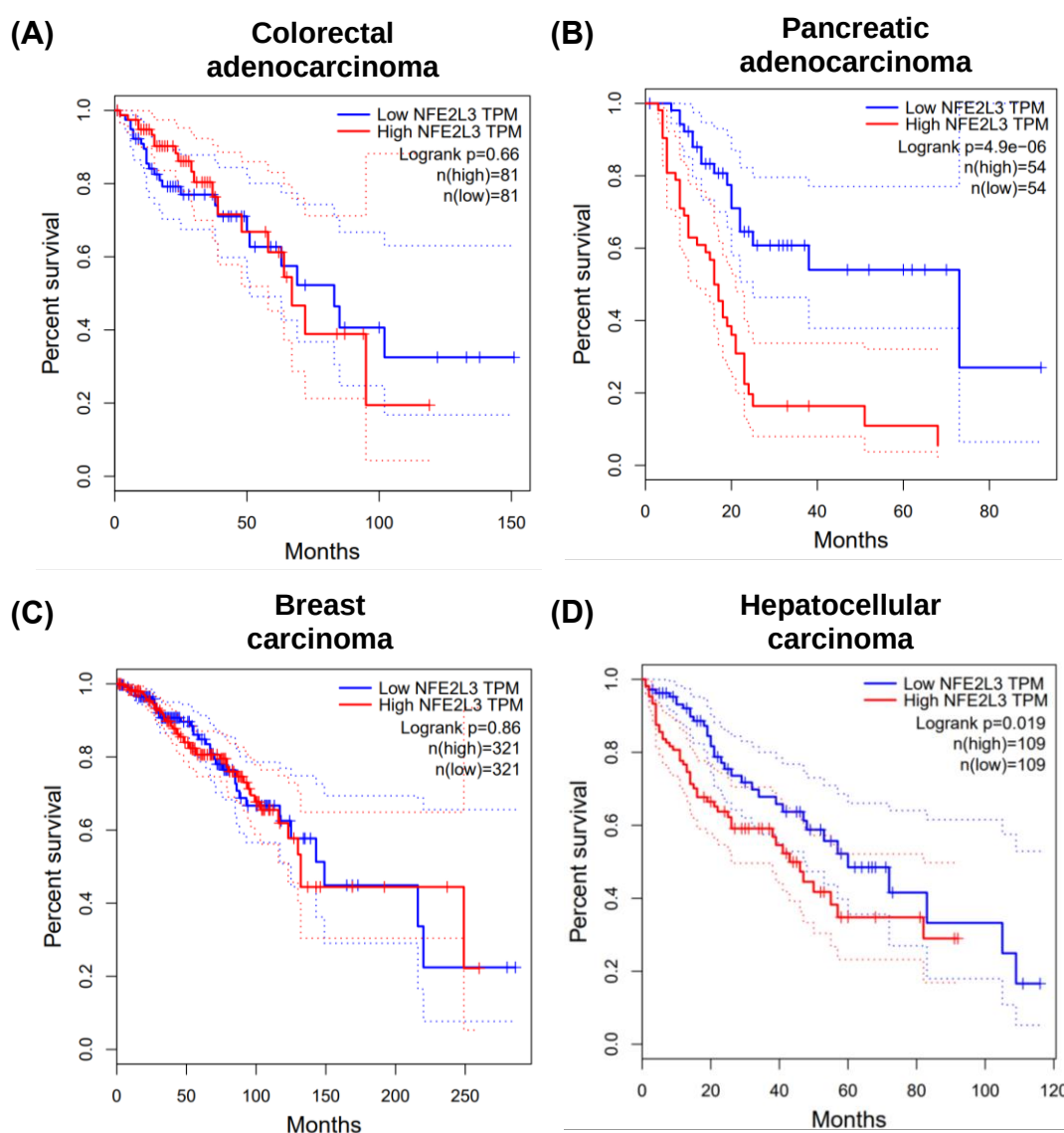


Figure 6.3 Overall survival of colorectal, pancreatic, breast and liver cancer patients with high and low NFE2L3 expression. Kaplan-Meier curves displaying percentage overall survival of patients with low (<25% percentile) and high (>75% percentile) NFE2L3 expression from TCGA datasets of **(A)** colorectal adenocarcinoma, **(B)** pancreatic adenocarcinoma, **(C)** breast carcinoma and **(D)** hepatocellular carcinoma. Data was accessed via the TCGA database and was analysed via the GEPIA webserver. Dotted lines represent the 95% confidence interval (CI). Statistical analysis was conducted using the log-rank test via GEPIA, where a Logrank $P \leq 0.05$ was considered statistically significant.

As the data presented from the TCGA and GEO databases suggests that NFE2L3 expression is elevated in colorectal adenocarcinoma (**Figures 6.1, 6.2A**) and may be associated with poorer overall survival (**Figure 6.3A**), we next studied NFE2L3-related gene expression in patient-derived samples of colorectal adenocarcinoma. To achieve this, RNAseq data from Dr Mark Williams laboratory (University of East Anglia, Norwich) was analysed to investigate whether NFE2L3 is differentially expressed in their system of patient-derived and patient-matched colonic organoids and tumouroids (**Figure 6.4**). In this culture system, tissue biopsies from colorectal adenocarcinoma patients, of both tumour and adjacent healthy tissue is collected, and colonic crypts of healthy tissue and cells from the tumour epithelia are isolated. These are then cultured in Matrigel Matrix, where they then form either colonic organoids (healthy tissue) or tumouroids, as detailed in Palaez-Llaneza *et al.*⁵⁵⁵. RNA is then isolated from all samples (including isolated crypts, isolated tumour epithelia, organoid and tumouroid), which is subjected to RNA sequencing and subsequent differential gene expression analysis using DESeq2 (see **Chapter 2.2.13**).

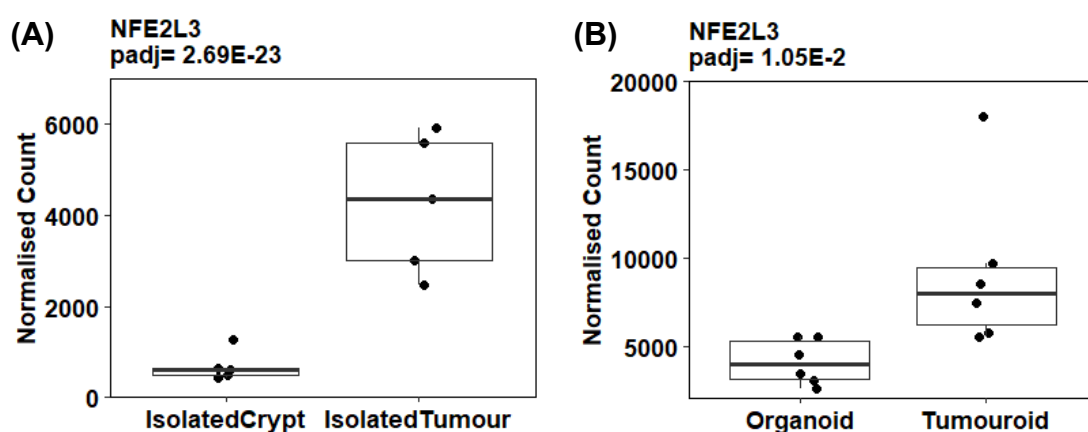


Figure 6.4 NFE2L3 mRNA is significantly upregulated in patient-derived and patient-matched colorectal cancer samples. RNAseq data collected from patient colorectal adenocarcinoma tumours and patient-matched adjacent healthy tissue was analysed for NFE2L3 mRNA expression in **(A)** isolated colonic crypts vs isolated tumour epithelia, and **(B)** colonic organoids vs tumouroids (passage <10). Data is representative of tissue derived from 6 individual colorectal cancer patients (N=6). Counts were normalised and P adjusted (Padj) values were derived from differential gene expression analysis in DESeq2. Data analysis and presentation was conducted by Sean Tattan.

Firstly, the expression of NFE2L3 was studied in both isolated colonic crypts vs isolated tumour tissue, and in colonic organoids (N=6) vs tumouroids (N=6). As shown in **Figure 6.4A**, NFE2L3 expression is significantly increased in isolated colonic tumour epithelia compared to isolated colonic crypts. Additionally, NFE2L3 is also significantly elevated in patient-derived tumouroids compared to organoids (**Figure 6.4B**), suggesting that the expression pattern of NFE2L3 is not affected by culture conditions.

Next, differential gene expression analysis of RNAseq data from the colorectal cancer tissue and patient-matched normal adjacent tissue was conducted to study Nrf3-related genes in colorectal cancer patient samples (**Figure 6.5**).

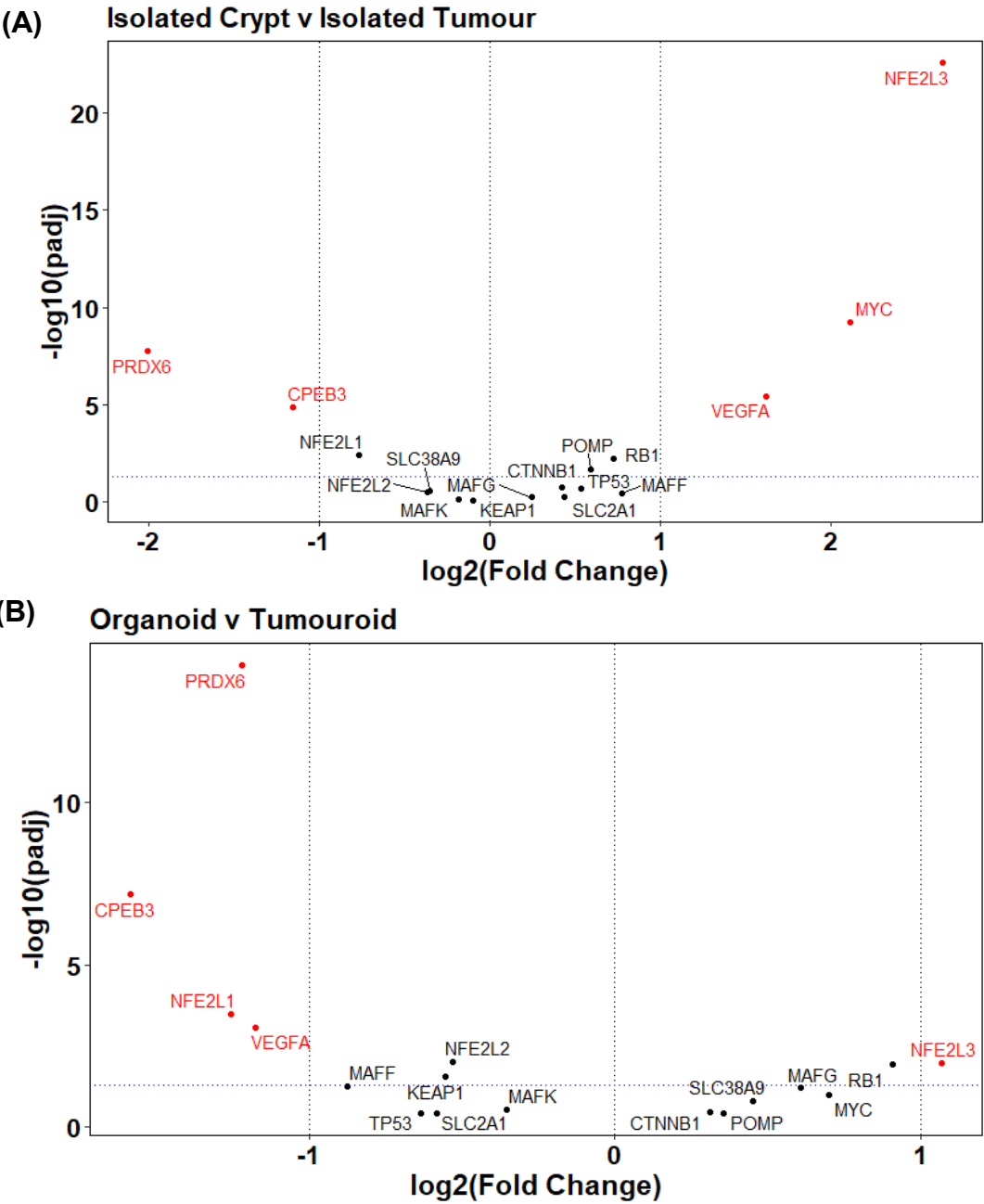


Figure 6.5 Differential gene expression of the NFE2 family members and their target genes in colorectal cancer. (A) Volcano plot depicting a differential gene expression analysis (undergone in DESeq2) of patient derived isolated colonic crypts (N=6) vs isolated tumour epithelia (N=6), and (B) patient derived colonic organoids vs colonic tumouroids (N=6, passage <10). Positive log2(fold changes) depict upregulation in tumouroids, and negative values depict downregulation in tumouroids with respect to organoids. Blue dotted line represents P adjusted (Padj) value of 0.05, genes presented in red possess Padj values <0.05 and > +/- 2 fold change. Data analysis and presentation was conducted by Sean Tattan.

As shown in **Figure 6.5**, NFE2L3 was the most significantly upregulated gene in both isolated tumour epithelia vs healthy colonic crypts, and in tumouroids vs organoids, compared to other Nrf3-related genes. Expression of the other NFE2 family members: NFE2L1 and NFE2L2, was significantly decreased in tumouroids compared to organoids (**Figure 6.5B**).

Furthermore, Nrf3 target genes that Nrf3 negatively regulates were shown to be significantly downregulated, such as PRDX6 (peroxiredoxin 6) in both isolated tumour epithelia and cultured colonic tumouroids (**Figure 6.5B**). Nrf3 target genes that have been shown to be upregulated by Nrf3 induction, such as the proteasome-related gene POMP (proteasome maturation protein)¹⁹⁷, was upregulated in isolated tumour epithelia and tumouroids, compared to adjacent healthy tissue (**Figure 6.5**). Unexpectedly, CPEB3 (cytoplasmic polyadenylation element binding protein 3), which is known to be upregulated by Nrf3 was found to be downregulated in both isolated tumour epithelia and tumouroids compared to isolated crypts and organoids (**Figure 6.5**). Notably, some differences in gene expression were observed between isolated colonic tumour epithelia and cultured colonic tumouroids, for example VEGFA (vascular endothelial growth factor alpha) was significantly upregulated in isolated tumour epithelia, but was downregulated in tumouroids (**Figure 6.5**), suggesting regulation of some Nrf3 target genes may be influenced by culture conditions imposed in the present study.

6.3.2 NFE2L3 is highly expressed in breast, prostate, liver, colorectal and pancreatic cancer cell lines

To further explore the role of Nrf3 in cancer, NFE2L3 expression was studied via RT-qPCR using RNA isolated from a variety of cancer cell lines, including melanoma (M202, A375), lung (A549, H292), breast (MDA-MB-231, MCF-7), prostate (LNCAP), colorectal (HCT116, CACO-2), liver (HEPG2), leukaemia (THP-1) and pancreatic (MIAPACA2) cells. The immortalised embryonic kidney cell line HEK293 was included here as a control, as it has previously been reported to show high NFE2L3 expression in the literature¹⁹⁶.

As shown in **Figure 6.6**, NFE2L3 expression was highest in the MDA-MB-231 breast cancer cells, followed by the CACO-2 colorectal cancer cell line. The lowest expression was observed in the M202 melanoma cell line (**Figure 6.6**). NFE2L3 expression also differed between cancer cell lines of the same organ origin, for example, HCT116 cells expressed significantly lower levels of NFE2L3 than the CACO-2 colorectal cell line

(Figure 6.6). A similar trend was observed in the breast cancer cell lines, with the MCF-7 cells displaying significantly lower NFE2L3 expression than MDA-MB-231 cells (Figure 6.6). On the other hand, the lung (A549 and H292) and melanoma (M202, A375) cancer cell lines did not show significant difference in NFE2L3 expression (Figure 6.6).

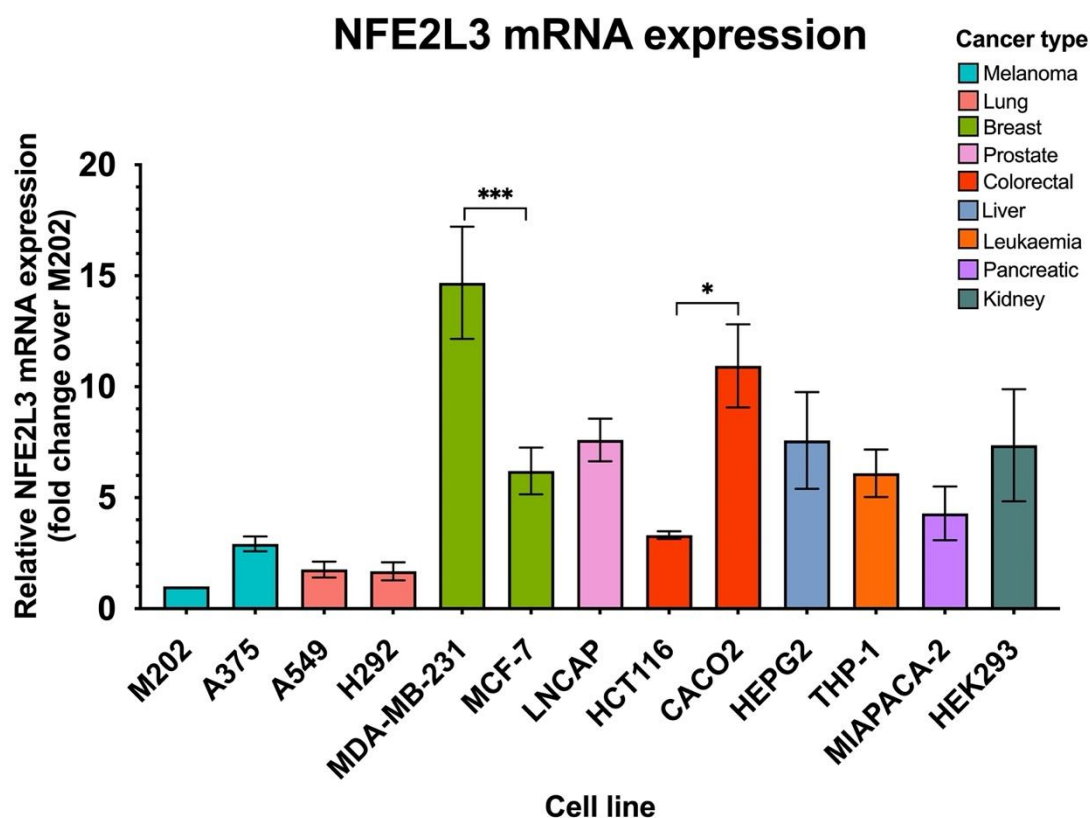


Figure 6.6 NFE2L3 mRNA is differentially expressed in a panel of cancer cell lines. RNA was extracted from 1×10^6 cells from a panel of melanoma, lung, breast, prostate, colorectal, liver, leukaemia, and pancreatic cancer cell lines, and HEK293 cells. cDNA was synthesised from the isolated RNA and mRNA expression was measured via RT-qPCR. mRNA expression was normalised to GAPDH and is presented as mean expression (fold change over the lowest expressing cell line, M202) $\pm$ SEM (N=3). Statistical analysis was conducted via one-way ANOVA with post-hoc Tukey test (* $P \leq 0.05$, *** $P \leq 0.001$).

6.3.3 Nrf3 protein is highly expressed in colorectal, pancreatic and breast cancer cell lines

High mRNA levels do not always correlate directly with high levels of gene expression at the protein level⁵⁴⁰, hence we next studied the protein expression of Nrf3 in cancer cell lines via western blot analysis. However, a key concern in Nrf3 research is the validity of commercially available anti-Nrf3 antibodies. Currently, the most commonly cited antibody in the literature is the anti-Nrf3 rabbit polyclonal antibody from Prestige Antibodies (HPA055889), which was developed and validated in conjunction with the Human Protein Atlas. Additionally, Prof Akira Kobayashi's lab (Doshisha University,

Japan), who first identified Nrf3¹⁹⁵ and have published several papers in this area, have also generated their own antibody against Nrf3²⁰². To select the best antibody for our research and address the concerns surrounding antibody specificity, we compared the HPA055889 antibody with the antibody from the Kobayashi lab (**Figure 6.7**). As Nrf3 expression is highest in the placenta, a commercially available placental tissue lysate was used as a positive control in this experiment. Three main forms of Nrf3 exist in the cell; a full-length glycosylated form (observed at ~140 kDa), a full length de-glycosylated form (~100 kDa) and a cleaved de-glycosylated form (~76 kDa)^{101,199}. As displayed in **Figure 1.5**, Nrf3 is sequestered in the ER in its full length (~140 kDa and ~100 kDa) forms, and can only enter the nucleus once cleaved and deglycosylated, meaning that only the ~76 kDa form is able to bind to the ARE and regulate gene transcription¹⁹⁹.

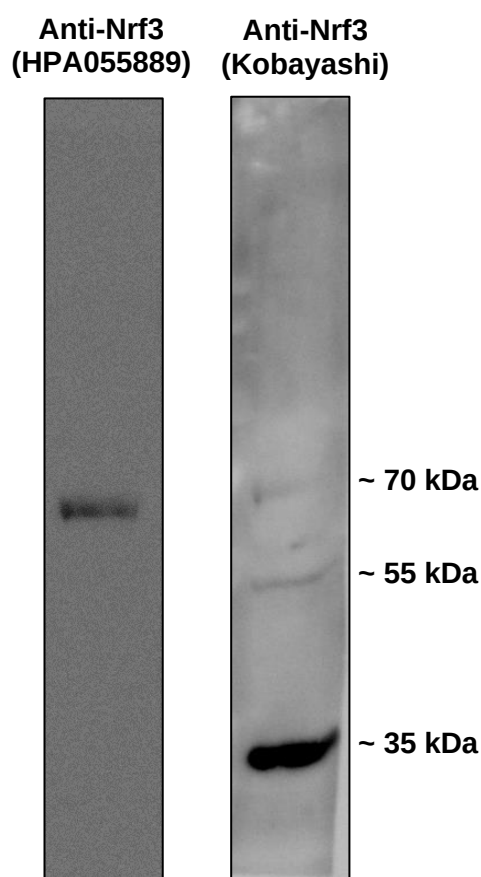


Figure 6.7 Nrf3 antibodies detect proteins of differing sizes in placental tissue. Placental tissue lysate was prepared to 40 µg total protein per gel lane and proteins were separated by SDS-PAGE. Western blot analysis was conducted using the Prestige Antibodies HPA055889 (1:500) and Kobayashi group (1:2000) antibodies (N=1).

As shown in **Figure 6.7**, the commercially available HPA055889 antibody only recognised one band at approximately 70 kDa for the placental lysate, which may indicate the presence of the cleaved form of Nrf3, as discussed in the literature¹⁹⁹. In contrast, the antibody from the Kobayashi group detected a weak band at 70 kDa and

indicated stronger bands at 55 and 35 kDa (**Figure 6.7**). Additionally, the antibody from the Kobayashi group displayed high background interference, as shown by the dark blotches in **Figure 6.7**. Despite attempts to remedy this, including the addition of further blocking steps with goat serum and increased washes with TBST and 5% milk, the interference issue persisted (data not shown). As the HPA055889 antibody is the most widely cited in the literature, and did not cause background interference, compared to the Kobayashi antibody, we therefore selected this antibody for further experiments.

Using the HPA055889 antibody, among the same panel of cancer cell lines explored previously (**Figure 6.6**), the highest Nrf3 protein expression was observed in the MCF-7 breast, HCT116 colorectal and MIAPACA2 pancreatic cell lines, as shown in **Figure 6.8**. On the other hand, the M202 melanoma, MDA-MB231 breast and CACO-2 colorectal cancer cell lines expressed very small amounts of Nrf3. All cell lines expressed proteins with a molecular weight of approximately 100 kDa, which is the observed molecular weight of the full length deglycosylated form of Nrf3 in SDS-PAGE gels¹⁹⁹. However, some cell lines such as HCT116 and HEK293 cells also displayed bands at an apparent molecular weight of 130 kDa (**Figure 6.8**), possibly indicating the presence of full length glycosylated Nrf3¹⁹⁹. Furthermore, the HEK293 and M202 cells displayed a band at approximately 70 kDa, likely indicating the cleaved form of Nrf3, although this band was very weak in M202 cells (**Figure 6.8**).

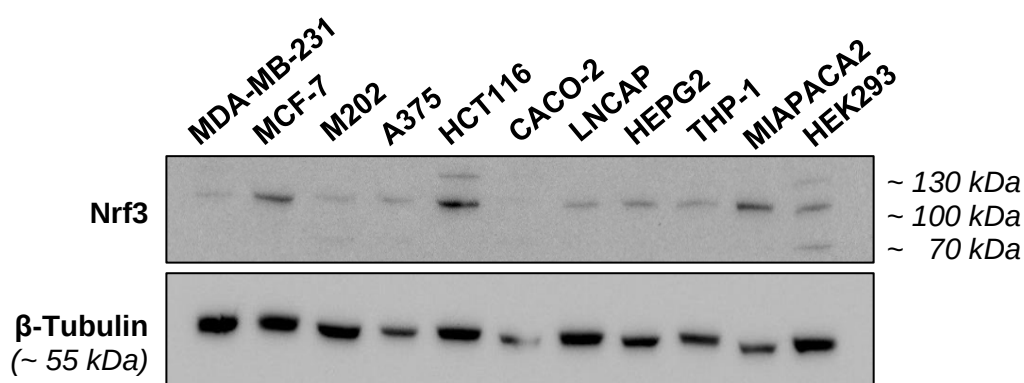


Figure 6.8 Nrf3 is highly expressed in breast, colorectal and pancreatic cancer cell lines. Representative western blot images of Nrf3 expression in a panel of melanoma, lung, breast, prostate, colorectal, liver, leukaemia, and pancreatic cancer cell lines, and HEK293 cells. Whole cell lysates were prepared from 1×10^6 cells, proteins were separated by SDS-PAGE and Nrf3 expression was measured via western blot analysis. β -Tubulin was included as a loading control. Images are representative of three individual experiments (N=3).

6.3.4 Optimisation of Nrf3 knockdown with siRNA

To further explore the role of Nrf3 in cancer, and determine its potential as a therapeutic target, we next studied the effects of Nrf3 knockdown on cancer cell proliferation and migration. As Nrf3 has been shown to be upregulated in patient samples of pancreatic cancer (**Figures 6.1, 6.2 and 6.3**), and the MIAPACA2 pancreatic cancer cell line showed high expression of Nrf3 at both the mRNA and protein level (**Figures 6.6 and 6.8**), MIAPACA2 cells were selected for further experiments. To achieve knockdown of Nrf3 in MIAPACA2 cells, DharmaFECT SMARTpool siRNA was utilised, which contains a mix of 4 siRNA's designed against a specific target, that have been previously authenticated by Dharmacon. Firstly, experiments were conducted to select the best concentration of siRNA to induce Nrf3 knockdown. To do so, MIAPACA2 cells were incubated with 5-100 nM of control or Nrf3 siRNA for 48 h and Nrf3 mRNA expression was measured via RT-qPCR (**Figure 6.9**).

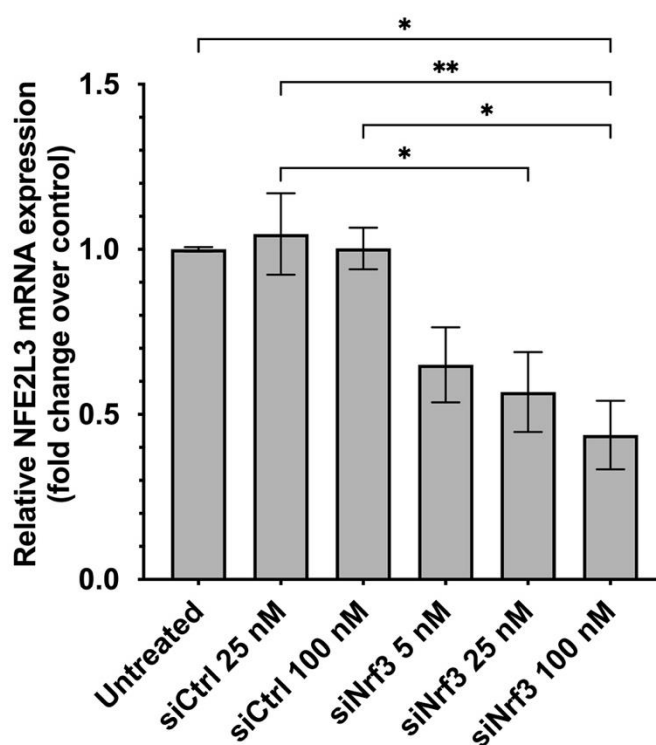


Figure 6.9 NFE2L3 knockdown in MIAPACA2 cells via siRNA. NFE2L3 mRNA expression following incubation of MIAPACA2 cells with 25 nM or 100 nM control (siCtrl) or 5 nM, 25 nM or 100 nM Nrf3 (siNrf3) siRNA, as measured by RT-qPCR. mRNA expression was normalised to GAPDH and is presented as mean expression (fold change over untreated control) $\pm$ SEM (N=3). Statistical analysis was conducted via one-way ANOVA with post-hoc Tukey test (*P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001, ****P $\leq$ 0.0001).

Compared to untreated control, the non-targeting control siRNA (siCtrl) did not affect Nrf3 mRNA levels (**Figure 6.9**). However, siRNA designed against Nrf3 (siNrf3) suppressed Nrf3 mRNA expression in a concentration-dependent manner in MIAPACA2

cells, with the greatest reduction observed with 100 nM siNrf3 (**Figure 6.9**). As the greatest repression of Nrf3 mRNA expression was observed with 25 nM and 100 nM siNrf3, these concentrations were selected to confirm whether the Nrf3 siRNA also reduced Nrf3 protein expression in the MIAPACA2 cells (**Figure 6.10**).

As shown in **Figure 6.10**, 100 nM siNrf3 significantly decreased Nrf3 protein expression compared to untreated cells and control siRNA, via western blot. Furthermore, no inhibition of Nrf3 protein expression was observed with the control siRNA (**Figure 6.10**). From these experiments, a concentration of 100 nM siRNA was taken forward for further experiments.

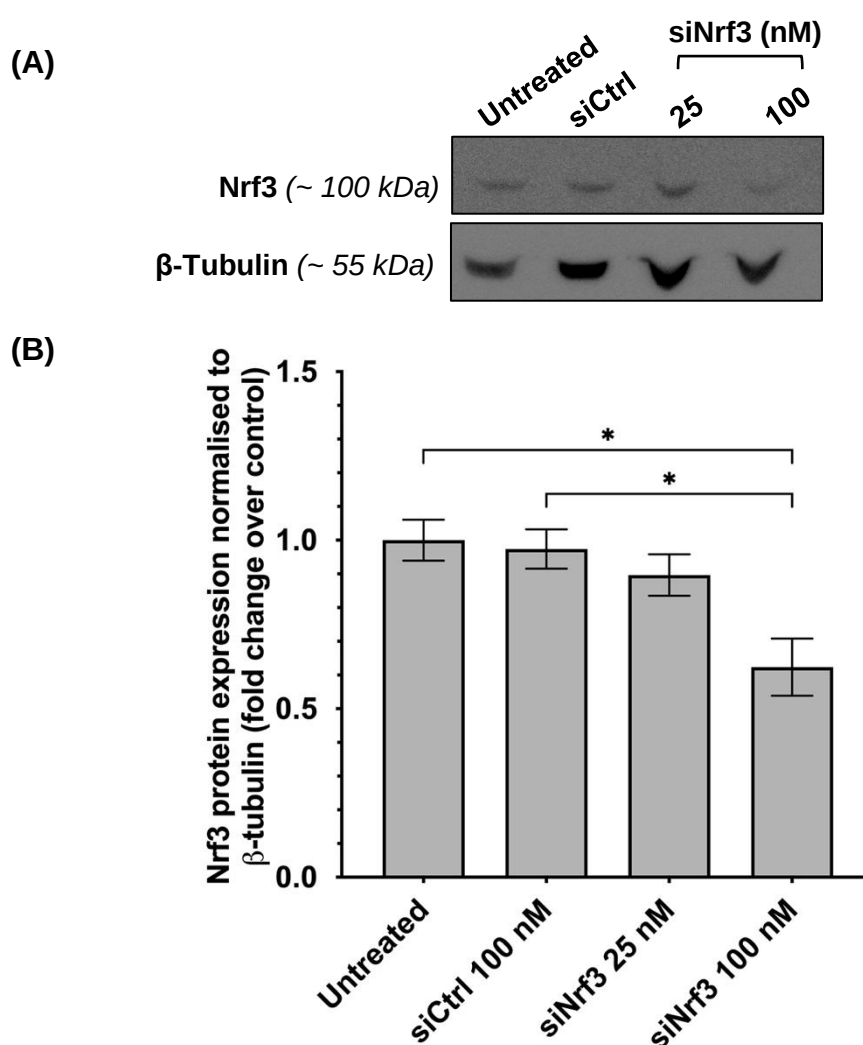


Figure 6.10 Nrf3 knockdown of MIAPACA2 cells via siRNA. **(A)** Representative western blot images of Nrf3 protein expression in MIAPACA2 cells treated with siCtrl or siNrf3. MIAPACA2 cells (1×10^6 /well) were treated with control (siCtrl) or Nrf3 (siNrf3) siRNA for 72 h prior to preparation of whole cell lysates. Protein expression was measured via SDS-PAGE and western blot analysis, with β-Tubulin included as a loading control. **(B)** Densitometry analysis of western blot images normalised to β-Tubulin. Data is presented as mean expression (fold change over untreated control) ± SEM (N=3). Statistical analysis was conducted via one-way ANOVA with post-hoc Tukey test (* $P \leq 0.05$).

6.3.5 Nrf3 knockdown via siRNA does not reduce cell proliferation of MIAPACA2 cells

Next, the effect of Nrf3 knockdown on MIAPACA2 cell proliferation was explored. To measure this, MIAPACA2 cells were treated with control or Nrf3 siRNA for 72 h and cell proliferation was measured via MTS assay. **Figure 6.11** shows that, compared to the untreated cells, the control siRNA did not affect cell proliferation. Additionally, Nrf3 siRNA only slightly reduced cell viability of MIAPACA2 cells, displaying a 6% reduction in cell viability, which was not statistically significant (**Figure 6.11**).

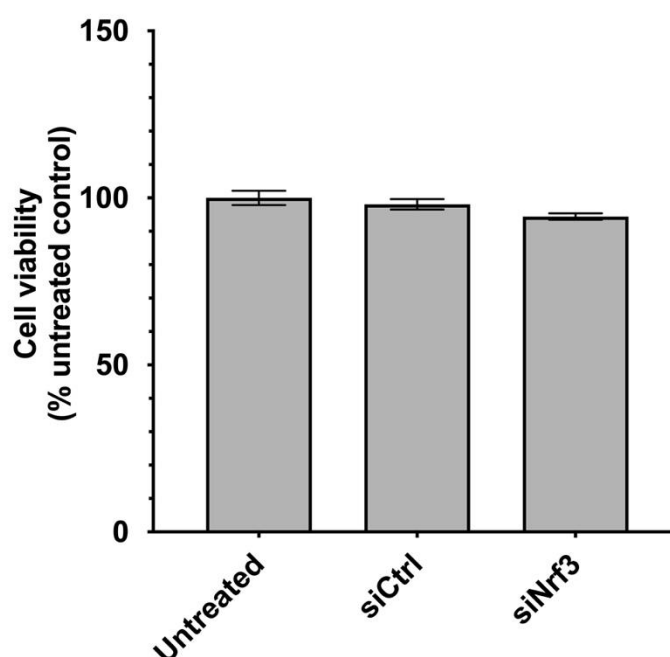


Figure 6.11 Nrf3 knockdown does not affect cell proliferation of MIAPACA2 cells. MIAPACA2 cells (6×10^3 /well) were treated with 100 nM control (siCtrl) or 100 nM Nrf3 (siNrf3) siRNA for 72 h and cell viability was measured via MTS assay. Results are presented as mean cell viability (% untreated control) $\pm$ SEM (N=3) and statistical analysis was conducted via one-way ANOVA with post-hoc Dunnett's test.

6.3.6 Knockdown of Nrf3 via siRNA reduces cell migration of MIAPACA2 cells

Several studies have reported that knockdown of Nrf3 can reduce migration of colorectal²¹², gastric⁴⁰⁸, eosophageal⁴¹⁰, liver⁴⁰⁴ and pancreatic²⁰⁹ cancer cells. To explore whether Nrf3 knockdown in MIAPACA2 cells could exert such effects, cell migration was studied via wound scratch assay. Firstly, Nrf3 knockdown was performed via incubation of MIAPACA2 cells with control or Nrf3 siRNA for 48 h. MIAPACA2 cells were then scratched and bright field microscopy images were taken of the cells at both 0 h and 24 h post-scratch to determine whether cells had migrated into the scratch area

(Figure 6.12A). ImageJ was then used to calculate the scratch area from the bright field microscopy images (Figure 6.12B).

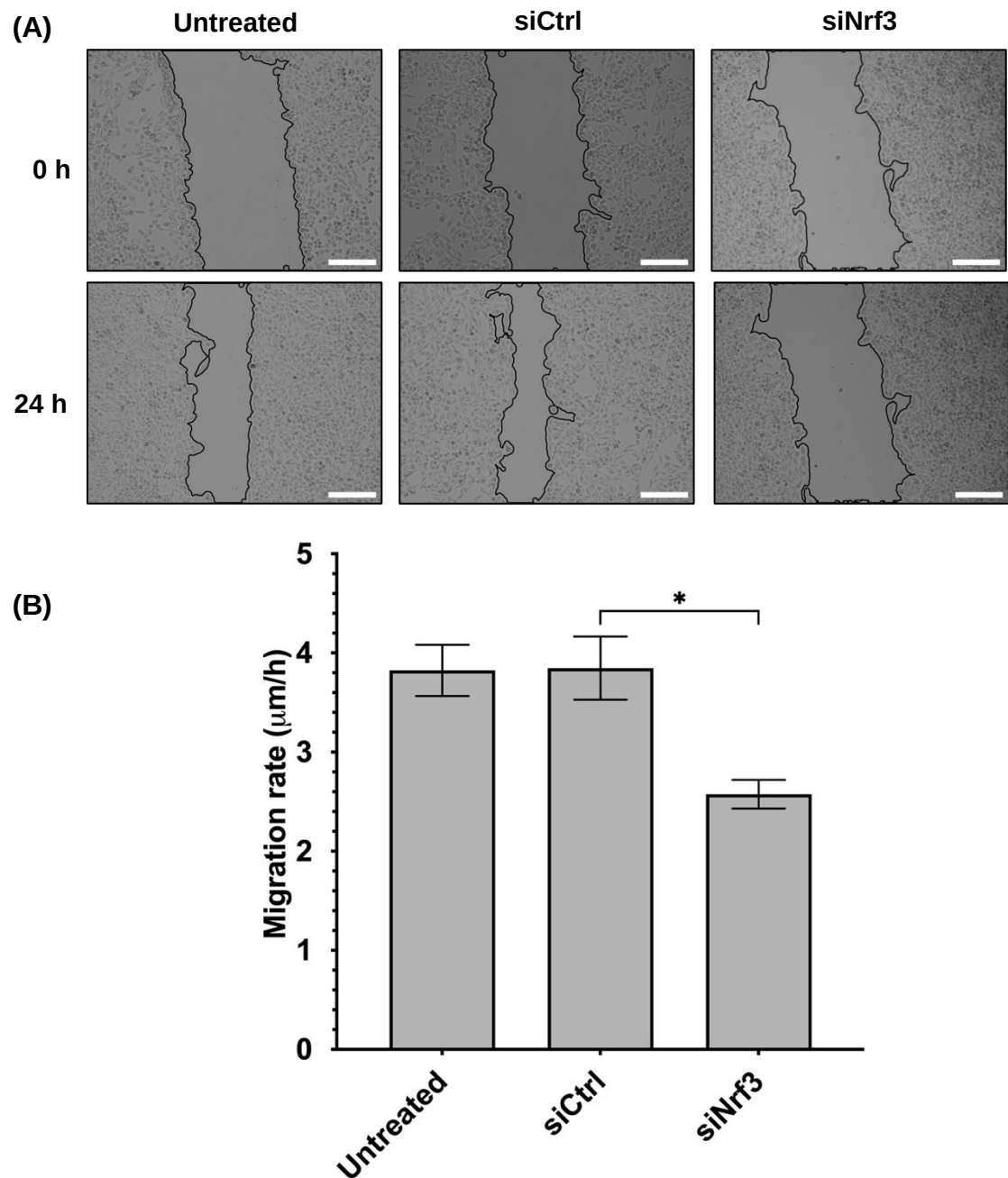


Figure 6.12 Nrf3 knockdown inhibits the migration of MIAPACA2 cells. (A) Representative bright field microscopy images of MIAPACA2 cells treated with siCtrl or siNrf3 at 0 h and 24 h after cell scratching. MIAPACA2 cells (1×10^5 /well) were treated with 100 nM control (siCtrl) or 100 nM Nrf3 (siNrf3) siRNA for 48 h. Cells were scratched, and cell migration rate was measured via ImageJ analysis of bright field microscopy images of the scratch area at 0 h and 24 h. Scale bar 100 μm . **(B)** Cell migration rate of MIAPACA2 cells treated with siCtrl or siNrf3. Results are presented as mean migration rate ($\mu\text{m/h}$) $\pm\text{SEM}$ (N=3) and statistical analysis was conducted via one-way ANOVA with post-hoc Dunnett's test. *P < 0.05.

As shown in **Figure 6.12B**, no difference was observed in migration rate of untreated MIAPACA2 cells (3.82 $\mu\text{m/h}$), compared to MIAPACA2 cells treated with 100 nM siCtrl (3.85 $\mu\text{m/h}$). However, treatment with 100 nM Nrf3 siRNA significantly reduced the migration rate of MIAPACA2 cells to 2.58 $\mu\text{m/h}$ (**Figure 6.12B**), suggesting that Nrf3 knockdown inhibits cell migration of MIAPACA2 cells.

6.4 Discussion:

Nrf3 is a member of the Cap 'N' Collar family, known mostly due to its relation to its sister protein Nrf2, which is widely researched in the context of oxidative stress, inflammatory disease and cancer¹¹⁹. Despite being identified not long after Nrf2, little is known about the physiological function of Nrf3. In the context of cancer however, increasing evidence suggests that Nrf3 may play a role in tumour promotion, metastasis and chemoresistance in several cancer types¹⁹⁷, implying it may be a novel therapeutic target in malignant disease. In the present study, publicly available cancer databases, such as the TCGA and GEO, were utilised to investigate Nrf3 expression in patient tumours across a variety of cancer types. Nrf3 and Nrf3-target gene expression was also found to be differentially expressed between patient-derived healthy and tumour tissue of colorectal cancer patients in 3D cultures of colonic organoids and tumouroids, which may also serve as a screening platform for potential inhibitors of Nrf3. Furthermore, as no *in vitro* studies to date have explored Nrf3 expression in multiple cancer types, here we present the first comparative study of Nrf3 expression across a panel of cancer cell lines, to inform further studies of Nrf3 in a variety of tumour types. Finally, we also demonstrated the potential therapeutic advantage of inhibiting Nrf3 in pancreatic cancer, using *in vitro* knockdown studies in MIAPACA2 pancreatic cancer cells.

Nrf3 cDNA was initially cloned in 1999¹⁹⁵ and like other CNC family members, binds as a heterodimer with sMaf proteins, such as MafG and MafK^{195,196,198}, to regulate expression of genes containing ARE sequences in their promoter regions¹⁹⁸. Currently the transcriptional role of Nrf3 is under researched however a transcriptional profiling study by Ibrahim *et al.*¹¹⁶ found that Nrf3 only influences 1.5% of the active transcriptome. In cancer, Nrf3 expression has been observed to be elevated in several tumour types, including those of colorectal^{202,212,400,401}, liver^{403,404}, ovarian⁵⁵⁴, oesophageal⁴¹⁰, gastric^{408,553}, kidney⁴⁰⁵, pancreatic²⁰⁹ and bladder⁴⁰⁹ cancers. Additionally, several of the Nrf3 target genes currently identified, including DUX4²¹², UHMK1²⁰², POMP¹⁹⁷, VEGFA²⁰⁹, CPEB3⁴¹³ and NQO1¹⁹⁸ are known to promote several of the hallmarks of cancer including uncontrolled cell proliferation, evasion of apoptosis¹⁹⁷, metastasis,

angiogenesis and chemoresistance¹⁹⁷. Several knockdown studies of Nrf3 have also found that Nrf3 repression can reduce cancer cell proliferation, migration and inflammation *in vitro*^{197,206,209,212,402,413} and inhibit tumour growth *in vivo*^{400,410}.

In the present study, Nrf3 expression was explored in patient tumour samples from a variety of cancers, using publicly available RNAseq and microarray datasets from the TCGA and GEO. Analysis of patient data from the TCGA showed that Nrf3 is significantly upregulated in 17/31 cancer types, with colon adenocarcinoma, rectal adenocarcinoma and testicular germ cell tumours displaying the highest expression of Nrf3 (**Figure 6.1**). Further analysis of patient-matched normal and tumour tissue from GEO datasets for colorectal adenocarcinoma, pancreatic ductal adenocarcinoma, breast carcinoma and hepatocellular carcinoma patients found that no significant difference in Nrf3 expression was observed between healthy and tumour tissue of hepatocellular carcinoma or breast carcinoma patients in the GEO datasets (**Figures 6.1 and 6.2**). These results conflict with previous reports from Yu *et al.*⁴⁰³ and Ren *et al.*⁴⁰⁴, who observed that Nrf3 is upregulated in hepatocellular carcinoma, with increases correlating with higher tumour grade and reduced overall survival of patients. Additionally, these studies also found that knockdown of Nrf3 in hepatocellular carcinoma cells inhibited cell proliferation and migration, induced cell cycle arrest, and reduced the expression of Wnt/ β -catenin target genes, such as cyclin D1, c-myc and TCF4^{403,404}. This therefore suggests that Nrf3 plays a role in Wnt signalling in hepatocellular carcinoma, which is known to be aberrantly activated in approximately 30-40% of hepatocellular carcinoma patient tumours⁵⁵⁶. The results from the present study and from the literature therefore suggest that while Nrf3 does not appear to be significantly upregulated in all patient hepatocellular carcinoma tumours, likely due to patient-to-patient variability, it may be a useful therapeutic target in advanced stages of hepatocellular carcinoma and could repress further metastasis due to its cross-talk with the Wnt pathway. In breast carcinoma, our data also conflicts with a study by Sun *et al.*⁴¹¹, who found that Nrf3 is downregulated in breast cancer. Although Nrf3 expression in our study did not correlate with overall survival, Sun *et al.* also observed that low Nrf3 expression correlated with better overall survival in patient data from the Human Protein Atlas⁴¹¹. It is possible therefore that Nrf3 may play different roles in different tumour types, however as the study by Sun *et al.* is the only other research currently published on Nrf3 in breast carcinoma, further research is required to fully understand Nrf3's potential role in this tumour type.

One of the first associations between Nrf3 and cancer was discovered in colorectal cancer²⁰¹. As shown in **Figures 6.1 and 6.2**, NFE2L3 was found to be upregulated in patient tumours of colorectal adenocarcinoma via TCGA and GEO dataset analysis.

Furthermore, although colorectal adenocarcinoma patients with high NFE2L3 expression appeared to show lower percent survival than those with low NFE2L3 expression, such correlation was not significant, in contrast to a previous report by Chen *et al.*⁴⁰¹. Despite this, several studies have found that knockdown of Nrf3 inhibits cancer cell growth both *in vitro* and *in vivo*, suppresses tumour inflammation, induces cell cycle arrest and apoptosis, and sensitises colorectal tumours *in vivo* to 5-fluorouracil (5-FU)^{212,400,402}. To further explore the potential role of Nrf3 in colorectal adenocarcinoma, we studied expression of Nrf3 and Nrf3-related genes in patient-matched healthy and tumour tissue of colorectal cancer, in collaboration with Dr Mark Williams' laboratory. In this culture system, colorectal tumour and patient-matched adjacent healthy tissue are first isolated and are subsequently cultured to form colonic organoid and tumouroid structures⁵⁵⁵. This system is especially valuable as it not only allows for a platform to study the mechanism of Nrf3 in colorectal cancer but may also serve as a screening tool for potential Nrf3-targeting cancer therapeutics.

As shown in **Figure 6.4-6.5**, RNASeq data from colorectal cancer patients showed that NFE2L3 and Nrf3 target genes VEGFA and POMP were significantly upregulated in isolated colonic tumour epithelia vs healthy colonic crypts, and in colonic tumouroids vs organoids. Furthermore, in isolated tumour epithelia, the proto-oncogene MYC was also upregulated (**Figure 6.5**). As a downstream gene of the Wnt/ β -Catenin pathway, which is commonly dysregulated in colorectal cancer, MYC expression is often upregulated in colorectal cancer²¹⁰. Interestingly, Nrf3 possesses a Wnt response element (WRE) in its promoter region, which suggests that Nrf3 is a downstream target gene of the Wnt/ β -Catenin signalling pathway in colorectal cancer²¹⁰, and could promote cell proliferation via Wnt signalling. We also observed that both NFE2L1 and NFE2L2 were downregulated in isolated tumour epithelia and colonic tumouroids (**Figure 6.5**). While one report has suggested that Nrf3 may compete with Nrf2 for binding to ARE of target genes²¹¹, interactions between Nrf3 and Nrf2 are not well researched. Furthermore, although Nrf3 has been shown to repress NFE2L1 translation via the inhibition of polysome formation on NFE2L1 mRNA⁴¹³, this is reported to be achieved via the induction of CPEB3. Conversely, data from the present study found CPEB3 to be decreased in tumour epithelia and tumouroids (**Figure 6.5**), suggesting that Nrf1 downregulation in tumours is not solely dependent on the CPEB3-Nrf3 axis.

Taken together, data from the TCGA, GEO and patient-derived colorectal cancer tumour tissue demonstrated that Nrf3 is upregulated in several cancer types, associated with poorer overall survival. This therefore provided a compelling rationale to investigate Nrf3 expression and assess its value as a potential therapeutic target in *in vitro* studies using

cancer cell lines. As displayed in **Figure 6.6**, NFE2L3 mRNA expression was highest in MDA-MB-231 breast, CACO-2 colorectal, HEPG2 hepatocellular cancer cell lines. Here, the embryonic kidney cell line HEK293 was included as a control, as it has been previously found to possess high expression of NFE2L3²⁰¹. Among this panel, NFE2L3 was shown to be low expressed in both melanoma (M202, A375) and lung cancer (A549, H292) cell lines for the first time. Although no studies have been conducted on the role of Nrf3 in lung cancer, in melanoma, Nrf3 expression appears to decrease as tumour grade increases, with the lowest expression observed in metastatic melanoma⁴¹². This may suggest that Nrf3 may be useful as a prognostic marker in melanoma.

To further validate the results observed in **Figure 6.6**, we next aimed to compare Nrf3 expression at the protein level in cancer cell lines via SDS-PAGE western blotting. Notably, in the literature, 3 bands are typically observed for Nrf3 in SDS-PAGE, corresponding to the three forms of Nrf3: glycosylated full length Nrf3, deglycosylated full length Nrf3, and cleaved Nrf3, which are typically observed at 140, 100 and 70 kDa, respectively^{101,199}. Although these bands do not match the predicted molecular weight of Nrf3, which is expected at 76 kDa, it is not unusual that molecular weights observed in SDS-PAGE vary from their predicted value; for example, such an effect is found with Nrf2, likely due to its migration pattern through SDS-PAGE gels, wherein it is observed at approximately 100 kDa, compared to the expected molecular weight of 62 kDa⁴⁴⁷. As bands for full length Nrf3 are typically observed at 100-130 kDa in the literature, it is therefore possible that Nrf3 also possesses an unexpected migratory pattern.

When completing these experiments however, a key issue that we faced was the limited availability and reliability of commercial Nrf3 antibodies. Here, antibodies from a commercial source and from the Kobayashi laboratory were tested for use in western blot analysis. Both antibodies were tested using a commercially available placental tissue lysate, as Nrf3 is most abundant in the placenta¹⁹⁶. When compared, the HPA055889 produced a clearer western blot image as the Kobayashi antibody appeared to display non-specific binding to the blotting membrane, indicated by blotches on the western blot image (**Figure 6.7**). This issue persisted despite attempts to block non-specific binding, such as preparing the antibody in 5% BSA in TBST rather than 5% skimmed milk (data not shown), inclusions of additional blocking steps with goat serum, and pre-incubating the membrane in BSA prior to the transfer step (data not shown). Additionally, while the Kobayashi antibody detected 3 bands for Nrf3 in the placental lysate, the molecular weight of these bands differs greatly from that observed in the literature, with bands observed at ~70, 50 and 35 kDa (**Figure 6.7**). In contrast to this, the HPA055889 antibody only displayed one clear band at ~70 kDa (**Figure 6.7**). A potential explanation

for this is that the placental lysate may mostly contain cleaved Nrf3, which may explain the bands at ~70 kDa for both antibodies. Furthermore, the presence of the lower bands observed at 50 and 35 kDa in the Kobayashi antibody could either be a result of non-specific antibody binding or could indicate further cleaved or degraded forms of Nrf3. As the HPA055889 antibody showed clearer western blot images and produced stronger bands that matched the molecular weight observed in the literature, we therefore selected this antibody for future experiments.

Using the HPA055889 antibody, we found that among a panel of 10 cancer cell lines derived from breast, melanoma, colorectal, prostate, liver, leukaemia and pancreatic tumours, all 10 displayed some expression of Nrf3, although the highest expression of the predominant form of Nrf3 (indicated by bands at ~100 kDa) was observed in MCF-7 breast, HCT116 colorectal and MIAPACA2 pancreatic cancer cell lines (**Figure 6.8**). Interestingly, only the HEK293 and M202 cells showed expression of the ~70 kDa isoform, which is believed to be cleaved Nrf3. Such a result does not appear to be unusual however, as other studies have also struggled to obtain strong bands for cleaved Nrf3, both in whole cell lysates and in cytoplasmic and nuclear fractions⁴¹². This could be due to the short half-life of cleaved Nrf3, which possesses a quick turn over rate of 20 minutes compared to that of 40 minutes for full length Nrf3¹⁹⁹. On the other hand, the more stable form identified at ~130 kDa was only present in low abundance in two cell lines: HCT116 and HEK293 (**Figure 6.8**), suggesting that the deglycosylated full length Nrf3 isoform (~100 kDa) is the predominant form in all of the cancer cell lines studied here. Furthermore, Nrf3 protein expression does not appear to directly correlate with NFE2L3 mRNA expression observed in **Figure 6.6**, possibly due to epigenetic changes or post-translational modifications of Nrf3.

To explore Nrf3 as a potential therapeutic target in cancer, we next selected a cell line that displayed high mRNA and protein expression of Nrf3 and was also upregulated in patient tumours. As we observed that Nrf3 was significantly upregulated in GEO datasets of pancreatic ductal adenocarcinomas (**Figure 6.1** and **6.2**) associated with poorer overall survival (**Figure 6.3**), we selected the MIAPACA2 pancreatic cancer cell line for further experiments. In this study, we found that Nrf3 knockdown in MIAPACA2 cells minimally reduced cell proliferation by 6% in MTS assays, suggesting that Nrf3 does not influence cell proliferation in these cells. Despite this, knockdown of Nrf3 did appear to have an inhibitory effect on the migration of MIAPACA2 cells via wound scratch assay (**Figure 6.12**). This is supported by a study conducted by Wang *et al.*²⁰⁹, wherein Nrf3 knockdown did not affect cell proliferation of pancreatic cells, but repressed cell migration. Additionally, Wang *et al.* also found that knockdown of Nrf3 inhibited cell

migration and expression of angiogenesis-related genes such as VEGFA²⁰⁹. This suggests that Nrf3 inhibition in pancreatic cancer may be a useful tool in suppressing tumour metastasis and could be used as a potential adjuvant chemotherapy alongside traditional treatments such as 5-FU and gemcitabine.

At present, no inhibitors of Nrf3 have been identified and validated. Hence, future research could investigate methods to inhibit Nrf3 signalling in cancer via the use of small molecule or peptide inhibitors. One potential small molecule is the DDI2 protease inhibitor nelfinavir, which prevents Nrf1 proteolytic cleavage and subsequent translocation into the nucleus¹⁹⁷. As Nrf1 and Nrf3 are structurally similar and share the same mechanism of DDI2-induced cleavage at their NHB2 domains (**Figure 1.5**), nelfinavir has also been proposed as a potential Nrf3 inhibitor. Despite this, no studies have thoroughly evaluated its efficacy and initial experiments in the present study did not find any effects on Nrf3 expression (**Figure 8.9**). Inhibitors of the NGLY1 enzyme, which mediates both Nrf1 and Nrf3 deglycosylation at their NST domains, have also been proposed as potential Nrf3 inhibitors. While Tomlin *et al.*⁴¹⁵ found that a small molecule inhibitor of NGLY1, named WRR139, could repress Nrf1 deglycosylation and subsequent nuclear translocation, no studies have yet reported any effects of WRR139 treatment on Nrf3. We also propose that peptide protein-protein interaction inhibitors, such as the MafG peptide mimic described in **Chapter 4**, could also be studied as possible inhibitors of Nrf3/sMaf interactions and downstream signalling in cancer.

While Nrf3 inhibitors may be useful in studying Nrf3's role in cancer, a key limitation in the Nrf3 field is that the mechanisms of Nrf3 induction and release from the ER are still largely unknown. As demonstrated by Chenais *et al.*¹⁹⁶ and Nouhi *et al.*¹⁹⁹, ER stress inducing agents (such as tunicamycin and thapsigargin), TNF- α , and the proteasome inhibitor MG-132 can induce Nrf3 cleavage and translocation into the nucleus in choriocarcinoma (BeWo and JAR) and colorectal (HCT116 and DLD-1) cancer cell lines^{196,199}. However, as displayed in **Figures 8.9-8.10**, in preliminary experiments, TNF- α stimulation had no effects on Nrf3 expression in either HCT116 or MIAPACA2 cells. Additionally, MG-132 did not appear to induce Nrf3 protein expression in either of the cell lines but did induce Nrf2 (**Figure 8.9**). Hence further work is required to fully validate mechanisms of Nrf3 induction to better understand Nrf3's general physiological function in non-malignant cells, as well as its activation within cancer cells. Another limitation of this study was the lack of commercially available anti-Nrf3 antibodies. While we identified that the HPA055889 antibody from Prestige Antibodies was suitable for our research, large delays in the availability of this antibody greatly limited our studies. As emphasised

in a review by Kobayashi *et al.*¹⁹⁷, there is therefore a strong need for better Nrf3 reagents in order for this field to progress at an advanced pace.

A further limitation is that, while the differential gene expression profiles for the patient-derived tumour and healthy tissue in **Figure 6.5** were largely consistent, some differences in gene expression were observed between isolated tumour epithelia and cultured colonic tumouroids. For example, VEGFA was significantly downregulated in tumouroids vs organoids, whereas it was upregulated in isolated tumour epithelia vs colonic crypts (**Figure 6.5**). As the tumour epithelia is sequenced immediately after isolation, these samples more accurately representative gene expression in patient tumours, whereas tumouroids are susceptible to gene expression changes induced by laboratory culture conditions. Additionally, genes that crosstalk with the tumour microenvironment such as VEGFA, which interacts with endothelial cells to promote angiogenesis, may be downregulated in tumouroids compared to isolated tumour epithelia, as tumouroid culture lacks surrounding stromal cells. While this platform of patient-derived tissue cultured in a 3D environment presents benefits over 2D *in vitro* cell culture of cancer cell lines, further study of the role of Nrf3 using *in vivo* models is warranted.

In conclusion, the present study found that Nrf3 may be a valuable therapeutic target in several cancer types, particularly in those where Nrf3 is upregulated and is associated with poor prognosis, such as in colorectal, pancreatic, and hepatocellular carcinomas. This study also provides novel data on Nrf3 expression in cancer cell lines, that may inform future *in vitro* studies of Nrf3 signalling in cancer. Furthermore, here we report for the first time that Nrf3 and Nrf3-target gene are upregulated in human patient-derived colonic organoids and tumouroids. Not only does this data validate our analysis of TCGA and GEO datasets for colorectal cancer but may also serve as a useful screening platform for future Nrf3-targeting therapies, in combination with the cancer cell line data obtained here. We also demonstrated for the first time that Nrf3 knockdown in MIAPACA2 pancreatic cancer cells could reduce cell migration, suggesting that Nrf3 may be a novel therapeutic target in pancreatic cancer. Further studies could therefore focus on the effect of Nrf3 knockdown on Nrf3 target gene expression, and on other hallmarks of pancreatic cancer, including apoptosis, EMT (epithelial to mesenchymal transition) markers and chemoresistance to commonly used chemotherapies such as gemcitabine.

Chapter 7. General discussion

The transcription factor Nrf2 has been widely studied for its role in mediating the oxidative stress response, and its implication in a range of diseases linked to chronic inflammation, and oxidative stress¹¹⁹. When activated, Nrf2 can translocate to the nucleus and form heterodimers with sMaf proteins, such as MafG, to induce cytoprotective and anti-inflammatory gene expression. As a result of this cytoprotective role, activators of the Nrf2 pathway have long been explored for their therapeutic use in chronic inflammatory diseases, with one activator (dimethyl fumarate; DMF) currently licenced for use in the UK to treat multiple sclerosis and psoriasis²⁴². Activation of the Nrf2 pathway can also provide a chemopreventive role against cancer development and is known to be involved in the mechanism of action of numerous chemopreventive compounds, such as oltipraz, sulforaphane and curcumin⁴³³. On the other hand, Nrf2 also possesses a “dark side” in cancer, wherein aberrant activation of the Nrf2 pathway promotes tumorigenesis and chemoresistance^{268,511}. Moreover, Nrf2 overexpression has been observed in a multitude of patient tumours, including lung, breast, ovarian, liver, pancreatic and melanoma, and is generally associated with poorer overall survival rates²⁷². Increasing evidence also suggests that this “dark side” can contribute to the majority of the hallmarks of cancer, including mechanisms that promote chemoresistance²⁵⁸.

Inhibitors of the Nrf2 pathway have therefore been proposed as potential anti-cancer agents that could repress tumour growth, metastasis and chemoresistance³⁸⁸. Such inhibitors are not yet approved for clinical use however, and have not been compared for their activity. In this study therefore, we compared small molecule and natural product inhibitors of Nrf2 (brusatol, luteolin, ML385, retinoic acid, triptolide, trigonelline and clobetasol) for their ability to repress Nrf2 signalling, cancer cell proliferation and sensitise cells to standard of care chemotherapies in both non-small cell lung cancer (NSCLC), and melanoma. Two novel methods of targeting Nrf2 were also explored, including use of a Nrf2 inhibitor-peptide drug conjugate, and a novel MafG peptide mimic designed to block the Nrf2/MafG protein-protein interaction (PPI).

In NSCLC patient tumours, Nrf2 is commonly overexpressed, often due to point mutations in Nrf2's negative regulator Keap1²⁶⁹. Several Nrf2 inhibitors have been studied in NSCLC (as summarised in **Table 1.5**), however many of these inhibitors have only been explored in single studies or show conflicting activity in at different concentrations, wherein they may also activate Nrf2. In the present comparative study, brusatol and triptolide consistently demonstrated the best inhibitory effects on Nrf2 protein expression and downstream gene activity in Nrf2-overexpressing A549 NSCLC cells, as supported by their nanomolar potencies observed in additional studies^{332,334,335,368,370,373}. Conversely, the Nrf2 inhibitors, clobetasol and trigonelline,

demonstrated very little effects on Nrf2 signalling in NSCLC, contrary to previous reports^{343,365,442}.

Additionally, ML385, which is the only small molecule reported to directly inhibit the Nrf2/ARE interaction, did not display any anti-proliferative effects on Keap1-deficient A549 cells (<100 μ M), nor did it inhibit Nrf2 protein expression or downstream gene activity (e.g., NQO1). These results contradict Singh *et al.*³⁵⁹, who initially identified ML385, and demonstrated its activity to specifically target Keap1-deficient cells, such as A549. On the other hand, experiments conducted in M202 melanoma cells in this study found that ML385 did appear to repress Nrf2 protein expression, NQO1 activity and cell migration, suggesting that its activity may be cell-type dependent. Currently, ML385 is sold commercially as an Nrf2 inhibitor and is routinely used as a control in Nrf2 studies^{249,470,471}, however further investigation into the activity of ML385 is warranted as its activity as an inhibitor appears to vary between cell types.

Herein, we also report that the Nrf2 activator DMF, which was initially included as a control in these experiments, was able to repress Nrf2 protein expression at high concentrations (e.g., 30 μ M). Alternatively, at lower concentrations (e.g., 10 μ M), DMF induced Nrf2 target gene expression and activity in both NSCLC and melanoma cells, implying that its effects may be concentration dependent. Similar effects have also been observed by Saidu *et al.*⁵⁴⁶ in CACO-2 colorectal cancer cells, however, this contradicts DMF's proposed mechanism of action to induce Nrf2 signalling. Furthermore, in a recent study by Li *et al.*⁴⁶⁰ DMF was found to also repress melanoma cell proliferation and sensitise cells to the BRAF inhibitor vemurafenib. Currently, the mechanism in which DMF modulates Nrf2 is not understood, though Saidu *et al.*⁵⁴⁶ observed that DMF repressed expression of DJ-1, a protein that acts to stabilise Nrf2 by interrupting its association with Keap1. While DMF did not affect DJ-1 protein expression at concentrations reported to inhibit Nrf2 in the present study, DMF has been found to repress the activity of several other signalling pathways, including NF- κ B²⁴², which contributes to its anti-inflammatory effects in non-malignant conditions and may also act to suppress Nrf2 due to their cross-talk mechanisms. Hence, although increasing evidence suggests that DMF could be used as an anti-cancer agent⁴⁴⁹, further research is required to confirm its mechanism of action and activity at different concentrations in both malignant and non-malignant cells.

Aside from NSCLC, Nrf2 has been implicated in several tumour types. While Nrf2 has been observed to be upregulated in several studies of melanoma patient tumours, its role in melanoma is not well characterised, with conflicting reports on its role in

melanoma tumour initiation and progression⁵²⁷. Hence, in this study, the expression and role of Nrf2 in melanoma was explored. Nrf2 expression was observed to be increased in six out of nine melanoma cell lines, compared to normal primary melanocytes, although, Nrf2 mRNA expression in patient melanoma tumours from The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) datasets show opposing results regarding whether NFE2L2 is upregulated or downregulated at the mRNA level. Currently, it is thought that Nrf2 expression in melanoma may be decreased in initial stages of tumorigenesis, which results in a weakened defence against DNA damage⁵⁴³. As melanoma progresses however, cells may increase Nrf2 expression to promote rapid tumour growth, metastasis and chemoresistance^{315,533,534}. We therefore propose that while Nrf2 may not always be increased in all melanoma tumours, it may be a useful therapeutic target in tumours with high Nrf2 expression (for example in advanced stages of disease), or where tumours have become resistant to standard of care chemotherapy.

Herein, we observed that Nrf2 inhibition using small molecule and natural product inhibitors could not only repress cell proliferation and Nrf2 signalling but also slowed the migration rate of A375 and M202 melanoma cells. Of these inhibitors, the greatest inhibitory effects on Nrf2 signalling and cell migration were observed in brusatol, luteolin and triptolide, all of which could also sensitise the BRAF-mutant A375 cells to vemurafenib, a BRAF inhibitor used to treat BRAF-mutant melanomas. This is supported by George *et al.*⁵⁵⁷, wherein luteolin demonstrated anti-proliferative, anti-migratory and pro-apoptotic activity in A375 melanoma cells by repressing the expression of matrix metalloproteinase-2/9 (MMP-2/9) via inhibition of the PI3K/AKT pathway. We therefore suggest that luteolin may be useful as an adjuvant therapy in malignant melanoma treatment. To further explore this, additional studies using samples from patient melanoma tumours and matched healthy tissue, and *in vivo* models of melanoma, are necessary to further confirm the findings in the present *in vitro* study.

In addition to luteolin, in both NSCLC and melanoma, brusatol and triptolide consistently repressed Nrf2 signalling, cell proliferation and cell migration, with potencies in the nanomolar range. While brusatol may serve as a useful positive control in experiments of Nrf2 inhibition, its clinical use is greatly hampered by its wide off-target effects, largely due to its ability to act as a global inhibitor of protein synthesis and thus affect many off-target proteins³⁸⁴. Increasing research has demonstrated triptolides ability to induce apoptosis and repress cancer cell growth *in vitro* and *in vivo*, as also demonstrated in the present study wherein it could repress 3D A549 tumouroid growth and sensitise 2D cultures of A549 cells to the antimetabolite drug, gemcitabine. However triptolide also

possesses off-target effects due to its role in repressing RBP1 subunit of RNA polymerase II⁴⁸⁰, and has been shown to demonstrate several organ toxicities⁴⁷⁷. As a highly potent inhibitor of both Nrf2 and cell proliferation however, triptolide appears to be an ideal candidate for inclusion in a targeted delivery system such as an antibody or peptide-drug conjugate.

Although previous research has utilised triptolide in an EGFR-targeting antibody drug conjugate⁴⁹⁸, in this study, a peptide-drug conjugate of triptolide was designed and synthesised, which provides a cheaper alternative to antibody-drug conjugates. To target NSCLC cancer cells, which often overexpress EGFR⁴⁹⁹, this conjugate utilised a peptide that binds to the ligand binding domain of EGFR⁴⁸⁹. Although this conjugate was not as potent as unconjugated triptolide, as is also observed with the triptolide-cetuximab antibody-drug conjugate⁴⁹⁸, inclusion of the EGFR-targeting peptide compared to a scrambled peptide showed increased potency of anti-proliferative effects in A549 NSCLC cells.

One drawback of this design, however, is that EGFR is also expressed on non-malignant cells. Hence, improvements to the triptolide-peptide drug conjugate could be made, such as using a different peptide that could more specifically target cancer cells. Although such targets are difficult to identify, as we found that triptolide could also induce anti-cancer effects in melanoma, one such potential target could be the melanocortin 1 receptor (MC1R), which is localised mainly in the skin and is upregulated in melanoma⁵⁵⁸. Furthermore, other methods of targeting triptolide to cancer cells, such as through nanoparticle delivery or antibody drug conjugates are being explored⁴⁸², alongside derivatives of triptolide that show reduced toxicities in clinical trials, such as the triptolide pro-drug Minnelide⁴⁸³. Targeted delivery of small molecule and natural product Nrf2 inhibitors could therefore serve as a novel mechanism of Nrf2-targeting in Nrf2 addicted tumours.

Another novel mechanism of Nrf2 inhibition is the use of peptide molecules to inhibit the Nrf2/sMaf PPI. Currently, Nrf2 inhibitors mostly consist of small molecule and natural products; however, very few small molecules have been identified thus far, and of the natural products found, many are either expensive to isolate, have off-target effects, or may paradoxically both inhibit and activate Nrf2 depending on their concentration²⁵⁶. Although previously believed to be undruggable, PPI inhibitors based upon peptides are gaining interest, especially to target transcription factors³⁹¹. Peptides are not only cheap to synthesise but can also mimic the native structure of proteins and bind to highly hydrophobic regions in the interface of PPIs to repress protein interactions³⁸⁹.

Herein, we describe a novel peptide that mimics the bZIP domain of MafG, which represses Nrf2-ARE binding activity, NQO1 and HO-1 mRNA expression, and sensitises A549 NSCLC cells to the chemotherapeutic agent gemcitabine. At present, only three studies have explored the use of peptide inhibitors of Nrf2/MafG interaction, all of which rely on either mimicking the bZIP domain of Nrf2 or creating an artificial tethered dimeric inhibitor of Nrf2/MafG³⁹³⁻³⁹⁵. Of these studies, only one peptide described in 2023 by Modi *et al.*³⁹⁵ was found to be cell permeable and specific for the Nrf2/MafG interaction. In the present study, while we explored the biological effects of the MafG peptide mimic on A549 cells, further studies of this peptide aim to characterise the peptide's α -helicity and ability to disrupt Nrf2/MafG binding using techniques such as circular dichroism and surface plasmon resonance, respectively.

As these peptides are designed based upon the native structure of the bZIP domains of either MafG or Nrf2, this method of targeting Nrf2 may be more specific than that of the small molecule and natural product inhibitors, which often display off-target effects, such as in the case of brusatol. One limitation of studies using peptide inhibitors, at present, is the lack of data concerning their effects in non-malignant cells. Hence, further research is also required on their selectivity for malignant cells to fully characterise their use in treating Nrf2 overexpressing tumours. Additionally, the ability of Nrf2/MafG peptides to display anti-tumour activity *in vivo* has also not yet been investigated. Despite this, in Phase I clinical trials, a p53 peptide mimic that inhibits the p53/MDM2 PPI has shown promising results in leukaemia³⁹², emphasising the promise of peptide-based PPI inhibitors in cancers.

Nrf3, a sister protein of Nrf2, has also been implicated in tumorigenesis and chemoresistance in colorectal, liver, thyroid, oesophageal, pancreatic, gastric, kidney and ovarian cancers^{209,212,399,400,403,406,409,551}. Although the normal physiological function of Nrf3 is not fully understood, Nrf3 is known to regulate the transcription of genes involved in proteostasis and cell cycle progression¹⁹⁷. In the present study, Nrf3 was investigated for its role in colorectal, breast, liver and pancreatic cancers and siRNA (short interfering RNA) knockdown was utilised to study the effects of genetic Nrf3 inhibition in a human pancreatic cell line. Using TCGA and GEO data, Nrf3 mRNA was found to be increased in colorectal and pancreatic patient tumours compared to matched healthy tissue. This was further supported by RNA sequencing data of patient-derived colonic organoids and tumours, wherein NFE2L3 was differentially expressed alongside other Nrf3 target genes such as VEGFA. Although increasing evidence suggests that overexpression of Nrf3 in cancer contributes to increased tumour growth, a lack of understanding of the mechanisms underpinning Nrf3's induction and overexpression in

cancer provide a stumbling block to fully understanding the therapeutic benefit of inhibiting Nrf3. Hence, further studies could explore the mechanisms of Nrf3 induction, for example with other common stress inducers such as pro-oxidants or pro-inflammatory stimuli. Moreover, additional studies could also aim to identify other Nrf3 target genes, to better elucidate both its normal physiological role and its involvement in tumorigenesis. As no Nrf3 inhibitors have yet to be identified, genetic inhibition via RNA interference remains to be the main method through which Nrf3 inhibition is studied. Although in the current study, knockdown of Nrf3 in the high-Nrf3 expressing MIAPACA2 pancreatic cell line had little effects at repressing cell proliferation, it significantly reduced MIAPACA2 cell migration, suggesting that Nrf3 inhibition could be used to prevent pancreatic cancer cell migration. This supports other Nrf3 knockdown studies in liver, colorectal and gastric cancer cell lines^{212,403,404,408}, which resulted in decreased cell proliferation and sensitised cells to chemotherapeutic agents such as 5-fluorouracil⁴⁰⁰. While siRNA-based treatments, such as loaded nanoparticle delivery, are being developed as potential cancer therapeutics, the focus in the Nrf3 field currently remains around identifying small molecule inhibitors.

One mechanism of Nrf3 inhibition that has been proposed is the use of small molecule DDI2 protease inhibitors, to prevent proteolytic cleavage and subsequently nuclear translocation of Nrf3. Nelfinavir, which is a HIV protease 1 inhibitor that also inhibits DDI2, can prevent proteolytic cleavage of Nrf1⁴¹⁴, thus could have activity to also repress Nrf3 due to their shared mechanism of proteolytic processing. Additionally, NGLY1 inhibitors, such as the small molecule WRR139, can prevent Nrf1 deglycosylation, resulting in decreased Nrf1 nuclear translocation⁴¹⁵. As Nrf1 and Nrf3 share a very similar structure and mechanisms of processing, nelfinavir and WRR139 may also prevent Nrf3 nuclear translocation. Although preliminary experiments in the present study were not able to find any such activity, experiments using a wider range of treatment times and concentrations of inhibitors could be conducted to explore whether either nelfinavir or WRR139 could modulate Nrf3, as they do with Nrf1.

Aside from identifying small molecule inhibitors of Nrf3, it is possible that the MafG peptide mimic described in the present thesis could also inhibit other CNC bZIP proteins that form interactions with MafG, such as Nrf3. Future work could therefore explore the use of PPI inhibitors, such as the MafG peptide described herein. Although 2D cell cultures often serve as the initial method of drug-screening, potential Nrf3 inhibitors could also be screened in the culture system of patient-derived organoids and tumouroids, as utilised in the present thesis. Additionally, further research in the O'Connell lab aims to

explore whether Nrf3 knockdown sensitises pancreatic cancer cells to standard of care chemotherapy agents, such as with gemcitabine or capecitabine.

In conclusion, this study investigated several mechanisms of inhibiting Nrf2 in NSCLC and melanoma tumours and explored the potential therapeutic benefit of targeting Nrf3 in cancer, as summarised in **Figure 7.1**. This included the first comparative study of seven small molecule and natural product inhibitors of Nrf2 to investigate their anti-cancer effects in both NSCLC and melanoma. Among these inhibitors, brusatol, luteolin and triptolide emerged as the best inhibitors of Nrf2 signalling, cell proliferation and migration in NSCLC and melanoma cells, and could also sensitise cells to standard of care chemotherapies. To reduce its off-target effects and limit systemic toxicity, triptolide was conjugated to an anti-EGFR peptide, resulting in the synthesis of a novel triptolide-peptide drug conjugate to target EGFR and Nrf2-overexpressing tumour cells.

This study also described a novel Nrf2/MafG PPI peptide inhibitor, based upon the bZIP domain of MafG, that significantly reduced Nrf2 downstream gene expression and sensitised NSCLC cells to the chemotherapeutic agent gemcitabine. This not only presents a mechanism to inhibit Nrf2 but also could be utilised to interrupt the transcriptional activity of other MafG-binding bZIP transcription factors, such as Nrf3. Finally, Nrf3 was studied for its involvement in a variety of cancers and, for the first time, Nrf3 knockdown was shown to repress MIAPACA2 pancreatic cancer cell migration. This therefore suggests that Nrf3 inhibition may be a useful therapeutic intervention in Nrf3-overexpressing tumours that warrants further research.

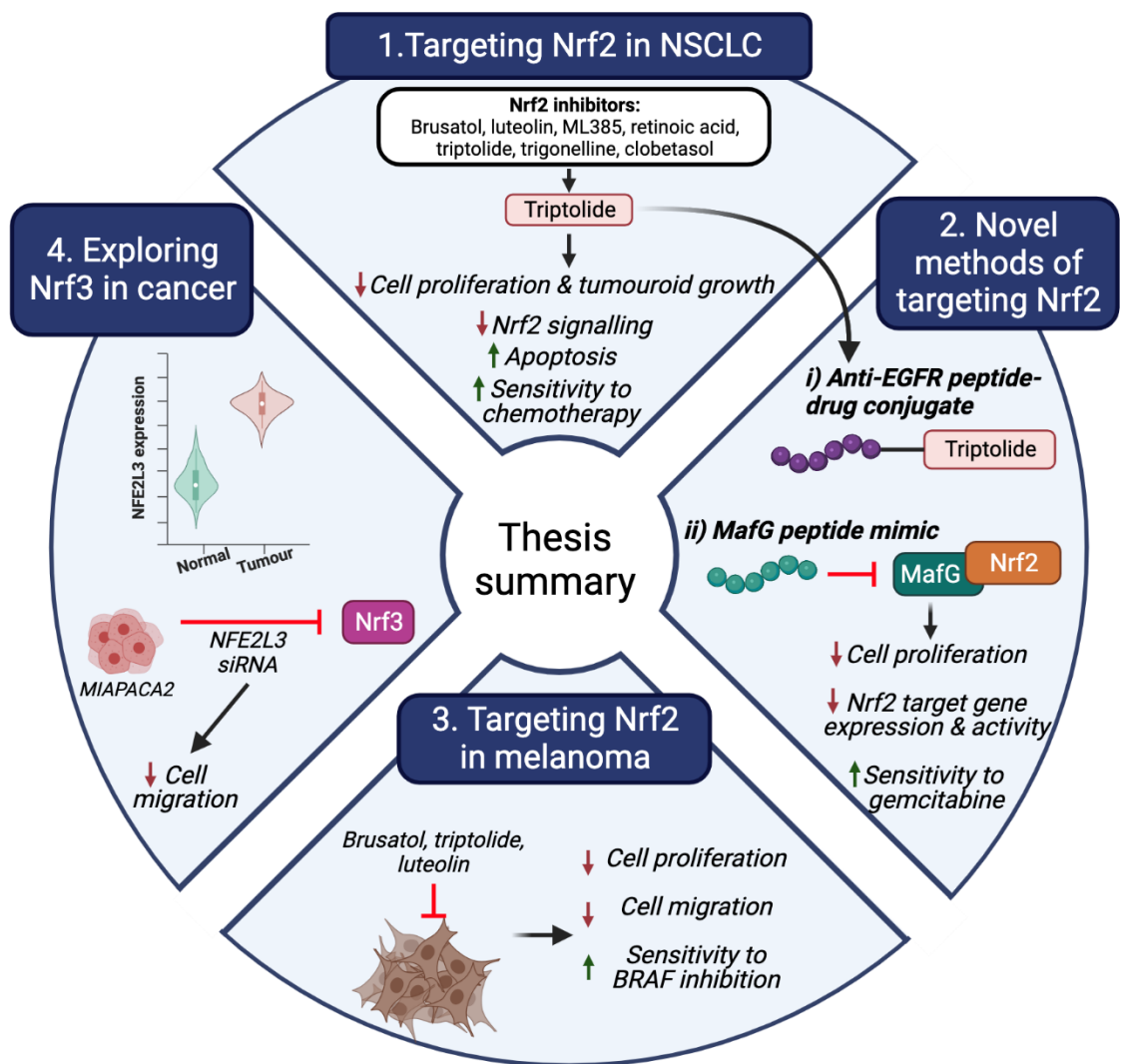


Figure 7.1 Thesis summary. Schematic depicting the key research outputs from each results chapter.

Chapter 8: Appendix

8.1 Recipes and buffers

8.1.1 Freezing media

10% DMSO in FBS (*1 mL/vial of cells*)
900 μ L FBS (filter sterilised with 0.45mm filter)
100 μ L sterile HybriMax DMSO

8.1.2 NQO1 activity assay

NQO1 cell lysis buffer:

0.8% digitonin in 2 mM EDTA (pH 7.8)*
80 mg digitonin
10 mL 2 mM EDTA
**(Heat to 95°C to dissolve prior to use and allow to cool to room temperature).*

NQO1 Reaction Buffer:

For one 96-well plate (200mL reaction buffer/well)
500 μ L 1M Tris-HCl, pH 7.4
134 μ L 100mg/mL Bovine Serum Albumin
134 μ L Tween 20
10 μ L 7.5mM Flavin adenine dinucleotide disodium salt hydrate (FAD)
134 μ L 150mM glucose-6-phosphate
12 μ L 50mM NADP+
20 μ L 2kU/mL Yeast glucose-6-phosphate dehydrogenase
41 mL 5mg/mL Thiazolyl Blue Tetrazolium Blue (MTT)
18 mL deionised water
20 μ L 50mM Menadione (added just before addition of buffer to plate)

8.1.3 Western immunoblotting:

1X MOPS running buffer:

40 mL NuPage 20X MOPS SDS running buffer
760 mL deionised water

10X Transfer buffer:

24.26 g Tris Base
112.6 g Glycine
1 g SDS
Top up to 1 L with deionised water

1X Transfer buffer:

100 ml 10x Transfer Buffer
700 ml deionised water
200 ml methanol

20X TBST:

48.4 g Tris Base

160 g NaCl

62 ml 5M HCl

20 ml Tween 20

Adjust pH to 7.6 and top up to 1 L with deionised water

1X TBST:

50 mL 20X TBST

950 mL deionised water

Blocking solution (5% milk in TBST):

20 g skimmed milk powder

400 ml 1X TBST

8.1.4 Nrf2 DNA-binding ELISA***Buffers for nuclear extraction:*****1X ice-cold PBS/ Phosphatase Inhibitor Solution:**

For one sample:

0.6 mL Nuclear extraction PBS (10X)

5.28 mL distilled water

0.12 mL Nuclear extraction phosphatase inhibitor (50 X)

1X Complete Hypotonic Buffer:

For one sample:

25 µL Nuclear Extraction Hypotonic Buffer (10X)

5 µL Nuclear extraction phosphatase inhibitor (50x)

2.5 µL Nuclear extraction protease inhibitor (100X)

217.5 µL distilled water

Complete Nuclear Extraction Buffer:

For one sample:

25 µL Nuclear Extraction Buffer (2X)

1 µL Nuclear extraction phosphatase inhibitor (50x)

0.5 µL Nuclear extraction protease inhibitor (100X)

5 µL 10 mM DTT

18.5 µL distilled water

Buffers for Nrf2 DNA-binding ELISA (Active Motif)**Complete Lysis Buffer:**

For 8 wells (1 strip)

0.1 µL DTT

0.12 µL Protease Inhibitor Cocktail

89 µL Lysis Buffer

Complete Binding Buffer:

For 8 wells (1 strip)

0.3 μ L DTT

3.6 μ L Herring Sperm DNA

356.1 μ L Binding Buffer

1X Wash Buffer:

For 8 wells (1 strip)

16.2 mL distilled water

1.8 mL 10X wash Buffer

1X Antibody Binding Buffer:

For 8 wells (1 strip)

180 μ L 10X Antibody Binding Buffer

1.62 mL distilled water

8.2 Experimental

8.2.1 RT-qPCR optimisation

8.2.1.1 Amplification efficiencies

A key component of the method is the use of efficient reaction conditions. To calculate the reaction efficiencies of RT-qPCR reactions, one method is to create serial dilutions of a known cDNA concentration and plot a linear regression based upon the obtained Ct values vs the Log (cDNA) concentration for each set of primers. The amplification efficiency is then calculated based upon the correlation coefficient (R^2) and the slope (M) of the curve. Using this method, a slope of -3.32 is considered to be 100% efficient, meaning that the PCR product doubles with every cycle, hence allowing for the application of the $\Delta\Delta$ CT method of relative gene quantification. Here, amplification efficiencies were calculated for each gene investigated using RT-qPCR (**Table 8.1**).

Table 8.1 Standard curves used to calculate the amplification efficiency of genes used in RT-qPCR. Linear regression curves were plotted from the Ct values of RT-qPCR reactions with NFE2L2, NFE2L3, HO-1, NQO1, PARK7 and GAPDH primers using serial dilutions of cDNA (-), presented here as Log values.

Gene	Amplification efficiency standard curves
NFE2L2	
NFE2L3	
HO-1	
NQO1	
DJ-1 (PARK7)	
GAPDH	

8.2.1.2 cDNA calibration curves

To select an appropriate cDNA concentration for RT-qPCR reactions, the Ct values of 5 dilutions of cDNA template were plotted against cDNA concentration for each gene of interest. As displayed in **Figure 8.1**, as cDNA concentration increases, the Ct value decreases due to the increase in the amount of cDNA template available for amplification. Using these cDNA calibration curves; a concentration of cDNA template was selected for each gene investigated to provide optimal reaction efficiency.

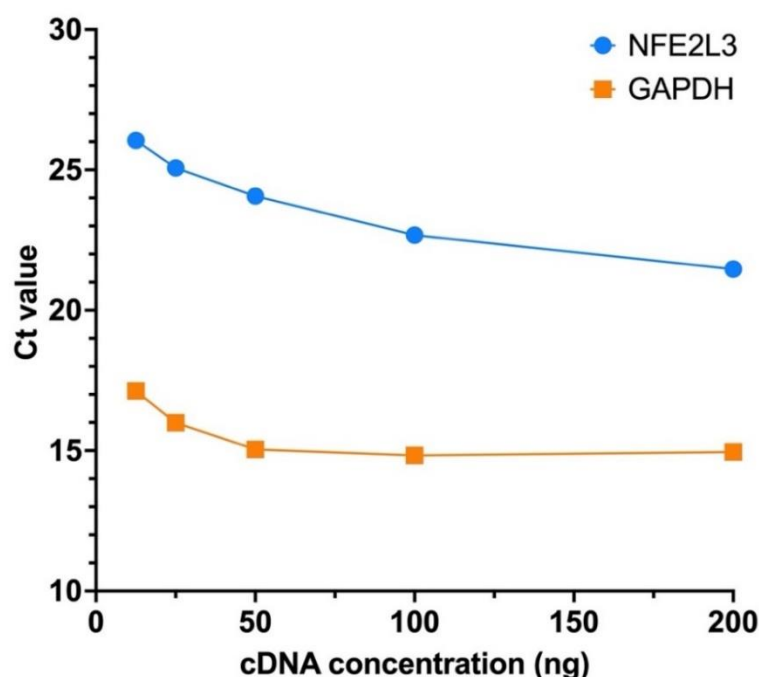


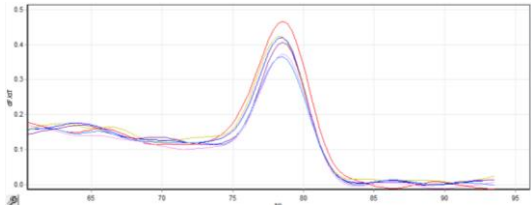
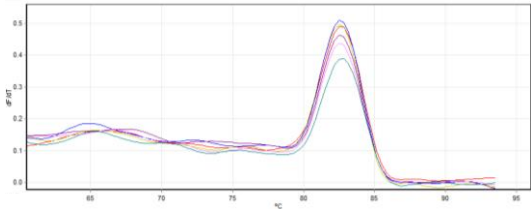
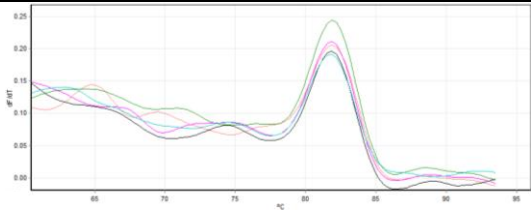
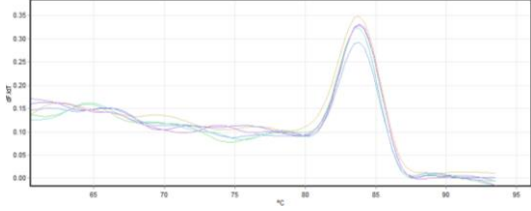
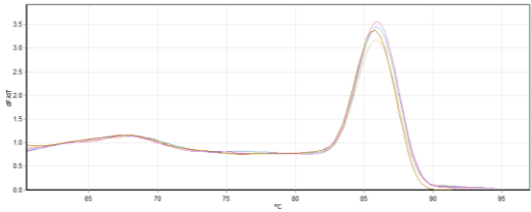
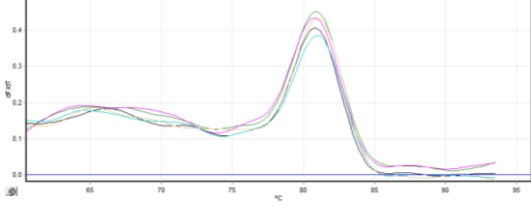
Figure 8.1 Representative cDNA calibration curve for RT-qPCR reactions using HCT116 cells. To determine the correct concentration of cDNA to use for RT-qPCR reactions, 200 ng RNA from unstimulated HCT116 cells was used to synthesise cDNA. Serial dilutions of cDNA were then prepared and NFE2L3 and GAPDH mRNA expression was measured via RT-qPCR. Cycle threshold (Ct) values were calculated to determine the effect of increasing cDNA concentration on gene amplification.

8.2.1.3 Dissociation curves

Following RT-qPCR, a melt-curve analysis is often carried out to identify the presence of any artifacts or primer-dimers that can occur due to poor primer design or reaction efficiency. Using incremental increases in temperature, changes in the fluorescence intensity of DNA intercalator dyes, such as SYBR green, can be detected which can indicate the dissociation between double-stranded DNA and DNA dyes. This allows for quantification of the melting point (T_m) of the PCR product, with several peaks representing either primer-dimer formation or formation of several PCR products,

thereby suggesting a lack of reaction specificity. In the present study, melt-curve analyses were carried out for each gene studied as displayed in **Table 8.2**.

Table 8.2 Dissociation curves of genes used in RT-qPCR.

Gene	Dissociation curves
NFE2L2	
NFE2L3	
HO-1	
NQO1	
DJ-1 (PARK7)	
GAPDH	

8.2.2 Concentration and time response of DMF treatment on Nrf2 and DJ-1 protein expression in A549 cells

As the Nrf2 activator DMF unexpectedly inhibited Nrf2 in **Figure 3.3**, we investigated the effects of DMF on Nrf2 protein expression in A549 cells at a range of concentrations (10-30 μ M) and treatment time-points (4-20 h), via western blot (**Figure 8.2**). The greatest induction of Nrf2 protein expression was observed at 4 h treatment with 10 μ M DMF and 4 h treatment with 20 μ M or 30 μ M repressed Nrf2 expression (**Figure 8.2**). On the other hand, all other concentrations did not appear to affect Nrf2 protein expression (**Figure 8.2**).

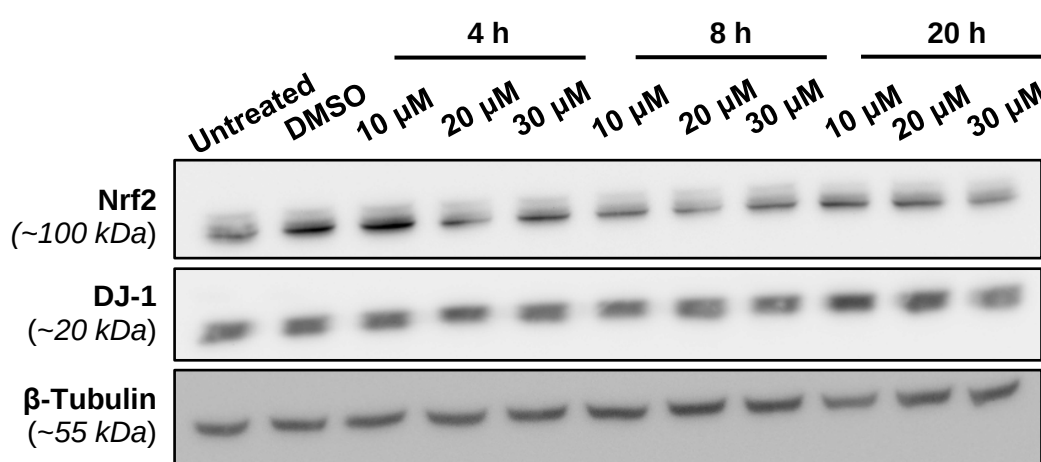


Figure 8.2 The effects of increasing concentration and treatment time of DMF on Nrf2 and DJ-1 expression in A549 cells. 1×10^6 A549 cells were treated with 10-30 μ M DMF for 4-8 h, prior to cell lysis. Nrf2 and DJ-1 expression was analysed via SDS-PAGE and western blot analysis, with β -Tubulin included as a loading control. Images are representative of three individual experiments (N=3).

8.2.3 Optimisation of A549 tumouroid culture experiments

In order to conduct the A549 tumouroids experiments in Chapter 3, culture conditions were first optimised, as described in **Figure 8.3**.

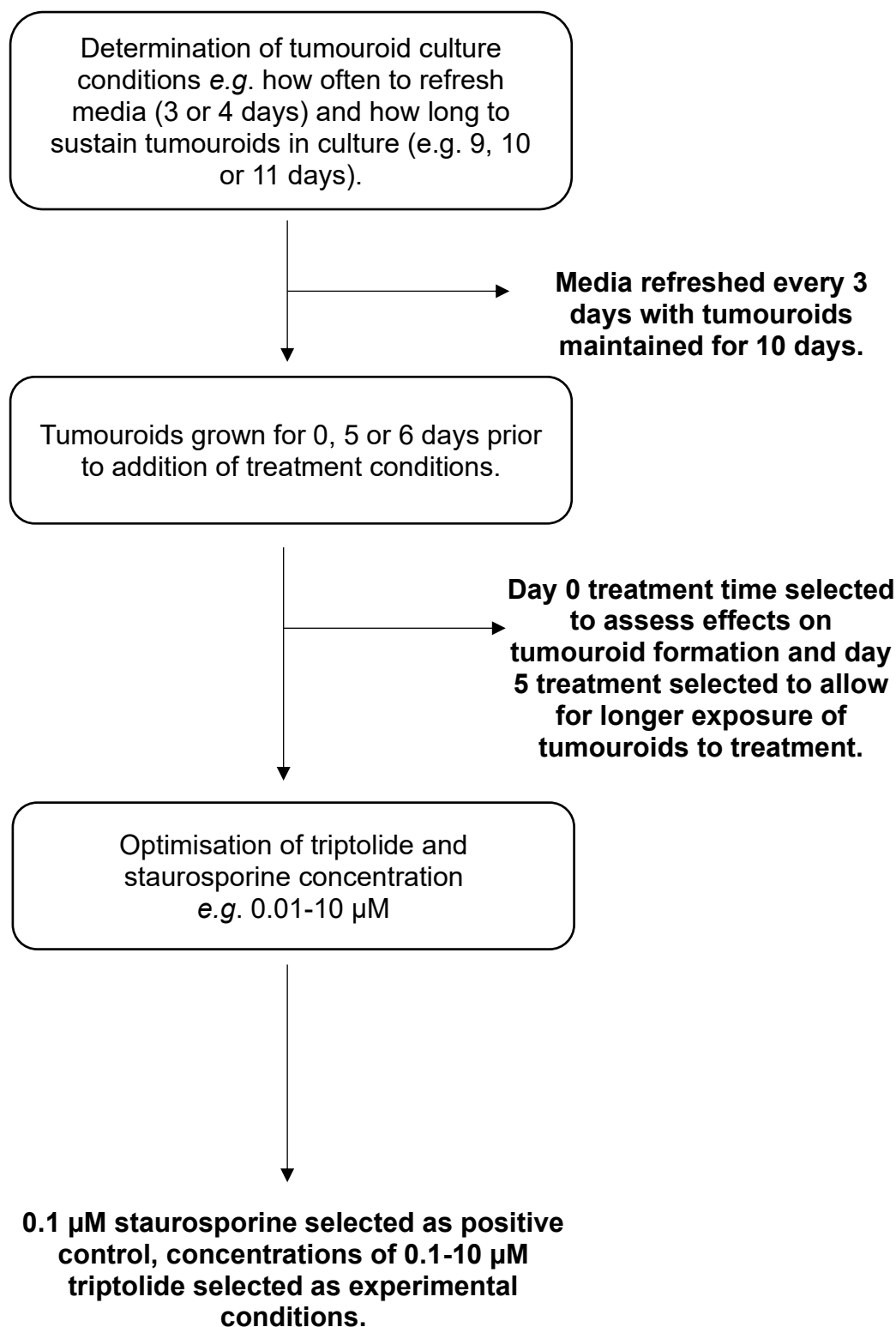


Figure 8.3 Schematic depicting the optimisation of A549 tumouroid culture and treatment conditions. Culture conditions for tumouroids were optimised according to initial experiments to determine the experiment length, treatment time points and treatment concentrations.

8.2.4 Analytical analysis of triptolide-peptide drug conjugates

To confirm the correct products were isolated for the synthesis of the triptolide peptide-drug conjugates in **Chapter 4**, reaction products were analysed via proton (^1H) and carbon (^{13}C) nuclear magnetic resonance (NMR), high-resolution mass spectrometry (HR-MS), MALDI-TOF (matrix-assisted laser desorption/ionisation- time of flight) mass spectrometry, and high-performance liquid chromatography (HPLC). All analysis is courtesy of Dr Marco Cominetti.

Following the synthesis of SU-TP (as described in **Figure 4.4**), HR-MS confirmed the presence of a product at a mass to charge ratio (m/z) of 461.1802, similar to that of the expected calculated m/z value of 461.1812 (**Figure 8.4**). As m/z can be considered to be the molecular mass + 1 ($[\text{M}+\text{H}]^+$), this confirmed the isolation of a product with the desired molecular mass. ^1H -NMR and ^{13}C -NMR were then conducted to confirm the identity of the product isolated as SU-TP (**Figure 8.5**).

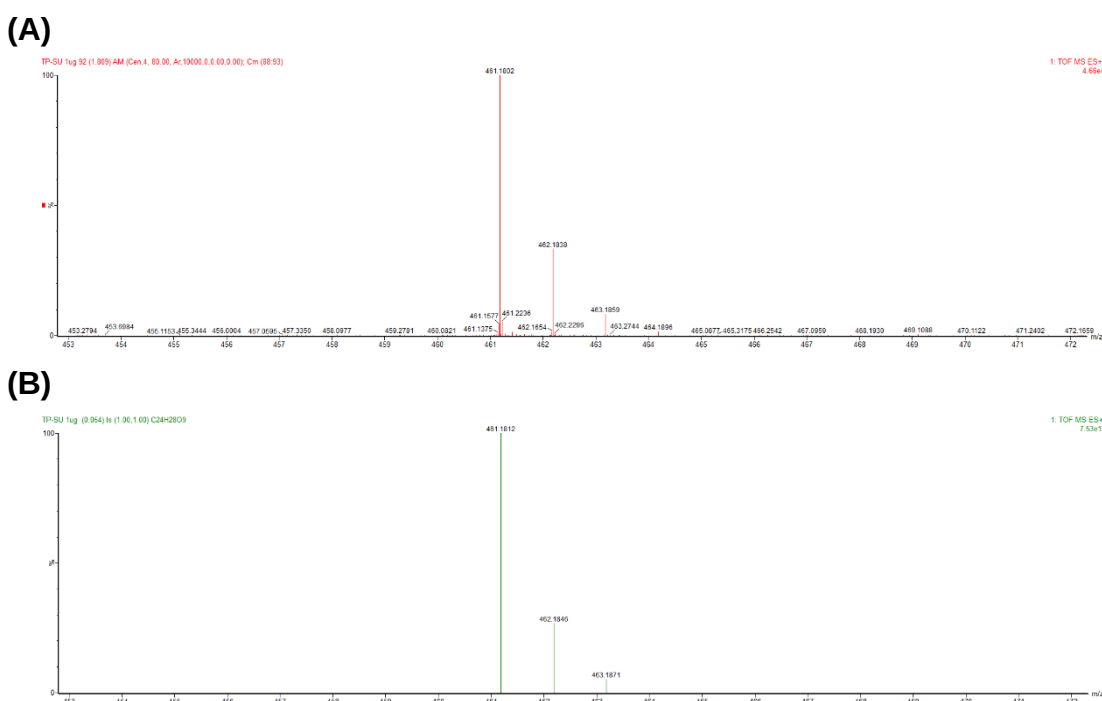


Figure 8.4 The high-resolution mass spectrometry of SU-TP. Mass spectrometry of the SU-TP was carried out using MALDI-TOF analysis to confirm the correct molecular weight of the product was isolated. **(A)** Spectra of the SU-TP product was compared to **(B)** spectra of the calculated m/z (mass to charge ratio) for the product.

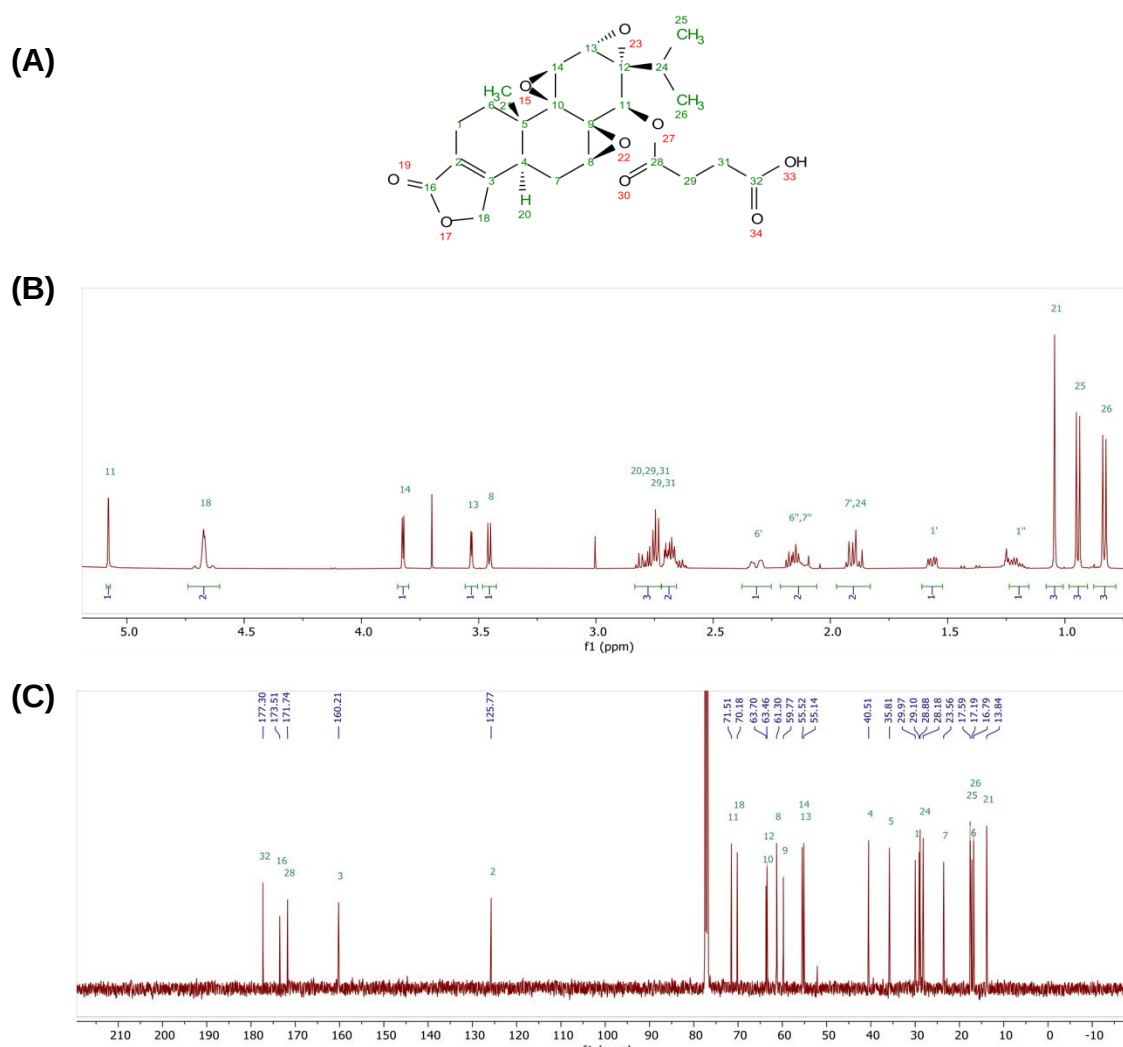
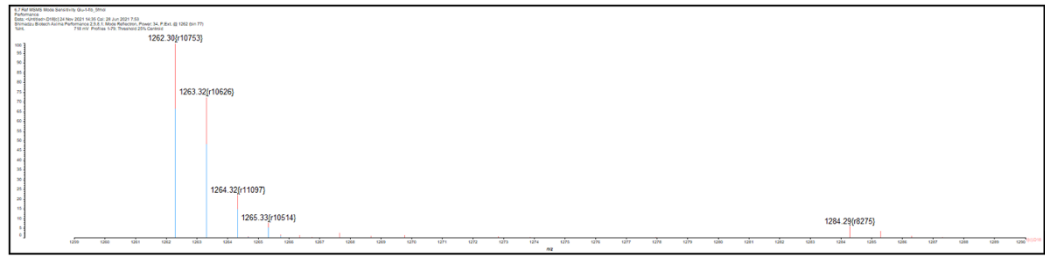


Figure 8.5 ^1H -NMR and ^{13}C -NMR spectra of the synthesised SU-TP product. (A) Legend for SU-TP NMR assignment. (B) ^1H -NMR spectra of SU-TP in CDCl_3 measured at 500 MHz. (C) ^{13}C -NMR spectra of SU-TP in CDCl_3 measured at 500 MHz. Both spectra are numbered according to the assignment legend in (A). Abbreviations: NMR; nuclear magnetic resonance, SU; succinate, TP; triptolide.

SU-TP was then conjugated to the AEYLRK and RALELK peptides (as described in **Figure 4.4**). To confirm the correct synthesis and isolation of Ac-AEYLRK-(SU-TP)- NH_2 and Ac-RALELK-(SU-TP)- NH_2 , MALDI-TOF mass spectrometry and HPLC were carried out. As displayed in **Figure 8.6**, MALDI-TOF confirmed the presence of a product at a $[\text{M}+\text{H}]^+$ m/z of 1262.30 (calculated $m/z=1262.63$), which was subsequently analysed with HPLC, displaying a singular product peak for Ac-AEYLRK-(SU-TP)- NH_2 with a retention time of 11.806 min. The same analysis was carried out for Ac-RALELK-(SU-TP)- NH_2 , which displayed $[\text{M}+\text{H}]^+$ m/z value of 1212.65 (calculated $m/z=1212.24$) and a single product peak with a retention time of 8.925 min (**Figure 8.7**). Together these analyses indicate the successful isolation of the desired products at purities of $\geq 95\%$.

(A)



(B)

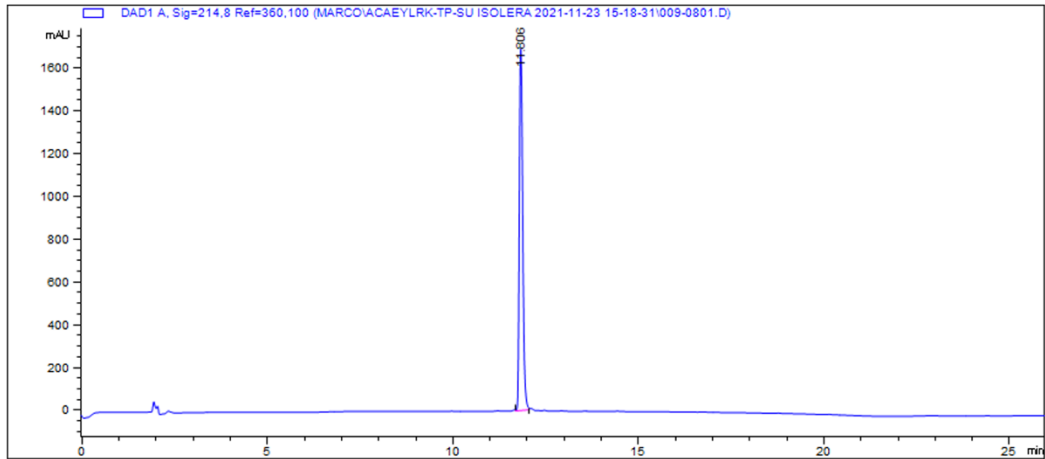
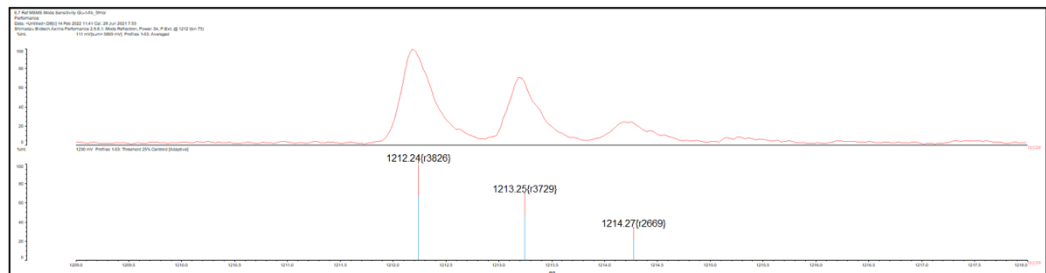


Figure 8.6 MALDI-TOF and HPLC analysis of Ac-AEYLRLK(SU-TP)-NH₂. (A) MALDI-TOF spectra and (B) HPLC trace of Ac-AEYLRLK(SU-TP)-NH₂.

(A)



(B)

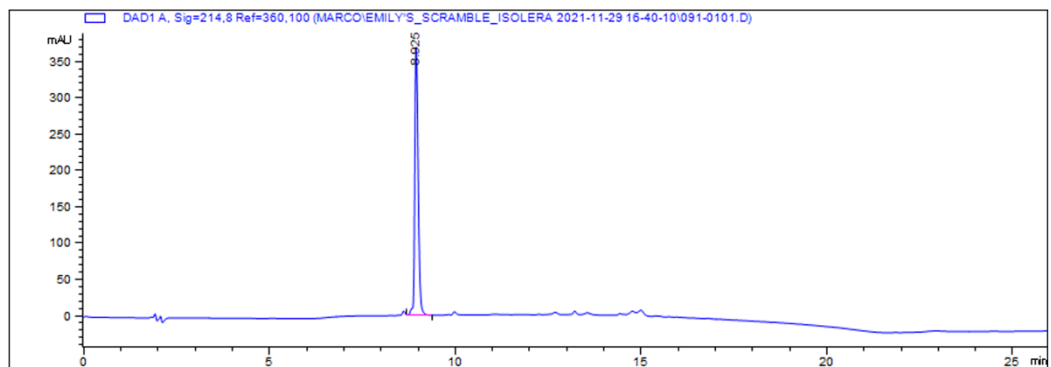


Figure 8.7 MALDI-TOF and HPLC analysis of Ac-RALELK(SU-TP)-NH₂. (A) MALDI-TOF spectra and (B) HPLC trace of Ac-RALELK(SU-TP)-NH₂.

8.2.5 Effect of vemurafenib on cell proliferation of A375 melanoma cells

To select a concentration of vemurafenib in the experiments conducted in **Chapter 5**, the anti-proliferative effects of several concentrations of vemurafenib were first determined via MTS assay and an IC_{50} was calculated. As shown in **Figure 8.8**, an IC_{50} value of 0.12 μ M was observed, hence concentrations below this value were selected for further experiments.

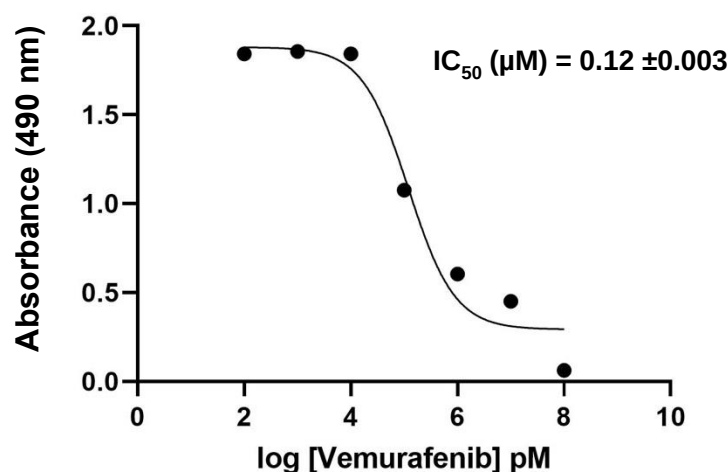


Figure 8.8 The anti-proliferative effects of vemurafenib on A375 cells. Concentration response curve of A375 cells (5×10^3) treated with a serial dilution of vemurafenib. Cells were treated with vemurafenib for 72 h and cell proliferation was measured via MTS assay. The IC_{50} value was calculated using Graphpad Prism 9 from the concentration response curves and is presented as mean IC_{50} value (μ M) $\pm$ SEM. Results are representative of three individual experiments (N=3).

8.2.6 Nrf3 induction

As the mechanisms of Nrf3 induction are not well-characterised, to better study the role of Nrf3 induction in cancer cells, we aimed to induce Nrf3 with TNF- α (tumour necrosis factor alpha) and the proteasome inhibitor molecule MG-132, as this has been previously found to induce Nrf3 by Chenais *et al.*¹⁹⁶ and Nouhi *et al.*¹⁹⁹ Hence, we stimulated HCT116 colorectal and MIAPACA2 pancreatic cell lines with either 100 ng/mL TNF- α or 10 μ M MG-132 for 16 and 6 h, respectively, as described in the literature^{196,199}.

As displayed in **Figures 8.9** and **8.10** however, in preliminary experiments, neither TNF- α or MG132 had any effects on Nrf3 expression in MIAPACA2 and HCT116 cells. We also studied the effects of the DDI2 protease inhibitor nelfinavir (which is reported to inhibit Nrf1 proteolytic cleavage) on MIAPACA2 Nrf3 expression, however no effects were observed (**Figure 8.9**).

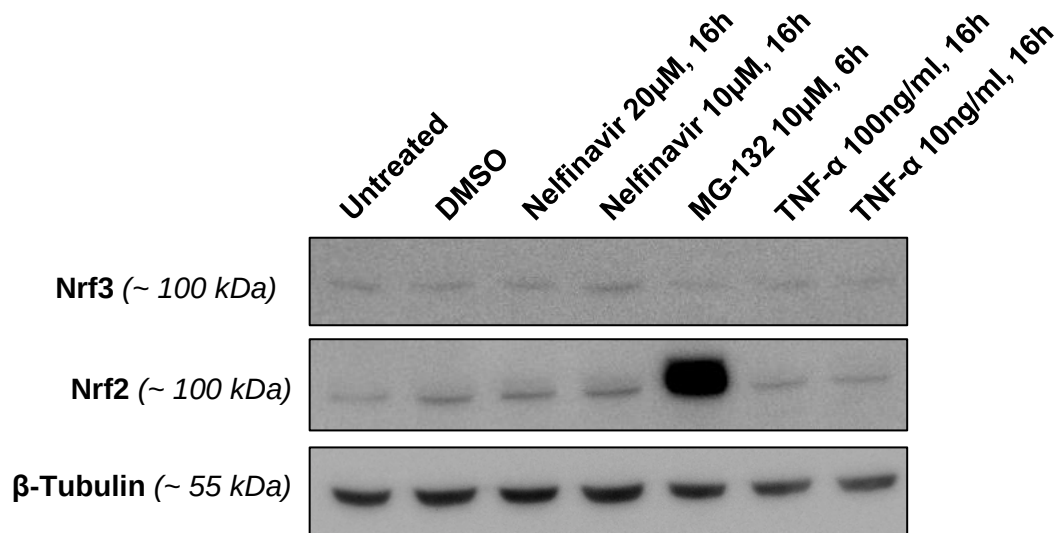


Figure 8.9 Nrf3 expression in MIAPACA2 cells following stimulation with nelfinavir, MG-132 or TNF- α . MIAPACA2 cells (1×10^6 /well) were stimulated with 20 μ M nelfinavir, 10 μ M MG-132 or 100 ng/mL TNF- α for 6 or 16 h. Cells were then lysed and Nrf3 and Nrf2 protein expression was measured via SDS-PAGE and western blot analysis. β -Tubulin was included as a loading control. Image is representative of one experiment (N=1).

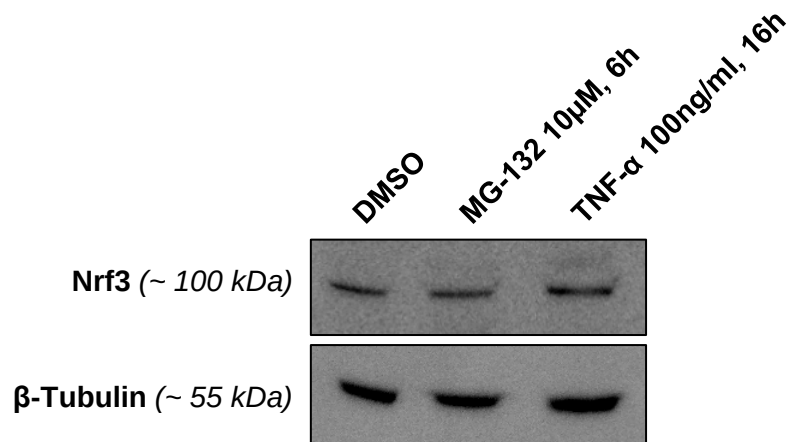


Figure 8.10 Nrf3 expression in HCT116 colorectal cancer cells following stimulation with MG-132 or TNF- α . HCT116 cells (1×10^6 cells/ well) were stimulated with 10 μ M MG-132 or 100 ng/mL TNF- α for 6 or 16 h, respectively. Cells were lysed and Nrf3 protein expression was measured via SDS-PAGE and western blot analysis. β -Tubulin was included as a loading control. Image is representative of one experiment (N=1).

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